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Protective Effects of Melatonin as an Antioxidant and Immunomodulator in Burn Injury of Rats

by

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A Thesis Submitted for the Degree of Doctor of Philosophy

School of Nursing

The Hong Kong Polytechnic University

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ABSTRACT

Multiple organ dysfunction or failure (MOD, MOF) and serious infection are the main complications in major burn and account for most of the deaths in severely burned patients. Major burn may induce obvious oxidative damages in remote organs, which are closely associated with MOD occurring at the early stage of burn. Furthermore, major burn also predominantly impairs the cell-mediated immunity (CMI), leading to increased susceptibility to infections. The present study aims to investigate the putative protective effects of melatonin, a very potent antioxidant and immunomodulator proved in many other animal models, on MOD and depressed CMI using a 30% total body surface area (TBSA) rat burn model. The results were as followed:

(1) The first study was carried out in 30 male Sprague-Dawley rats to observe the changes of haematocrit, plasma total protein (TP) concentrations and plasma malondialdehyde (MDA) levels at 6 h postburn. The significantly increased haematocrit and decreased TP concentrations indicated the existence of hemoconcentration and hypoproteinemia following the 30% TBSA burn. Melatonin treatment (10 mg/kg, given i.p. immediately postburn) was found to reduce the increased plasma levels of MDA as well as the formation of tissue oedema in the lung and burned skin. These results suggested that melatonin might act as an

antioxidant and exert some beneficial effects on burn-induced tissue damages.

(2) The protective effects of melatonin on oxidative stress in burn injury were further investigated in four remote organs (liver, kidney, lung and heart) at 6 h, 24 h and 72 h postburn by measuring the tissue levels of MDA and reduced glutathione (GSH). It was found that oxidative stress was evident within 24 h postburn in all tested organs, as evidenced by increased MDA levels and decreased GSH levels. In the kidney, the oxidative damages lasted up to 72 h following burn. The treatment with a single dose of melatonin (10 mg /kg, i.p.) significantly inhibited these alterations at 6 h postburn. However, repeated administrations of melatonin (10 mg/kg each time, given i.p. immediately postburn and then every 12 hours until 24 h and 72 h postburn) did not exert similar effect as the single injection.

To further explore the mechanisms responsible for the protective effect of melatonin, activities of three enzymes (glutathione peroxidase: GSH-Px; superoxide dismutase: SOD; myeloperoxidase: MPO), all of which are involved in burn-induced oxidative stress, were measured at 6 h postburn. GSH-Px and SOD (two main antioxidant enzymes) showed different responses to burn injury. The activities of GSH-Px were significantly decreased in all tested organs following burn, while SOD activities showed an increase in the kidney and the lung. Melatonin treatment (10 mg /kg, one dose) stimulated the liver GSH-Px and SOD activities, but had no significant effect in other three organs. In addition, MPO (an indicator for neutrophil infiltration) was found to increase markedly in all remote organs after burn. This implies that neutrophils are the very important source for the generation of free radicals. Increased MPO levels were partially alleviated by melatonin treatment.

In summary, melatonin may protect the vital organs against oxidative damages in burn injury. The antioxidant action of melatonin depends predominantly on its high free radical scavenging capacity, and in the liver, the antioxidant action is also enhanced by the stimulation of melatonin on GSH-Px and SOD activities. Melatonin therapy also decreases the neutrophil infiltration, thus preventing the neutrophil-mediated tissue damage in burn injury.

(3) The protective effect of melatonin on MOD was also assessed at 6 h, 24 h and 72 h postburn by measuring the serum levels of organ-specific markers (ALT, AST and ALP for liver dysfunction; creatinine and urea for kidney dysfunction; CK and LDH for heart dysfunction). The rises in these biomedical markers occurred as early as 6 h postburn and lasted up to 24 h postburn before the complete restoration at 72 h postburn. A single dose of melatonin exerted a significant protection against liver, kidney and heart dysfunction. Unfortunately, repeated administrations of melatonin had no effects. This failure is most likely due to the long injection interval (every 12 hours) relative to the very short half-life of melatonin.

(4) The improvement of melatonin on the depressed cellular immune response was also investigated in the present study. Severe immunosuppression was observed at Day 7 postburn, as evidenced by the significant inhibition in the ear swelling during the contact hypersensitivity reaction (CHR). Melatonin treatment (10 mg/kg, i.p. daily for 7 days) markedly enchanced the CHR but did not restore it completely. This result suggested another beneficial effect of melatonin as an immuno-enhancing agent in burn injury.

In conclusion, melatonin may exhibit significant protections against burn-induced MOD and immunosuppression, acting as a very effective antioxidant and immunomodulator. These beneficial effects together with the very low toxicity may enable melatonin to become a promising therapeutic agent of clinical significance in burn injuries.

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LIST OF ABBREVIATIONS

ADCC	antibody dependent cell-mediated cytotoxicity
AFMK	*N*1-acetyl-*N*2-formyl-5-methoxykynuramine
ALI	acute lung injury
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	one-way analysis of variance
ARDS	acute respiratory distress syndrome
ARF	acute renal failure
AST	aspartate aminotransferase
BSA	bovine serum albumin
BW	body weight
CAT	catalase
CHR	contact hypersensitivity reaction
CK	creatine kinase
Con A	concanavalin A
CTL	cytotoxic T cells
DNFB	2,4-dinitrofluorobenzene
DTH	delayed-type hypersensitivity
DTNB	5,5-dithiobis (2-nitrobenzoic acid)
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GSH	reduced glutathione

GSH-Px	glutathione peroxidase
GSH-Rd	glutathione reductase
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
H-E	hematoxylin-eosin
HETAB	hexadecyltrimethyl-ammonium bromide
HIOMT	hydroxyindole-*o*-methyltransferase
i.p.	intraperitoneal
i.v.	intravenous
IL-1	interleukin-1
IL-2	interleukin-2
IL-6	interleukin-6
IL-8	interleukin-8
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MDA	malondialdehyde
Mel	melatonin
MHC	major histocompatibility complex
MOD	multiple organ dysfunction
MOF	multiple organ failure
MPO	myeloperoxidase
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NAT	*N*-acetyltransferase
NO	nitric oxide
NOS	nitric oxide synthase

$O_2^{\bullet -}$	superoxide radical
$^{\bullet}OH$	hydroxyl radical
OFR	oxygen free radicals
PAF	platelet activating factor
PBS	phosphate- buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PMN	polymorphonuclear cells
PUFA	polyunsaturated fatty acid
RBC	red blood cell
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROO$^{\bullet}$	peroxyl radical
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
s.c.	subcutaneous
SCN	suprachiasmatic nucleus
SD	Sprague-Dawley
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SOD	superoxide dismutase
TBA	2-thiobarbituric acid
TBSA	total body surface area
TEP	1,1,3,3-tetraethoxypropane
Th	helper T cells

TNB	5,5-thio-2-nitrobenzoate
TNF-α	tumor necrosis factor-alpha
TP	total blood protein
Ts	suppressor T cells
vs	versus
XO	xanthine- oxidase

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Chapter 1

INTRODUCTION

1.1. Free Radicals and Antioxidative Defence System

1.1.1. Free Radicals and Reactive Species

Free radicals are often defined as any species capable of independent existence and containing one or more unpaired electrons in their outer orbitals (Halliwell and Gutteridge, 1985). Because of their high reactivity, free radicals can be very toxic to other molecules, especially those of biologically significance such as lipids, proteins and DNA, causing oxidative damages to cells and tissues.

Oxygen-centered free radicals (OFR) such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$) are the main radicals generated within aerobic cells when they utilize molecular oxygen as the basis of their metabolism. Some non-radical derivatives of oxygen such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) have the similar toxicity as OFR by acting as direct oxidizing agents or by converting into radicals, both of which are often collectively called reactive oxygen species (ROS). Main ROS as well as reactive nitrogen species (RNS) are listed in Table 1.1 (Halliwell, 1997).

Table 1.1 Reactive oxygen and nitrogen species of biological importance (from Halliwell, 1997)

Reactive Oxygen Species (ROS)	
Radicals	Non-radicals
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $^{\bullet}OH$	Hypochlorous acid, HOCl
Peroxyl, $ROO^{\bullet}$	Hypobromous acid, HOBr
Hydroperoxyl, $HOO^{\bullet}$	Ozone, O_3
Alkoxyl, $RO^{\bullet}$	Singlet oxygen, $^1\triangle g$
Reactive Nitrogen Species (RNS)	
Radicals	Non-radicals
Nitric oxide, $NO^{\bullet}$	Peroxynitrite, $ONOO^-$
Nitrogen dioxide, $NO_2^{\bullet}$	Alkyl peroxynitrites, ROONO
	Nitrous acid, HNO_2
	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-

1.1.2. Main Reactive Species and Their Generation *in Vivo*

1.1.2.1. Main Reactive Species

The main reactive species generated from oxygen include $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$. $O_2^{\bullet -}$ is a radical with relatively low reactivity, however it is easily catalyzed by superoxide dismutase (SOD) to H_2O_2 (Equation 1), the latter is a very active and toxic molecule (Fridovich, 1983; Deby and Goutier, 1990).

H_2O_2 is a main non-radical reactive species generated by many oxidation pathways. It acts as an oxidant for many biological molecules, especially those containing -SH groups, iron-sulfur clusters and reduced haem moieties (Svistunenko et al., 1997). In the

presence of transition metals Fe^{2+} or Cu^{2+}, H_2O_2 can be reduced to $^{\bullet}OH$ via the Fenton reaction (Equation 2). Therefore, its cytotoxicity is partly due to the generation of very toxic hydroxyl radicals (Ignatowicz and Rybczynska, 1994).

$^{\bullet}OH$ is the most toxic radical ever known because of its very high reactivity. It can react with almost everything it encounters. Fenton-like reactions are the main source of $^{\bullet}OH$ *in vivo*. $^{\bullet}OH$ is usually generated at the sites where transition metals are bound, it either reacts with a functionally important biomolecule close to its birthplace or wastes itself on the unimportant targets equally close by. Therefore, both the generation of $^{\bullet}OH$ and its damage to cells are site-specific (Halliwell and Gutteridge; 1986; Saran and Bors, 1990).

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad \text{(Equation 1)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad \text{(Equation 2, Fenton reaction)}$$

1.1.2.2. Generation of Main Reactive Species *in Vivo*

ROS are essential intermediates to many normal biological processes. They can be generated by many different pathways, at least including the activity of mitochondrial electron transport chain; respiratory burst of phagocytic cells; various oxidases and oxygenase systems. Transition metals also play a very important role in free radical's formation.

(1) Activity of mitochondrial electron transport chain

This process requires four electrons to reduce a molecule of O_2 to water, and also produces ATP (Ho et al., 1995). Cytochrome oxidase keeps all the partially reduced oxygen intermediates tightly bound to its active center, and in general there is little leakage of electrons. However, some other components of the electron transport chain

can leak electron directly onto oxygen. Since oxygen can accept one electron at a time, $O_2^{\bullet -}$ is generated. Therefore, $O_2^{\bullet -}$ can be considered as a 'normal' by-product of aerobic respiration, and its production increases with increased oxygen consumption, e.g. during exercise (Alessio, 1993).

(2) Respiratory burst of phagocytic cells

During the phagocytosis of neutrophils (a main component of phagocytic cells) and macrophages, NADPH-dependent oxidase bound to the membrane is activated and instantly reduces oxygen to $O_2^{\bullet -}$ and H_2O_2. This reaction is accompanied with increased oxygen uptake and consumption, and is often referred to "respiratory burst". Because of the low reactivity, $O_2^{\bullet -}$ and H_2O_2 are only partly responsible for the destruction of bacteria or other foreign particles. Phagocytosis also leads to the release of myeloperoxidase (MPO) in the neutrophil's cytoplasm, an enzyme which utilizes H_2O_2 and chloride to form hypochlorous acid (HClO) (equation 3). The latter is a very strong oxidant capable of oxidizing many biological molecules and enhancing the bactericidal activity (Babior, 1978; Takeshige et al., 1989).

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^- \quad \text{(Equation 3)}$$

(3) Multiple oxidase and oxygenase systems

Various oxidases and oxygenases use oxygen directly *in vivo*, and this is an important source for ROS. The most well-known of these enzymes is xanthine/xanthine oxidase system. Xanthine oxidase (XO) exists in normal tissues in the form of xanthine dehydrogenase which does not generate any radicals, however, it can be transformed into XO by tissue ischemia, limited proteolysis or oxidation of sulphydryl groups (Chen et al., 1995). Tissue ischemia results in increased degradation of ATP and subsequent

accumulation of its derivative hypoxanthine, and also activates XO, the latter oxidizes hypoxanthine to xanthine, and then to uric acid with the production of $O_2^{\bullet -}$ and H_2O_2 (Equation 4 and 5). Free radicals generated from XO play an essential role in the pathophysiology of ischemia and reperfusion damage (Parks and Granger, 1986).

$$\text{ATP} \longrightarrow \text{hypoxanthine} \xrightarrow{\text{XO}} \text{xanthine} \xrightarrow{\text{XO}} \text{uric acid} \quad \text{(Equation 4)}$$

$$\text{xanthine} + O_2 \xrightarrow{\text{XO}} O_2^{\bullet -} \quad \text{(Equation 5)}$$

In addition, many other oxidases such as D-amino acid oxidase and urate oxidase are powerful source of ROS (White, 1991). Arachidonic acid metabolism is accompanied by the generation and subsequent utilization of OFR. Microsomal enzyme systems in the liver can produce radical species directly during detoxification of toxic materials such as drugs and toxins (Halliwell and Gutteridge, 1986).

(4) Transition metals

Most of the free radical reactions *in vivo* are catalyzed by transition metals (mostly iron and copper) (Miller et al., 1990). These cations facilitate the electron transfer to biological macromolecules such as lipids, proteins and DNA. In the presence of $O_2^{\bullet -}$ and H_2O_2, these transition metals promote the formation of very toxic hydroxyl radical via the Fenton reaction, and also catalyze the decomposition of existing organic peroxides which are formed during the lipid peroxidation (Minotti, 1993). So transition metals play a very important part in free radical-mediated tissue injury, and the sequestration of free metals is an important mechanism for antioxidative defence. Fortunately, biological systems have developed both specific and nonspecific chelating proteins to make their bioavailability and cellular concentration under control.

1.1.3. Toxicity of Free Radicals

OFR and other reactive species can be generated both in normal and pathological circumstances. In normal condition, the production of OFR can be limited and removed rapidly by many different mechanisms collectively called antioxidative defence system, therefore, they can't result in cell and tissue damages. However, in many pathological conditions, e.g. inappropriate activation of phagocytic cells in inflammation, there are increased production of ROS and / or lowered antioxidative defence, a status often referred to as oxidative stress (Sies, 1993).

The toxicity of free radicals is due to their high reactivity. They are able to attack the very important macromolecules such as lipid, protein and DNA, leading to oxidative damages of cells and tissues by lipid peroxidation, protein oxidation and DNA damage.

(1) Lipid peroxidation

Many free radicals such as hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals can initiate the chain reaction of lipid peroxidation by attacking polyunsaturated fatty acid (PUFA) of the membrane (Babbs and Steiner, 1990; Dinis et al., 1993). The radical attack to PUFA produces an initial carbonyl radical, then the intermolecular action of two neighbouring radicals creates a new diene conjugate. The subsequent addition of oxygen to this molecule produces a peroxyl radical, which can propagate a chain reaction, amplifying the initial damage to the cell membrane. Peroxidation of PUFA results in the formation of lipid peroxides and hydroperoxides. Both are easily decomposed by Fe^{2+} / Fe^{3+} complex to form peroxyl and alkoxyl radicals and other toxic products, initiating new rounds of lipid peroxidation (Miller et al., 1990; Morel et al., 1990; Minotti, 1993).

Lipid peroxidation of the cell membrane destroys its structural and functional

integrity. It is the most important mechanism responsible for the cellular injury induced by free radicals (Halliwell, 1994).

(2) Protein oxidation

Different kinds of proteins (enzymes, receptors, channel proteins et al.) are critical targets for free radical attack. The damage of proteins is due to the peptide fragmentation, cross-linking or amino acid modifications, depending on the nature of proteins and the attacking radical species (Stadtman, 1986; Wolff and Dean, 1986; Davies, 1987).

The consequence of protein oxidation may be altered enzyme activity or abnormal membrane and cellular functions. The oxidative modification of membrane channel proteins may lead to intracellular calcium accumulation, the latter is a very important mechanism for cell damage and death. Oxidized proteins are much more susceptible to proteolysis (Wolff and Dean, 1986; Davies, 1987).

(3) DNA damage

DNA is also important target for free radical attack, resulting in nucleobase hydroxylation, cross-linking and single or double strand breakdown (Sonntag, 1987). The pyrimidine C_5 double bond is especially sensitive to hydroxylation. Oxidative alterations in DNA may influence gene expression and are responsible for mutations and carcinogenesis (Clemens, 1991). Extensive DNA damage can induce excessive activation of poly (ADP-ribose) polymerase, an enzyme which utilizes large amounts of NAD for its DNA repair activity, thus depleting the intracellular $NAD^+/NADH^+$ level. This will impair the ability of a cell to produce ATP, leading to energy deficiency and ultimately the cell death (Wiseman and Halliwell, 1996)

1.1.4. Antioxidant Defence System

To fight against the toxic actions of free radicals and reactive species, aerobic organisms have developed multiple defence systems, both enzymatic and non-enzymatic. Main antioxidant enzymes consist of SOD, glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd) and catalase (CAT). They interact in a co-operated manner with each other to effectively remove harmful reactive species (Figure 1.1). Non-enzymatic antioxidants are varied in nature and actions, e.g. glutathione, Vitamin A, C and E, uric acid and some plasma proteins. They usually exert an antioxidant role by directly scavenging free radicals and/or by keeping the transition metals under control.

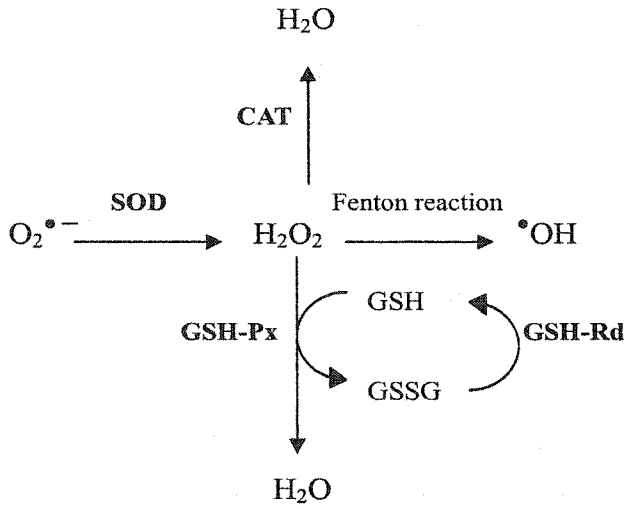


Figure 1.1 Co-operation between antioxidative enzymes.
SOD: superoxide dismutase; CAT: catalase;
GSH-Px: glutathione peroxidase; GSH-Rd: glutathione reductase.

1.1.4.1. Main Antioxidant Enzymes

(1) Superoxide dismutase (SOD)

SOD is a ubiquitous enzyme in all aerobic cells. It may catalyse the dismutation of

$O_2^{\bullet -}$ to H_2O_2 (Equation 1). This enzyme requires metal ions for its activity. Cu/Zn-SOD is found predominantly in the cytoplasm and Mn-SOD in mitochondria of mammalian cells. Fe-SOD exists mainly in prokaryotes (Bandy et al., 1990; Deby and Goutier, 1990; Levieux et al., 1991).

SOD protects cells against dangerous superoxide radical attack. Howver, the subsequent product of H_2O_2 is a very toxic molecule which can be removed by both glutathione peroxidase and catalase.

(2) Glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd)

Both GSH-Px and GSH-Rd are found in the cytosol and mitochondria of mammalian cells. GSH-Px is a selenium-dependent enzyme. It can effectively remove H_2O_2 and organic peroxides produced during lipid peroxidation with the consumption of reduced glutathione (GSH) (Equation 6 and 7). Maintenance of GSH level is essential for GSH-Px activity, and this can be obtained by oxidized glutathione (GSSG) reduction to GSH under the catalyzation of NADPH-dependent GSH-Rd (Equation 8) (Yu, 1994).

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-Px}} GSSG + 2\,H_2O \quad \text{(Equation 6)}$$

$$ROOH + 2GSH \xrightarrow{\text{GSH-Px}} GSSG + ROH + H_2O \quad \text{(Equation 7)}$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSH-Rd}} 2GSH + NADP^+ \quad \text{(Equation 8)}$$

(3) Catalase

Catalase is a haemoprotein mainly located in cell peroxisomes. It decomposes H_2O_2 into water and molecular oxygen (Equation 9). The affinity of catalase for H_2O_2 is low, so it requires a high H_2O_2 concentration to work fast. In normal body, low concentration of H_2O_2 can be detoxified effectively by GSH-Px, but in some pathological conditions, increased productions of H_2O_2 have to be removed by both

catalase and GSH-Px.

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2 \quad \text{(Equation 9)}$$

1.1.4.2. Main Non-enzyme Antioxidants

(1) Reduced glutathione (GSH)

GSH is a most important antioxidant within the cells. It is essential in keeping the activity of GSH-Px, thus removing H_2O_2 and organic peroxide effectively (Reed, 1986). GSH is also a scavenger for superoxide anions, and protect protein thiol groups from oxidation. In addition, GSH plays a major role in restoring other free radical scavengers and antioxidants such as Vitamin E and C to their reduced states (Ross, 1988).

(2) Vitamin E

Vitamin E is composed of different but closely related tocopherols. It is the major lipid-soluble chain breaking antioxidant in human plasma. Vitamin E can bind to the membrane and react with peroxyl or other radicals, forming a new vitamin radical which is not reactive to continue the chain reaction of lipid peroxidation. As a lipid soluble compound, Vitamin E plays a very important role in cell membrane protection (Thomas and Reed, 1990; Fuji et al., 1991).

(3) Vitamin C

Vitamin C (ascorbic acid) is reported to be the most effective water-soluble antioxidant in human plasma. It can react with many kinds of free radicals and toxic species such as superoxide, hydroxyl and peroxyl radicals, singlet oxygen and hydroperoxides to form the semidehydroascorbate radical. This radical is easily reduced

by GSH-mediated pathway or by specific NADPH-dependent enzyme. Vitamin C also acts synergistically with Vitamin E by reducing Vitamin E radicals to normal molecules (Bendich, 1986; Padh, 1991).

(4) Plasma proteins

Albumin is the main plasma protein and is reported to trap up to 50% peroxyl radicals in human plasma due to its SH-groups. Albumin is able to bind Cu^{2+} tightly and iron weakly. Cu^{2+} bound to albumin may still participate in Fenton reactions, but $^{\bullet}OH$ would be formed on the albumin surface and then scavenged by it. Damaged albumin can be quickly replaced. So the antioxidant role of albumin is partly due to a sacrificial mechanism (Wayner et al., 1987; Halliwell, 1994).

Caeruloplasmin is a copper-containing protein. It acts as an antioxidant by reacting directly with $O_2^{\bullet -}$ or by its ferroxidase activity, converting Fe^{2+} to less active Fe^{3+}. It is regarded as a physiological inhibitor in lipid peroxidation (Chaine et al., 1991)

Transferrin, lactoferrin and ferritin are specific iron-chelating proteins in the circulation, making iron unavailable in iron-catalysed radical reactions and lipid peroxidation (Bunker, 1992).

1.2. Basic Concepts, Organ and Immune Dysfunction in Major Burns

1.2.1. Size, Depth and Severity of Burn Injury

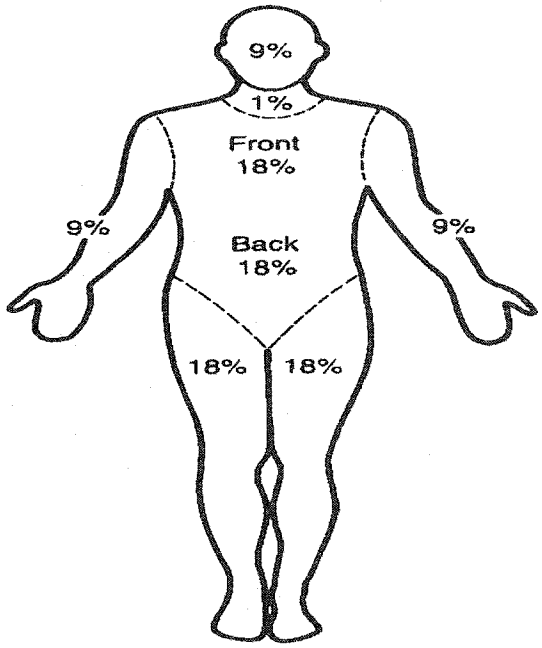
1.2.1.1.Burn Size

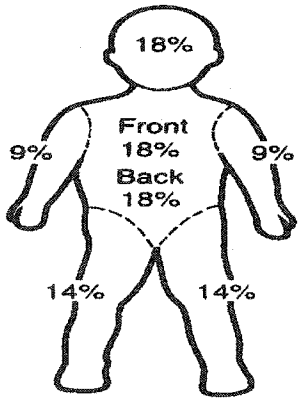
In adult burn patients, the size of burn is usually estimated by the Rule of Nines (Figure 1.2). In this rule, each arm and the head represent 9% of the body surface. The

anterior trunk, the posterior trunk and each leg represent 2× 9% respectively. The neck constitutes 1% of body surface. However, the Rule of Nines cannot be used in children below 12 years old, since their heads constitute a relatively larger, and the lower limbs, a relatively smaller part of the body surface (Mlcak and Buffalo, 2002).

In animals, the total body surface area (TBSA) is estimated according to their body weight or in combination with body length. In rats, TBSA can be calculated by Meeh's formula: $TBSA = KW^{2/3}$ (TBSA: cm^2; K: a constant, 9 to 10 for rats; W: body weight in gram) (Walker and Mason, 1968).

Adult body	% of total
Part	BSA
Arm	9%
Head	9%
Neck	1%
Leg	18%
Anterior trunk	18%
Posterior trunk	18%





Child body	% of total
Part	BSA
Arm	9%
Head and neck	18%
Leg	14%
Anterior trunk	18%
Posterior trunk	18%

Figure 1.2 Estimation of burn size using the Rule of Nines.

1.2.1.2.Burn Depth

The traditional classification of burn depth is first (1st), second (2nd) and third (3rd) degree, indicating the increasing depth of tissue destruction. It is more characteristic today to describe burn injury as superficial, partial thickness and full thickness burn, the latter better describes clinical findings and implies healing time and subsequent scarring.

The depth of burn is primarily estimated by histological examinations, but other clinical criteria, such as wound appearance, sensation (pinprick test), blanching under pressure and healing time, are usually used in combination (Table 1.2). The accurate estimation of burn depth is important for effective surgery intervention. Unfortunately, burn depth is seldom uniform, and the initial depth at the time of injury may change with time due to desiccation, infection and other factors, thus frequent observations of the burn wound are necessary (Richard and Staley, 1994).

1.2.1.3.Burn Severity

Burn severity may be affected by a lot of factors, including the size, depth and causes of burn, patient's age and pre-existing diseases as well as other accompanying injury, among which the size and depth of burn are the most important factors and are often used in combination to estimate the severity of burn. According to the severity, burn injury is also divided into three types: minor, moderate and major burn (Table 1.3). Minor and moderate burn have relatively smaller influences on body functions, and in most cases, only local wound cares are needed. However, major burn may result in a number of abnormalities and is closely related with high mortality following burn, therefore, both local and systemic management (e.g. fluid resuscitation and organ support) should be carried out immediately postburn (Hartford, 2002).

Table 1.2 Criteria for the depth of burn (from Richard and Staley, 1994)

Burn Classification	**Histological Depth**	**Wound Appearance**	**Sensation**	**Healing**
Superficial burn	Epidermis only	Redness Dry, no blisters Edema (variable)	Painful	3-7 days with peeling No scar May have discoloration
Partial thickness burn				
Superficial	Epidermis and some papillary dermis	Red color Blistered Moist blebs	Very painful, sensitive to temperature	7-21 days, no grafting No/minimal scarring Pigment changes
Deep	Epidermis, papillary and reticular layers of dermis	Pale or pink to cherry red Blisters are large	Less painful	21-35 days if no infection May develop severe hypertrophic scar
Full thickness burn	All skin elements, even down to subcutaneous tissues and organs	Mixed white, waxy, dark or charred Dry, leathery Usually no blisters Blood vessels visible under eschar	Little or no pain Anesthetic to temperature Hair pulls out easily	Large areas require grafting or require several months to heal Small areas may heal from edges after weeks

Table 1.3 Burn classification according to severity (from Hartford, 2002).

Minor burn
< 15% TBSA in adults
< 10% TBSA in children and the elderly
< 2% TBSA full-thickness burn in children or adults without cosmetic or functional risk to eyes, ears, face, hands, feet or perineum
Moderate burn
15-25% TBSA in adults with < 10% full-thickness burn
10-20% TBSA partial-thickness burn in children under 10 and adults over 40 years of age with < 10% full thickness burn
< 10% TBSA full-thickness burn in children or adults without cosmetic or functional risk to eyes, ears, face, hands, feet, or perineum
Major burn
> 25% TBSA
> 20% TBSA in children under 10 and adults over 40 years of age
> 10% TBSA full-thickness burn
All burns involving eyes, ears, face, hands, feet, or perineum that are likely to result in cosmetic impairment
All high-voltage electrical burns
All burn injury complicated by major trauma or inhalation injury
All poor risk patients with burn injury

1.2.2. Burn Shock and Organ Dysfunction in Major Burn

1.2.2.1.Pathophysiology of Burn Shock

Major burn may result in hypovolemic shock, which is characterized by the specific hemodynamic changes, including decreased plasma volume, cardiac output, urine output and an increased systemic vascular resistance with resultant reduced peripheral blood flow (Aulick et al., 1977; Lund and Reed, 1986). It is now recognized that burn shock is a complex process of circulatory and microcirculatory dysfunction due to the interplay of hypovolemia and multiple mediators released after burn injury (Demling, 1987; Youn et al., 1992).

The hypovolemia following burn results mainly from the marked increased capillary permeability. The latter is caused by both the direct heat-induced vascular damage and the release of many inflammatory mediators, including histamine, serotonin, kinins, prostaglandins, thromboxanes, OFR and cytokines (Hayashi et al., 1964; Pitt et al., 1987). The changes in capillary permeability are maximal 8-12 h following burn and then return to normal level within 24 to 36 hours post-burn. Increased capillary permeability leads to extensive loss of fluid and plasma proteins from the circulation into the extravascular spaces, causing severe burn wound oedema and subsequent hypovolemia (Harm et al., 1982). When burn injury exceeds 25% TBSA, there is also oedema formation in non-burned tissues and organs as the result of the sustained hypoproteinemia as well as increased capillary permeability (Demling et al., 1984).

Immediately following burn, there is immediate depression of cardiac output before any detectable reduction of plasma volume, this may be due to the presence of a circulating cardiac depressant factor which is released from burn wound and inhibits the myocardial contractility (Baxter et al., 1966). In addition, hypovolemia and increased

systemic vascular resistance also contribute to the reduced cardiac output (Hilton and Marullo, 1986; Crum et al., 1990).

Sympathetic stimulation and hypovolemia following burn result in the release of catecholamines, vasopressin and angiotensin II (Crum et al., 1990), all of which cause vasoconstriction and increase the systemic vascular resistance.

There are several organs (kidney, liver and gastrointestinal tract) particularly susceptible to ischemia and organ dysfunction. Renal ischemia can result directly from hypovolemia and increased renal blood vessel constriction (Holm et al., 1999). In major burn without fluid resuscitation, the urine output falls to a very low level or even to zero. The cells of the kidney may be so damaged by anoxia that they cannot recover even if the blood flow is recovered later on.

The microcirculation dysfunction is very significant in burn shock, and results from many factors, including microvascular constriction, increased blood viscosity and slow blood flow, aggregation of blood cells and platelets, and even the formation of microvascular thrombosis (Crum et al., 1990). These abnormalities in microcirculation aggravate the end-organ ischemia from hypovolemia and cardiac dysfunction. Without early and full fluid resuscitation, burn shock may result in organ dysfunctions and even death.

1.2.2.2.Mechanisms for Organ Dysfunction

Organ dysfunction is very common in major burn, and may occur alone but often in combination. The most severe situation is called multiple organ failure (MOF) with very high mortality. Severe shock is often related to the early organ dysfunction, and various infections often lead to its late occurrence.

The mechanisms responsible for organ dysfunction and MOF have not been fully

understood, however, it has been widely accepted that uncontrolled systemic inflammatory response and hypoxic tissue damage are the most possible causes.

(1) Systemic inflammatory response syndrome (SIRS)

SIRS is a term to describe a systemic inflammatory process, independent of its cause (infectious or non-infectious). Two or more of the following conditions must be fulfilled for the diagnosis of SIRS: (1) body temperature > 38 °C or < 36 °C; (2) heart rate > 90 beats/min; (3) respiratory rate > 20 /min or $PaCO_2$ < 32 mmHg; (4) leukocyte count > 12000/ul, < 4000/ul, or > 10% immature forms (Bone et al., 1992). SISR has a varied severity, and the most severe form induces organ dysfunction and MOF.

In severe burn injury, the most common cause of SIRS is the burn itself. Sepsis is also a major initiator. Recently, it has been found that enteric bacteria translocation and their products (e.g. endotoxin) occurring very early following burn are involved in the development of early SISR and organ dysfunction (Kelly et al., 1997).

SIRS-induced tissue damages are mediated by a number of inflammatory mediators, including pro-inflammatory cytokines, free radicals, arachidonic acid metabolites, platelet activating factor (PAF), nitric oxide and complement, among which pro-inflammatory cytokines and free radicals play most important roles.

Tumour necrosis factor-alpha (TNF-α) is released primarily by macrophages very soon following burn and modulates a variety of immunological and metabolic events (Spooner et al, 1992). The local released TNF-α may activate neutrophils and mononuclear phagocytes, and also serves as a growth factor for fibroblasts. However, the systemic effects of TNF-α are often a destructive cascade of events, including induction of fever, stimulation of acute phase proteins, activated coagulation cascade, myocardial suppression, induction of nitric oxide and subsequent hypotension,

catabolism of muscle and fat, and even apoptosis (Voss and Cotton, 1998). TNF-α is also a potent stimulus for the release of other inflammatory mediators, particularly interleukin-1 (IL-1) and interleukin-6 (IL-6).

The source and physiological effects of IL-1 are essentially identical to those of TNF-α (Van et al., 1999). However, IL-1 itself does not induce tissue injury or apoptosis but can potentiate the injurious effects of TNF-α. IL-6 is secreted by macrophages, endothelial cells, fibroblasts and T cells. IL-6 cannot induce tissue injury directly, but its presence in the circulation is a marker of on-going inflammation, and is often associated with poor outcome in severe burn patients. The primary effect of IL-6 is to induce the release of acute phase proteins from liver as well as serve as a growth and differentiation factor for B lymphocytes (Van et al., 1999).

Activated neutrophils are attracted to burn wound and nonburned tissues and organs by multiple mediators, e.g. interleukin-8 (IL-8), complement C3a and C5a, leukotrienes et al. The accumulation of these activated cells may release various toxic products such as free radicals, proteases, PAF and other substances, causing significant tissue injury (Weiss, 1989).

(2) Hypoxic tissue damage

The prolonged tissue hypoxia and the generation of toxic free radicals during reperfusion are another important mechanism for end-organ dysfunction. Tissue ischemia impairs the generation of ATP (a main energy source), resulting in inhibited Na-K ATPase activity and defective cellular functions. The tissues that are initially in shock and then reperfused produce large amount of OFR due to the increased XO activity (Parks and Granger, 1986). These toxic products may impair cellular functions, increase capillary permeability and promote the release of other inflammatory mediators,

leading to tissue damage and organ dysfunction. Antioxidant therapy seems beneficial in combination with fluid resuscitation (Schiller et al., 1993).

1.2.2.3.Manifestation of Main Organ Dysfunction

In severe burn with delayed or insufficient fluid therapy, various organ systems may show signs of dysfunction. The most frequently affected organs include the lung, the kidney, the liver and the heart.

(1) Lung

Most burn patients present with a certain degree of respiratory insufficiency and relative hypoxia, as evidenced by hyperventilation, increased oxygen consumption and alterations in blood gas test. Severe burns, especially those with inhalation injury or serious infections may lead to acute lung injury (ALI) and even acute respiratory distress syndrome (ARDS), the latter is associated with very high mortality rate (Suchyta et al., 1992).

ALI and ARDS are clinically manifested by dyspnea, severe hypoxemia and decreased lung compliance with radiographic evidence of diffuse bilateral pulmonary infiltrates (Doyle et al., 1995; Ware and Matthay, 2000). It is speculated that these characteristic changes may result from direct or mediator-induced lung endothelial injury, increased vascular permeability and lung oedema (Doyle et al., 1995). The treatment of ALI and ARDS is largely supportive, including mechanical ventilation, fluid management, surfactant therapy and anti-inflammatory strategies.

(2) Kidney

Kidney impairment ranges from a transient and relatively insignificant proteinuria

to acute renal failure (ARF). The latter is a potentially lethal complication in major burn, resulting in the failure of kidney to excrete nitrogenous waste products and to maintain fluid and electrolyte homeostasis (Thadhani et al., 1996).

ARF may be oliguric or nonoliguric according to the urine output, the differential diagnosis may refer to Table 1.4. In an oliguric state, functional (or prerenal) or organic (or renal) failure must be determined for different treatment strategies. Urine osmolarity and sodium concentration are often used for these determinations because of the ease of measurement (Table 1.5). Nonoliguric renal failure is not that detectable, however, the continuously increased blood creatinine and urea level during or after burn shock may be an important indicator for its occurrence. The initial care of patients with AFR is focused on reversing the underlying cause, and correcting fluid and electrolyte imbalance. Intermittent blood and peritoneal dialysis remain the main therapy for severe ARF (Conger, 1995).

Table 1.4 Differential diagnosis of oliguric and non-oliguric renal failure (from Shinozawa and Aikawa, 2002)

Examination	**Oliguric**	**Nonoliguric**
Urine volume (ml/24h)	< 400-500	> 500
Serum K (mEq/L)	Increased	Normal range
Serum creatinine (mg/dl)	Increased	Gradually increased
U_{Na} (mEq/L)	40-80	Various
C_{cr} (ml/min)	< 5	5~30

U_{Na}: urine sodium; C_{cr}: creatinine clearance

Table 1.5 Laboratory tests to distinguish prerenal from renal failure (from Thadhani et al., 1996)

Examination	**Functional (Prerenal)**	**Organic (Renal)**
Urine osmolarity (mosmol/L)	> 400	< 400
U_{Na} (mEq/L)	< 20	> 40

(3) Liver

After burn injury, a variable degree of liver injury is present with the release of many hepatic enzymes, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). AST and ALT are the most sensitive markers of hepatocyte injury, and the elevated serum levels are prompt following burn. Increased ALP level usually indicates the damage of biliary system, both intrahepatic and extrahepatic (Carter et al., 1986).

The liver has many functions, thus its injury is manifested by multiple alterations, including increased synthesis of acute phase proteins, reduction in constitutive hepatic proteins (e.g. albumin) and many coagulation factors (e.g. V, VII, IX and fibrinogen), reduced synthesis and secretion of bile salts and vitamins deficiency, especially fat-soluble vitamin A, E, D, K. In general, the liver dysfunction plays a crucial role in the outcome of burn injury. The early protections on liver functions are necessary in burn management.

(4) Heart

Severe burn injury affects heart performance in a number of ways. It reduces the heart preload through decreased plasma volume, and also inhibits the myocardial contractility by releasing a myocardial depressant factor, TNF-α and free radicals (Cioffi et al., 1986), leading to reduced cardiac output. These effects are most evident in the early stage of burn injury. Increased heart rate and abnormal rhythm can also exist and contribute to the cardiac dysfunction. Adequate fluid replacement is the most effective treatment.

1.2.2.4. Fluid Resuscitation in Burn Shock

Fluid resuscitation is aimed to maintain adequate organ perfusion of the patients throughout the initial 24 h to 48 h period of hypovolemia. It is the primary management for burn shock and organ dysfunction, and should be initiated as early as possible after burn. The fluid volume within the first 24 h postburn can be predicted by different formulas (Table 1.6), usually with one-half of total amount administered within first 8 h postburn. The composition of resuscitation fluid may vary from crystalloid, colloid or in combination, which make little difference in the first 24 h postburn. However, the optimizing fluid resuscitation should be individualized according to the patient's response to the therapy (Warden 1992).

Table 1.6 Formulas for estimating adult burn patient resuscitation fluid needs within first 24 h postburn (from Warden, 1992)

Colloid formula	**Electrolyte**	**Colloid**	**D5W**
Evans	Normal saline 1.0 ml/kg/%burn	1.0 ml/kg/%burn	2000 ml
Brooke	Lactated Ringer's 1.5 ml/kg/%burn	0.5 ml/kg	2000 ml
Slater	Lactated Ringer's 2 L/24h	Fresh frozen plasma 75 ml/kg/24h	
Crystalloid formula			
Parkland	Lactated Ringer's 4 ml/kg/%burn One-half of that volume in the first 8 hours postburn		
Modified Brooke	Lactated Ringer's 2 ml/kg/%burn	2 ml/kg/%burn	
Hypertonic saline formula			
Hypertonic saline solution (Monafo)	Volume to maintain urine output at 30 ml/h Fluid contains 250 mEq Na/L		
Modified hypertonic (Warden)	Lactated Ringer's + 50 mEq $NaHCO_3$ (180 mEq Na/L) for 8 hours to maintain urine output at 30-50 ml/h Lactated Ringer's to maintain urine output at 30-50 ml/h beginning 8 h postuburn		
Dextran formula (Demling)	Dextran 40 in saline - 2 ml/kg/h for 8 h Lactated Ringer's -volume to maintain urine output at 30 ml/h Fresh frozen plasma – 0.5 ml/kg/h for 18 h beginning 8 h postburn		

1.2.3. Impaired Cell-Mediated Immunity in Major Burn

1.2.3.1.Cell-mediated Immunity in Normal Body

The immune system defends the host against infections. Innate immunity serves as the first line of defence but lacks the specific protection against invading pathogens. In contrast, adaptive immunity, including cell-mediated immunity mainly via T cells and humoral immunity via B cells, enable the body to recognize the specific foreign antigens.

The cell-mediated immunity plays a central part in the host defence mechanism. It is initiated by antigen presenting cells (macrophages, dendritic cells and B cells) when they present specific antigen-MHC complex to antigen-specific T cells. Activated T cells lead to the release of a T cell growth factor interleukin-2 (IL-2), which stimulates the subsequent proliferation and differentiation of T cells into two kinds of effector cells. Helper T (Th) cells secrete a number of cytokines, activating various phagocytic cells and enabling them to phagocytose and kill microorganisms (especially bacteria and protozoa) more effectively. Cytotoxic T cells (CTL) participate in the cell-mediated immune reaction by killing altered self-cells, e.g. the virus-infected cells and tumor cells (Goldsby et al., 2000).

1.2.3.2.Cellular Immunodeficiency in Major Burn

Major burn predominantly impairs the cell-mediated immunity, causing increased susceptibility to infection (Mannick, 1993). The cellular immunodeficiency has been evidenced by inhibited lymphocyte proliferation (Renk et al, 1982; Singh et al., 1986), suppressed delayed cutaneous hypersensitivity (Cetinkale et al., 1993), increased

allograft rejection time (Ninnemann et al., 1978).

Cellular immunodeficiency occurs as early as one day but reaches maximum about one week following burn. It results from a complex interaction of immune cells and a number of immunoregulatory mediators, among which the arachidonic acid cascade and the cytokine cascade play leading roles. The functions of macrophages and T cells, which serve as main immune cells in the cellular response, are also significantly impaired.

(1) The arachidonic acid cascade

PGE_2, a major arachidonic acid metabolite, shows a significant increase in the circulation soon after burn, and this increase lasts up to several weeks. Increased PGE_2 production impairs the antigen presentation of macrophages, leading to reduced IL-2 secretion and inhibited T cell activation (Grbic et al., 1991). Suppression of PGE_2 production and activity by indomethacin (a cyclooxygenase inhibitor) has been shown to improve survival and immune function after burn injury (Horgan et al., 1990).

(2) The cytokine cascade

Following burn, induction of a number of cytokines rapidly occurs, the best studied of these are IL-2, IL-1, IL-6 and TNF-α.

IL-2 is released primarily from T cells and acts as a key factor for T cell activation and proliferation. It also increases NK cell activity and ADCC. Major burn leads to persistently decreased IL-2 production and IL-2 gene expression, which correlate with increased mortality (Wood et al., 1984; Teodorczyk-Injeyan et al., 1989).

IL-1, IL-6 and TNF-α are the main pro-inflammatory cytokines involved in the systemic inflammatory response. These cytokines are important immunomodulators at

low concentrations, however, their overproduction usually exhibit immunosuppressive activities in severe burn.

IL-1 is a very important local immunomodulatory agent in normal cellular response. It may act as a strong co-stimulator for T cell activation, promoting IL-2 synthesis and T cells blastogenesis (Drost et al, 1993). Although these functions are advantageous, excessive and prolonged production of IL-1 following burn is always harmful, especially in combination with TNF-α (O' Sullivan and O' Connor, 1997).

IL-6 serves as a growth and differentiation factor for B cells (Van et al., 1999). The induction of IL-6 production is as early as 1 h postburn. High concentration of IL-6 exhibits the immunosuppressive activity possibly due to the activation of TGF-β and increased PGE_2 product (Zhou et al., 1991). It is also a marker for tissue injury and a poor prognosis in severe burn.

Increased TNF-α occurs rapidly after trauma but is cleared soon from the circulation. Excessive TNF-α appears to be associated with propagation of sepsis as a poor prognosis (Marano et al., 1990). Anti-TNF antibody therapy has been shown to improve survival from sepsis, but the clinical relevance remains to be proven (Exley et al., 1990).

(3) Macrophages

Defective macrophage functions have been suggested as a trigger mechanism for suppressed cellular response following burn. Severe burn impairs the antigen presentation capacity of macrophages, leading to suppressed T cell activation and IL-2 release (Unanue et al., 1984). This is possibly mediated by increased production of PGE_2, which reduces the expression of MHC II molecules on the macrophage surface. On another hand, over-activation of the macrophages leads to extensive release of

inflammatory mediators, including PAF, pro-inflammatory cytokines (IL-1, IL-6, TNF-α) and PGE_2 (O' Sullivan and O' Connor, 1997). Increased PGE_2 and pro-inflammatory cytokines are the main mediators responsible for immunosuppression in severe burn.

(4) T cells

T cell is the central part of cell-mediated immunity. Their normal functions depend on proper activation, proliferation and differentiation, and balanced cytokine release. Following severe burn, all these steps seem defective due to a variety of endogenous factors, including impaired antigen presentation, unbalanced cytokine cascade or other inflammatory mediators, e.g. PGE_2 (O' Sullivan and O' Connor, 1997).

Initially, it was suggested that the immunosuppression following burn was caused by increased ratio of suppressor T (CD8) cells to helper (CD4) cells (Munster et al., 1976), and this has been confirmed (Hansbrough et al, 1984). Recent evidences show that altered cytokine profiles seem more important. For example, it has been found that reduced IL-2 secretion accounts to a large extent for the postburn immunosuppression, and excessively increased pro-inflammatory cytokines from T helper cells may lead to the failure of T cell activation in response to physiological stimuli possibly by overuse of T cells or some unknown alterations on intracellular or membrane physiology (Teodorczyk-Injeyan et al., 1991; Tolentino et al., 1991).

1.3. Melatonin: From Metabolism to Biological Functions

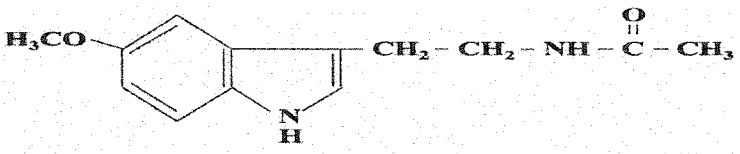
1.3.1. Biosynthesis of Melatonin

Melatonin (*N*-acetyl-5-methoxytryptamine) is a hormone mainly secreted from the pineal gland. It was first identified in bovine pineal extracts on the basis of its ability to

aggregate melanin granules and thereby lightened the colour of frog skin (Lerner et al. 1958 and 1959). The chemical structure of melatonin is illustrated in Figure 1.2.

Melatonin is synthesized from the amino acid tryptophan taken up by the pinealocytes from the blood (Figure 1.3). Tryptophan is firstly converted by tryptophan hydroxylase to 5-hydroxytrytophan, then decarboxylated to serotonin. The synthesis of melatonin from serotonin involves two distinct enzymes: *N*-acetyltransferase (NAT) and hydroxyindole-*o*-methyltransferase (HIOMT), both of which are largely confined in the pineal gland. Serotonin is N-acetylated by NAT to N-acetylserotonin, the latter is then methylated by HIOMT to form melatonin (Axelrod and Weissbach, 1960; Klein and Weller, 1970).

Although the pineal gland is a major source for the production of melatonin, it is also synthesized in other sites, including the retina (Pang and Allen, 1986), the Harderian gland (Menendez-Peleaz et al., 1991), the gut (Huether et al., 1992; Lee and Pang, 1993), bone marrow cells (Tan et al., 1999; Conti et al., 2000), and even some organs (liver, lung, brain and skin) known to have high oxygen radical formation (Huether et al., 1992; Yaga et al., 1993).



***N*-acetyl-5-methoxytryptamine**

Figure 1.3 Structure of melatonin (from Vijayalaxmi et al., 2002)

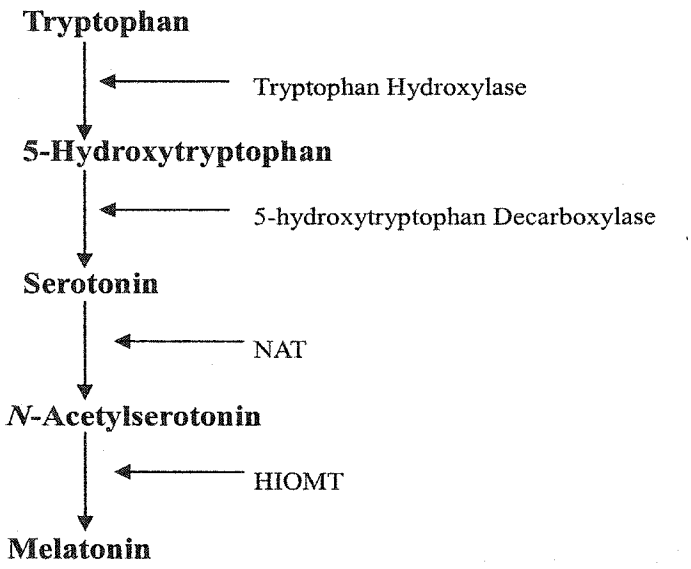


Figure 1.4 Biosynthesis of melatonin (from Vijayalaxmi et al., 2002)

1.3.2. Melatonin Secretion and Its Regulation

Once produced from the pineal gland, melatonin is quickly released into the bloodstream through passive diffusion. The secretion of melatonin and its plasma level have a significant circadian rhythm with very low plasma level in the daytime and peak level at night (Reiter, 1982; Laakso et al., 1994). This rhythm of melatonin can be found in all vertebrates and in some invertebrates.

The circadian rhythm of melatonin is modulated predominantly by day-light cycle (Laakso et al., 1994). The mammalian pineal gland is a neuroendocrine transducer. Photic information from the retina is transmitted through the suprachiasmatic nucleus (SCN) in the hypothalamus and the sympathetic nervous system to the pineal gland (Fung, 1987). The release of norepinephrine and its activation on α-1 and β-1 adrenergic receptors increase the activity of NAT, initiating the synthesis and secretion of melatonin (Pangerl et al, 1990). This neuronal system is activated by darkness and suppressed by light. The daily rhythm of melatonin secretion is also controlled by an

endogenous pacemaker located in SCN (Brezezinski et al., 1988).

The secretion and plasma level of melatonin is also affected by various other factors, including age, season, menstrual cycle, diseases et al. Melatonin plasma level is reduced considerably with age (Reiter, 1997). Nocturnal secretion of melatonin also changes in different seasons, possibly due to the resynchronization of seasonal light variations with the endogenous circadian pacemaker. Alterations in serum melatonin levels are found in a number of diseases, most of which demonstrate increased melatonin levels.

1.3.3. Absorption and Catabolism

Exogenous melatonin can be given by multiple routes, including oral, intraperitoneal (i.p.), intravenous (i.v.), subcutaneous (s.c.) and intranasal administration. Melatonin, being fat soluble, is usually absorbed rapidly, and its plasma levels increase strikingly as early as 30 min following injection. However, the absorption of melatonin after the subcutaneous injection will take a longer time (2 to 4 hours) to reach above the baseline plasma level (Lee et al., 1994).

The half life of plasma melatonin is relatively short, ranging from less than 30 min to about 1 hour (Mallo et al., 1990; Yeleswaram et al., 1997). For example, following i.v. injection of 3 mg/kg (5 mg/kg in rats), the half life of melatonin is about 19.8, 18.6 and 34.2 min in rats, dogs and monkeys respectively (Yeleswaram et al., 1997).

Melatonin is metabolized chiefly in the liver (Kopin et al., 1961; Lane and Moss, 1985; Young et al., 1985). Microsomal enzymes in hepatic cells metabolize melatonin to 6-hydroxymelatonin, the latter is subsequently conjugated with sulphate (70%) or

glucuronide (6%) to form water-soluble metabolites which are secreted in the urine. 6-hydroxymelatonin is the main metabolite of melatonin, and its urine concentration closely parallels to the serum melatonin level (Lynch et al., 1975). Small quantities of unmetabolized melatonin (<5% of that in the blood) may be excreted directly into the bile (Lane and Moss, 1985) or the urine (Tan et al., 1999b).

1.3.4. Cellular Localization

Melatonin has been shown to be localized in the membrane, cytosol, nucleus and mitochondria (Menendez-Pelaez and Reiter, 1993). This widespread subcellular distribution of melatonin is due to its high lipid-soluble property (Costa et al., 1995) as well as the existence of binding proteins within the cells. The melatonin levels in the subcelluar organelles can be higher than those in the blood, and the highest concentrations are found in the nucleus (Menendez-Peleaz and Reiter, 1993) and mitochondria (Acuna-Castroviejo et al., 2001). The nucleus contains many DNA molecules which are the most sensitive targets for free radical attack, whereas the mitochondria are the very important source of free radical generation. Thus, high levels of melatonin may be highly beneficial at these two sites.

1.3.5. Toxicity of Melatonin

The toxicity of melatonin has been tested in both animals and humans over a wide range of doses from physiological to high pharmacological concentrations. In general, no serious side effects or risks have been reported in association with the ingestion of melatonin. The doses of melatonin used in animals are up to 200 mg/kg in normal or pregnant rats and mice (Barchas et al., 1967; Blickenstaff et al, 1999;

Jahnke et al, 1999), or even 800 mg/kg in rabbits, cats and dogs (Barchas et al., 1967), no abnormalities have been reported.

In human studies, no adverse side-effects have been observed after oral administration of melatonin at different doses, e.g. 10 mg daily for 28 days (Sebra et al., 2000), even 1g daily for 30 days (Nordlund and Lerner, 1977). However, adminstration of melatonin still results in several unfavorable effects, such as hypothermia, decreased alertness, and possibly reproductive effects (Brzezinski, 1988), thus it has been suggested that people who take melatonin over long periods should be monitored for any potential unusual reactions (Arendt and Deacon, 1997).

1.3.6. Melatonin Receptors

The binding sites of melatonin seem to be broad, including cell membrane, cytosolic proteins (e.g. calmodulin) and the nucleus. Two types of melatonin receptors on the membrane have been identified: MEL1 (high-affinity, pmol) and MEL2 (low-affinity, nmol) (Morgan et al., 1994; Dubocovich, 1995). MEL1 receptor is G protein-coupled, and its activation results in the inhibition of adenylate cyclase activity in target cells (Ebisawa et al., 1994). MEL 1 receptors may be divided into two subgroups: MEL1a and MEL1b in several mammals, including humans. The MeL 1a receptor is expressed mainly in the hypophysial pars tuberalis and SCN (Reppert et al., 1994). The MEL 1b receptors are expressed mainly in the retina and, to a lesser extent, in the brain of birds and melanoma cells (Ying et al., 1993; Reppert et al., 1995). The MEL 2 receptors are coupled to the stimulation of phosphoinositide hydrolysis, but their distribution has not been determined.

Melatonin also has the ability to bind to the cytosolic calmodulin, affecting the calcium-dependent activity. For example, melatonin has been shown to inhibit the calmodulin-dependent nitric oxide synthase (NOS) through this mechanism (Bettahi et al., 1996).

Nuclear binding sites of melatonin are found almost in all cells. Melatonin may act as a ligand for two orphan receptors (alpha and beta) in the family of nuclear retinoid Z receptors. The binding is in the low nanomolar range, suggesting that some of the potential actions of melatonin may be mediated via the nuclear receptors (Becker-Andre et al., 1994; Carlberg and Wiessenberg, 1995).

1.3.7. Main Biological Functions

Melatonin may affect multiple biological processes, including antioxidative defence, immune response, sleep and circadian rhythm, sexual maturation and reproduction, aging and tumour growth. Recent research interests focus on its antioxidant and immunomodulating functions. These two functions are also involved in the anti-aging and anti-tumour effects of melatonin, therefore, more details about them need be introduced.

1.3.7.1. Antioxidant Function

Melatonin has been found to be a very effective antioxidant and protect cells, tissues and organs against oxidative damage in both *in vivo* and *in vitro* studies (Reiter et al., 1997). The antioxidant action of melatonin results predominantly from its strong free radical scavenging capacity, and is also partly due to its influences on some enzymes involved in the antioxidative defence.

(1) Free radical scavenger and its action mechanism

The first evidence of melatonin as a free radical scavenger came from the observation of Tan and coworkers using a cell free in vitro system (Tan et al., 1993a). They found that melatonin could detoxify highly toxic hydroxyl radicals and have greater efficacy than GSH (an endogenous antioxidant) and mannitol (an antioxidant found in plants). These observations have been repeatedly confirmed (Poeggeler et al., 1996; Bromme et al., 2000).

Melatonin also scavenges very toxic peroxyl radical produced in the lipid peroxidation of the cell membrane, and seems to have greater efficacy compared with Vitamin E, a main endogenous chain-breaking antioxidant (Pieri et al., 1994). In addition, melatonin has been shown to inhibit the cellular damage induced by singlet oxygen in two animal models (Kukreja et al., 1993; Cagnoli et al., 1995), suggesting that it may act as a singlet oxygen scavenger to eliminate its potential toxicity.

Excessive amount of nitric oxide has been proved to cause cytotoxic changes in cells. Nitric oxide may further react with superoxide radicals to form peroxynitrite anion (Pryor and Squadrito, 1995), the latter is very toxic reactive species. Melatonin at physiological concentrations may reduce the generation of nitric oxide by inhibiting the activity of NOS (Battahi et al., 1998), and to detoxify the peroxynitrite anion via a direct interaction (Gilad et al., 1997; Blanchard et al., 2000).

Melatonin is a highly electroreactive molecule. It acts primarily as a powerful electron donor and detoxifies electrodeficient reactive oxygen species (Poeggeler, et al., 1994 and 1996; Matuszak et al., 1997). After reacting with ROS, melatonin itself becomes a radical, the melatonyl cation radical, which has low reactivity, thus reducing the overall toxicity to cells. Once generated, the melatonyl cation radical

may be recycled back to melatonin (Mahal et al., 1999) or additionally reacts with singlet oxygen to produce N_1-acetyl-N_2-formyl-5-methoxykynuramine (AFMK), this oxidative product is also a potent free radical scavenger (Hardeland et al., 1993).

(2) Influences on enzymes

Besides the direct free radical scavenging ability, melatonin also acts as an antioxidant by stimulating the activities of several very important antioxidative enzymes, including SOD, GSH-Px and GSH-Rd (Reiter et al., 1997 a). Exogenous administration of melatonin has been found to increase the mRNA levels of Zn/Cu-SOD and Mg-SOD in rat brain cortex (Antolin et al., 1996; Kotler et al., 1998). Melatonin also stimulates the activity of GSH-Px and GSH-Rd (Barlow-Walden et al., 1995; Pablos et al., 1995 and 1998).

Different from the above-mentioned enzymes, melatonin at physiological concentration inhibits the NOS activity. NOS is the key enzyme catalyzing the generation of nitric oxide radical, leading to increased peroxynitrite anion product, thus it is usually considered as a pro-oxidative enzyme. These influences of melatonin on pro-oxidative and antioxidative enzymes additionally increase its protective ability toward free radical-mediated tissue damages.

(3) Other factors affecting antioxidant action of melatonin

The antioxidant action of melatonin *in vivo* may be affected by more factors, e.g. its metabolites and other derivatives, interaction with other antioxidants and lipid-soluble capacity. 6-hydroxylmelatonin, a main hepatic metabolite, has been found to be more effective as a free radical scavenger than melatonin itself (Pierrefiche et al., 1993). Even AFMK, an oxidative product of melatonin, is also a potent free radical

scavenger due to its iron chelating property (Tan et al., 1993 a, b). When applied in combination with classic antioxidant Vitamin E, C or metal desferrioxamine, melatonin is capable of exaggerating the efficiency of these molecules in protecting against oxidative damage (Gitto et al., 2001). Melatonin is a highly lipid-soluble molecule and can easily cross the cell membrane and other morphophysiological barriers, thus the application of melatonin *in vivo* is especially effective (Reiter, 1997b)

1.3.7.2. Immunomodulation Function

Melatonin stimulates the immune response in most conditions tested up till now, and this effect seems especially apparent in the immunodeficiency state. Pinealectomy and other methods which may inhibit melatonin synthesis (e.g. prolonged lighting) usually induce an immunosuppressive state, which can be counteracted by melatonin treatment (Rella and Lapin 1978; Del Gobbo et al., 1989), suggesting the physiological regulation of endogenous melatonin on the immune system.

Effect of exogenous melatonin on the immune response may be affected by a lot of factors, including species, doses, administration time, the pre-existing immune status (Skwarlo-Sonta, 2002). Maestroni and co-workers have contributed a lot to the early study of melatonin as an immunomodulator (Maestroni et al., 1986 and 1988; Maestroni and Conti, 1989 and 1991). They found that melatonin administration (0.1-10 mg/kg, s.c.) in the late afternoon counteracted the depression of T cell-dependent antibody production, T cell reaction as well as spleen and thymus cellularity induced by acute stress or corticosterone treatment in mice (Maestroni et al., 1986). Interestingly, melatonin injected in the morning did not produce similar effects. The immunoregulatory effects of melatonin have been extended to many other immunodeficiency states, such as those secondary to aging (Maestroni et al., 1988;

Caroleo et al., 1993), trauma-hemorrhage (Wichmann et al., 1996), viral diseases (Maestroni, 1989), and cancer (Vijayalaxmi et al., 2002). In general, melatonin seems to exert widely beneficial effects especially on the cellular immune responses, such as improved antigen presentation (Pioli et al., 1993), increased T cell proliferation and IL-2 product and enchanced ADCC (Maestroni, 1993).

It seems that one of the main targets of melatonin is thymus, the central organ of the immune system. Melatonin receptors have been found in a variety of immune cells. It has been suggested that the immunoenchancing effect of melatonin is most likely to be receptor dependent, and may be mediated by the release of Th cell-derived opioid peptide and lymphokines (e.g. IL-2 and interferon $-\gamma$) (Maestroni, 1993).

1.3.7.3.Other Functions

Besides acting as an antioxidant and immunomodulator, melatonin also affects many other biological functions or processes, including sleep and circadian rhythm, sexual maturation and reproduction, aging and tumour growth. These functions are briefly reviewed in Table 1.7 (Brzezinski et al., 1997).

Table 1.7 Other biological functions of melatonin (from Brzezinski et al, 1997)

Biological Process	Effect of Melatonin	Possible Mechanism	Evidence
Sleep	Increased sleep propensity Hypnotic effect	Hypothermic effect Receptor-mediated action on limbic system	Placebo-controlled clinical trials
Circadian rhythm	Controls of circadian rhythm Entrainment to light dark cycle	Melatonin secretion in response to neural input from eyes Receptor-mediated effects on neural and peripheral tissues	Studies in animals and humans on the effect of light and the light –dark cycle on the pattern of melatonin secretion
Sexual maturation and reproduction	Inhibition of reproduction	Hypothalamic-pituitary-gonadal axis's inhibition Influence on ovarian steroid genesis	Studies in animals and comparative clinical studies of melatonin secretion during puberty and in women with amenorrhea
Aging	Anti-aging effect	Scavenging of free radicals	In vitro and in vivo studies in animals
Tumor growth	Anti-tumor effect	Direct antiproliferative effect Enchanced immune response Scavenging of free radicals	In vitro and in vivo studies in animals In vitro studies of human neoplastic cells and cell lines, and a few clinical studies.

1.4. Aims of the Study

Burn injury is a worldwide problem with high mortality rate. Although burn management has been significantly improved during the last decades, the handling of multiple organ dysfunction or failure (MOD or MOF) and serious infections which are the main complications in burn injury, is still no easy task. It is generally accepted that burn-induced MOD results from the uncontrolled systemic inflammatory response as well as the hypoxic tissue damage. During these two processes, oxygen free radicals over-produced following burn injuries are the most important mediator. The supplement of classical antioxidant (e.g. SOD, Vitamins E and C, GSH) has been shown to provide some beneficial effects by reducing the free radical-mediated tissue damage.

Various infections are mainly secondary to the immunosuppression especially the depression in cell-mediated immunity after burn injury. However, the underlying mechanisms were not fully understood. Many efforts have been done to improve the immune defence ability, e.g. the surgical excision of burn wound, early organ support and nutrient supplement, and the use of immunomodulating drugs.

On the other hand, melatonin has been proved to be a potent antioxidant and may protect cells, tissues and organs against oxidative stress and damage induced by various stimuli. The antioxidant action of melatonin is mainly due to its very high free radical scavenging capacity, and is also enchanced by its stimulation on some important antioxidative enzymes. Moreover, melatonin also acts as an immunoenchancing agent in all conditions tested to date, and seems especially effective in the immunodeficiency states.

Our study aims to investigate the putative protective effects of melatonin in burn injury by acting as an antioxidant and immunomodulator. The antioxidant action of melatonin and its protection against burn-induced MOD are our specific concerns. To achieve this aim, three sets of experiments will be performed.

1. Experiment 1 is designed to confirm the oxidative stress following 30% major burn as well as the antioxidant role of melatonin.

2. Experiment 2 concentrates on the antioxidant action of melatonin as well as its protection against MOD following burn. The tissue levels of MDA and GSH in four remote organs were measured to estimate the burn-induced oxidative damage, while the enzyme activities were assessed to reveal the antioxidant mechanisms for melatonin. In addition, the serum levels of TNF-α and nitrite (a marker for NO production) were also measured to estimate their involvement in the protective effect of melatonin on MOD.

3. Experiment 3 is to study the immunomodulating effect of melatonin in burn injury.

Chapter 2

MATERIALS & METHODS

2.1. Chemicals

All chemicals and reagents used in the experiments are of analytical grade and are listed in Table 2.1

2.2. Equipment

All equipment and other materials used are listed in Table 2.2

2.3. Animals

Male Sprague-Dawley (SD) rats (250 – 300 g) were used in the experiments. All animals were purchased from The Chinese University of Hong Kong and maintained under a 12/12 h light-dark cycle (lights on 08:00 h). A standard rodent diet (No. 5001) and water were given *ad libitum*. After one week of acclimation, the animals were randomly assigned to different groups. The use of animals was approved by The Department of Health, Government of Hong Kong Special Administrative Region, China and The Animal Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University.

2.4. Burn Model

2.4.1. Induction of 30% Full-thickness Burn

A 30% TBSA, full-thickness burn model was used throughout the study as described by Peter et al. (1999). After anaesthesia with sodium pentobarbital (50mg/kg body weight, i.p.), the animals were shaved on the dorsum using an electronic clipper. 30% TBSA was calculated by Meeh's formula (k=10) and marked on the shaved back. This region was immersed through a square hole in a protective plastic template into boiling water for 15 s, thus creating a full-thickness and painless scald burn. All burned animals received 15 ml of 0.9 % saline as fluid resuscitation according to Parkland formula (4 ml/kg/ % burn) immediately following burn. The animals were decapitated under anaethesia at 6 h, 24 h and 72 h postburn respectively.

The animals in sham group were treated identically except for exposure to tap water and without fluid resuscitation. They were sacrificed immediately after sham operation.

2.4.2. Confirmation of Burn Depth and Size

The depth of burn wound was examined histologically under the microscope at 6 h postburn. The specimens from the center of dorsal skin were excised, fixed in 4% buffered formaldehyde, processed in an automatic tissue processor and embedded in paraffin. Microscopic sections (5 μm thick) were made perpendicular to the wound surface using a Leica microtome, and stained with standard hematoxylin-eosin (H-E) staining (Jimenez PA et al., 1999).

The size of burn was also estimated at 6 h postburn. The boundary of whole body skin and burned skin were scanned into the computer and analyzed with planimetry. The burned area was expressed in percentage of total body surface area.

2.5. Melatonin Treatment

Melatonin (Mel) was freshly prepared by dissolving in absolute ethanol, and then diluted ten-fold in 0.9% saline. The melatonin was given i.p. at a dose of 10 mg/kg body weight (b.w.) and a volume of 2 ml/kg b.w. each time. 10% ethanol in 0.9% saline (vehicle) was given to sham and burn controls in the same way as melatonin treatment.

2.6. Estimation of Biochemical Parameters in Plasma and Tissue Oedema Following Burn (Expriment 1)

2.6.1. Experimental Protocol

30 male SD rats (250-300 g) were assigned randomly to three groups: sham control (n = 9), burn control (n = 10) and burn + mel treatment (10 mg/kg, i.p. immediately postburn, n = 11). At 6 h following burn or sham operation, all animals were anaesthetized for sample collections and then sacrificed.

About 1.0 ml of blood (20U heparin per 1 ml blood) was taken from the heart. After haematocrit measurement, all blood samples were centrifuged at 4,500 rpm for 10 min, the plasma was used for determination of total protein concentrations and malondialdehyde levels. Liver, kidney, lung and burned skin were also collected to estimate the tissue oedema.

2.6.2. Measurement of Haematocrit

Haematocrit was measured in duplicate using a micromethod. The capillary haematocrit tube was filled with heparinized blood sample by capillary attraction. After

centrifugation at 6,500 rpm for 5 min, the value was read using a haematocrit reader.

2.6.3. Determination of Plasma Total Protein Concentrations

The total protein (TP) concentrations in the plasma were measured by the Biuret method. Biuret stock reagent was prepared by dissolving 45 g Na-K tartrate, 15 g $CuSO_4.5H_2O$, 5 g KI in 1 L of 0.2 N NaOH, and diluted five-fold (working solution) with 0.2 N NaOH immediately before assay. 0.1 ml of plasma or bovine serum albumin (BSA) standard was mixed with 1.9 ml of sulphate-sulphite solution (208 g Na_2SO_4, 70 g Na_2SO_3, 2 ml concentrated H_2SO_4 in 1L distilled water), then 5 ml of Biuret working reagent was added and mixed thoroughly. After incubation at 37 ^oC for 10 min, the absorbance was read at 555 nm spectrophotometrically. The sample TP concentrations were estimated from the standard curve, and expressed as g/100 ml plasma (Watabe, 2002).

2.6.4. Determination of Plasma Malondialdehyde

The plasma level of malondialdehyde (MDA) was measured using the method of Yagi (1976). 25 μl of plasma was added to 4.0 ml of N/12 sulphuric acid and mixed thoroughly. To this solution, 0.5 ml of 10% phosphotungstic acid was added to precipitate the lipids in the plasma. After standing at room temperature for 5 min, the mixture was centrifuged at 4,000 rpm for 10 min, and the supernatant was discarded. The sediment was suspended in 4.0 ml of distilled water and 1.0 ml of thiobarbituric acid (TBA) reagent [0.67 % TBA aqueous solution + glacial acetic acid, 1:1, v/v], and then heated at 95 ^oC for 60 min. After cooling with tap water, 5.0 ml of 1-butanol was added. The mixture was vortexed vigorously and centrifuged at 4,000 rpm for 15 min.

The organic layer was taken for fluorometric measurement at 515 nm excitation and 553 nm emission. 1,1,3,3-tetraethoxypropane (TEP) was used as a standard. The results were expressed as nmol MDA/ml blood.

2.6.5. Estimation of Tissue Oedema

About 0.2 g of fresh tissues from the liver, kidney, lung and burned skin were weighed (wet weight), wrapped in aluminum foil, and placed in an electric oven at 75 ^{o}C for drying for exact 72 hours. Afterwards, all tissues were re-weighed (dry weight), and the wet/dry weight (W/D) ratio was calculated to estimate the tissue oedema (Daizoh et al., 1994).

2.7. Effects of Melatonin on Burn-induced Oxidative Stress and Multiple Organ Dysfunction (Experiment 2)

2.7.1. Experimental Protocol

This study aimed to investigate the effects of single or repeated injections of melatonin on oxidative stress and multiple organ dysfunctions (MOD) in a 30% major burn animal model. The possible involvement of TNF-α and nitric oxide (NO) were also included. The animals were subjected to three kinds of groups: (1) sham control (n=9); (2) burn controls (Burn-6h, Burn-24h and Burn-72h, n=10/group); (3) melatonin-treated groups (Burn-6h + Mel; Burn-24h + Mel, Burn-72h + Mel, n=10/group).

Melatonin was given i.p. immediately following burn in all melatonin-treated animals at a dose of 10 mg/kg b.w. and a volume of 2 ml/kg b.w. each time. This treatment was repeated every 12 hours. At the time of sacrifice (6 h, 24 h and 72 h

postburn), the animals received one to six doses of melatonin respectively. The animals in burn controls were given vehicle (2 ml/kg b.w., i.p.) in the same way as melatonin treatment.

For each animal, both blood sample from the heart and tissue samples of liver, kidney, lung and heart were collected for different assays. About 2 ml of blood was centrifuged immediately at 4,500 rpm for 10 min. The serum obtained was divided into two parts, one part was analyzed within 24 h to estimate the organ dysfunctions, the other part was stored at –70 °C for determining the serum TNF-α and nitrite levels. Organ samples were dissected rapidly after the heart perfusion with ice-cold 0.9% saline (about 150 ml for 5 min), then rinsed in ice-cold normal saline, snap-frozen in liquid nitrogen and finally stored at –70 °C for later use. The experimental design is summarized in Figure 2.1.

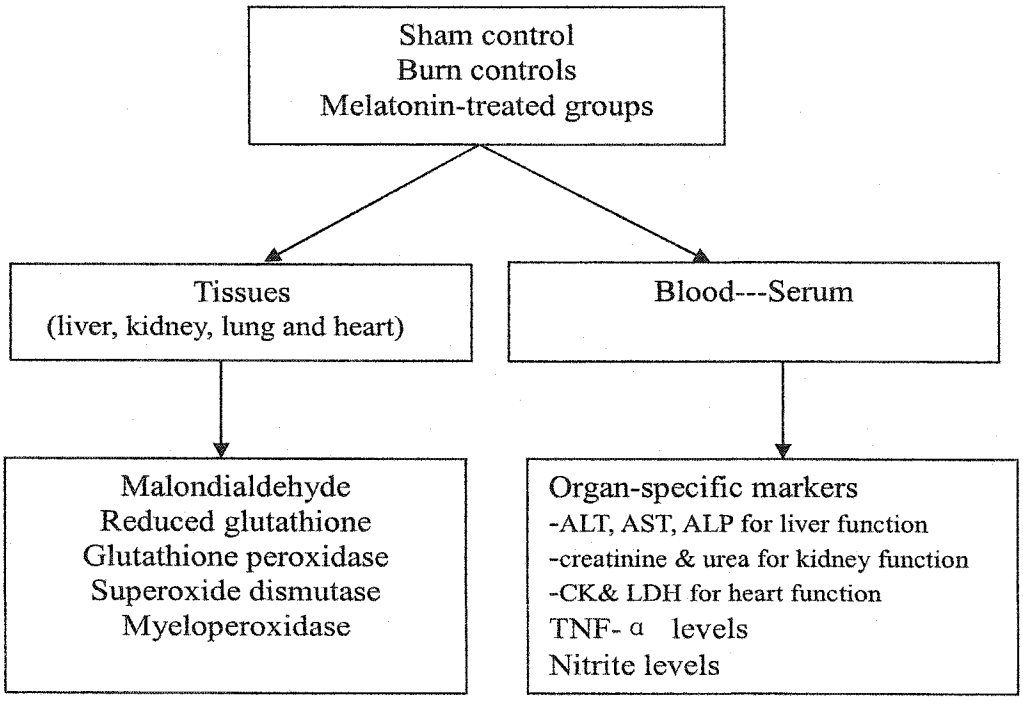


Figure 2.1 Summary for the design of Experiment 2.

2.7.2. Lowry Protein Assay

All samples were diluted fifty-fold in 50 mM phosphate buffer for protein assay as described by Lowry (1951), using BSA (0-400 μg/ml) as a standard. Briefly, alkaline copper reagent was freshly prepared by mixing 1 % $CuSO_4.5H_2O$, 2 % Na-K tartrate and 2 % Na_2CO_3 in 0.1 M NaOH at a ratio of 1:1:100. To each tube containing 100 μl of blank, standards and samples, 1 ml of alkaline copper reagent was added, and then 100 μl of Folin-Ciocalteu's reagent (diluted 1:1 immediately before use) was added and mixed vigorously. After incubation at room temperature for 15-20 min, the absorbance was measured at 650 nm spectrophotometrically. The protein concentration in samples was estimated from the standard curve.

2.7.3. Assays of Oxidative and Antioxidative Systems

2.7.3.1. Sample Preparation

About 0.1-0.2 g of tissues from lung, liver, kidney and heart were taken for different assays. Sample preparation was always carried out at – 4 °C, and the final samples were stored at –70 °C.

For asssays of MDA, GSH-Px and SOD, about 0.2 g tissue was homogenized in 10 volumes of ice-cold 50 mM potassium phosphate buffer (pH 7.4) using a homogenizer (30 s burst on setting 6). The homogenate was centrifuged at 3,000 rpm for 30 min. The resulting supernatant was collected and separated into two roughly equal aliquots: one part was stored for MDA and SOD assay, the other was centrifuged again at 13,200 rpm for 30 min. The final supernatant was stored for subsequent assay of GSH-Px activity (Hara et al., 1997).

For assays of GSH and GSSG, about 0.1 g tissue was homogenized in 5 volumes of ice-cold 50 mM phosphate buffer containing 1 % picric acid. The homogenate was centrifuged at 13,200 rpm for 30 min. The supernatant was used to measure total glutathione and GSSG with the difference calculated for the GSH content (Hara et al., 1997).

For MPO measurement, about 0.1 g tissue was homogenized in 20 volumes of 50 mM potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 13,000 rpm for 20 min. The pellet containing more than 90% of total MPO activity was collected. This washing step was repeated once for lung, twice for liver and kidney to remove any inhibiting substances in tissues. The final pellet was homogenized again in 10 volumes of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl-ammonium bromide (HETAB) and 10 mM EDTA. This HETAB-containing homogenate was subjected to one cycle of freezing / thawing and 60 seconds of sonication. After centrifugation at 13,000 rpm for 20 min, the final supernatant was stored for later use (Grisham et al., 1990).

2.7.3.2. Malondialdehyde (MDA) Levels

The tissue levels of MDA (a marker of lipid peroxidation) were measured according to the modified method of Ohkawa et al. (1979). The reaction mixture contained 0.1 ml of sample, 0.1 ml of 8.1% sodium dodecylsulfate (SDS), 0.75 ml of 20% acetic acid (pH 3.5) and 0.75 ml of 0.8 % TBA. The mixture was finally made up to 2.0 ml with distilled water and boiled at 100 °C for 60 min. After cooling with tap water, the reactants were supplemented with 0.5 ml of distilled water and 2.5 ml of 1-butanol and pyridine (15:1,v/v), shaken vigorously for 1 min, and centrifuged at 4,500 rpm for 10

min. Absorbance of the organic layer was measured at 532 nm spectrophotometrically. TEP was used as a standard. The results were expressed as nmol MDA/mg protein.

2.7.3.3. Reduced Glutathione (GSH) Levels

The tissue GSH levels were estimated by the difference between the total glutathione and GSSG.. Total glutathione was assayed as described by Griffth (1980). Three working solutions were prepared in stock buffer (125 mM sodium phosphate, 6.3 mM sodium-EDTA, pH 7.5) as follows:

Solution I: 0.3 mM NADPH

Solution II: 6.0 mM DTNB

Solution III: 50 units / ml glutathione reductase

For assay of total glutathione, 700 μl of solution I, 100 μl of solution II, 175 μl of stock buffer and 25 μl of samples or water were added to a cuvette with 1 cm light path. The reduction of 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoate (TNB) was monitored at 412 nm for 5 min. The standard curve was plotted in GSH equivalents (1, 2, 3, 4 nmol) against the rate of absorbance change (ΔA/min) at 412 nm. The results were expressed as nmol GSH /mg protein.

For GSSG measurement, the GSH present in the samples was readily derivatized by adding 2 μl of neat 2-vinylpyridine per 100 μl of solutions (samples and standards). After mixing vigorously for 1 min, the mixture was incubated at room temperature for 60 min. Aliquots of derivatized solution were assayed directly for GSSG as described for the total glutathione measurement.

2.7.3.4. Glutathione Peroxidase (GSH-Px) Activity

GSH-Px activity was determined according to the method of Flohe and Gunzler (1984). At the beginning of the experiment, the following solutions were combined and warmed to 25 °C:

500 μl phosphate buffer with 1 mM EDTA and 1 mM sodium azide (pH 7.0)

100 μl 2.4 U / ml glutathione reductase

100 μl 10 mM GSH

100 μl 1.5 mM NADPH in 0.1 % $NaHCO_3$

100 μl of sample or blank (phosphate buffer) was added to 0.8 ml of the above mixture and incubated at 25 °C for 5 min before initiating the reaction with 100 μl of 1.5 mM H_2O_2. The decrease in absorbance was monitored at 340 nm for 5 min. The net reaction rate for the sample was obtained by subtracting the rate for the blank sample and converted to NADPH oxidized using an extinction coefficient of 6220 /M/cm. The final GSH-Px activity was expressed as nmol NADPH oxidized /min/mg protein.

2.7.3.5. Superoxide dismutase (SOD) activity

SOD was assayed using the method of Flohe and Otting (1984). Two solutions were prepared in the assay:

Solution A: 0.76 mg xanthine in 10 ml of 0.001 M NaOH

24.8 mg cytochrome C in 100 ml of 50 mM phosphate buffer (pH 7.8)

Mix these two solutions before assay

Solution B: about 0.2 U/ml xanthine oxidase in 50 mM phosphate buffer (pH 7.8)

2.9 ml of solution A was placed into a 3 ml cuvette, then 50 μl of sample or SOD standard was added and warmed to 25 °C. The reaction was started by adding 50 μl of

solution B. The change in absorbance was monitored at 550 nm for 5 min. The standard curve was plotted as the reciprocal absorbance change per min (1/ ΔA min^{-1}) versus concentration of SOD standards. The SOD activity was expressed as U/mg protein.

2.7.3.6. Myeloperoxidase (MPO) Activity

MPO activity, a marker of neutrophil infiltration, was assayed as described by Grisham (1990). Briefly, 300 μl of MPO sample was added to 3 ml of reaction mixture (0.5 mM o-dianisidine in 50 mM phosphate buffer, pH 6.0). After starting the reaction by pipetting 300 μl of 0.006% H_2O_2, the change of absorbance at 460 nm was recorded for 5 min at 1 min interval. MPO activity was expressed as U/g tissue, using 1.13×10^4 as extinction coefficient for oxidized o-dianisidine.

2.7.4. Estimation of Liver, Kidney and Heart Dysfunctions

Liver dysfunction was assessed by measuring the rise in serum levels of alanine aminotransferase (ALT, a specific marker for hepatic injury), aspartate aminotransferase (AST, a nonspecific marker for hepatic injury), alkaline phosphatase (ALP, a marker of hepatobiliary damage).

Renal dysfunction was assessed by measuring the rises in serum levels of creatinine (an indicator of reduced glomerular filtration rate) and urea (an indicator of impaired excretory functions of the kidney and /or increased catabolism).

Integrity of cardiac cells was monitored by measuring serum creatine kinase (CK) and lactate dehydrogenase (LDH, also a nonspecific marker for hepatic injury).

About 2 ml of blood was collected from the heart and centrifuged immediately at

4,500 rpm for 10 min to separate serum. The serum samples were analyzed within 24 h using an automated clinical analyzer (Cobas Fara II). All the reagents used were BioSystems (U.S.A.) products (ALT: COD11533; AST: COD11531; ALP: COD11518; creatinine: COD11502; urea: COD11516; CK: COD11524; LDH: COD11580).

2.7.5. Determination of Serum TNF-α Levels

The content of serum TNF-α was determined by rat TNF-α (raTNF-α) Enzyme-Linked Immunosorbent Assay (ELISA) kit (Biosource, USA). An antibody specific for raTNF-α has been coated onto the wells of microtiter strips provided. When performing the assay, standards, controls and samples were pipetted into these wells, followed by the addition of biotinylated second antibody. During the first incubation, the raTNF-α antigen binds simultaneously to the immobilized antibody on one side, and to the biotinylated second antibody on a second site. After removing of excess second antibody, streptavidin-peroxidase (enzyme) was added. This enzyme was bound to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all unbound enzymes, a stabilized chromogen was added, which was acted by the bound enzyme to produce yellow color. The intensity of this colored product is directly proportional to the concentration of raTNF-α present in the original specimen. The absorbance of each well was measured at 450 nm using a microplate reader. The assay procedure was summarized in Figure 2.2.

The raTNF-α ELISA kit is very sensitive and specific. The minimum detectable dose of raTNF-α is < 4 pg/ml, and the cross-reactivity was only observed with mouse TNF-α (100%) and human TNF-α (0.15%).

To each well pre-coated with raTNF-α antibody

Add 50 μl of standards, controls & samples

Add 50 μl of biotinylated second antibody

Incubate for 90 min at RT

↓ aspirate and wash 4 times

Incubate 100 μl of streptavidin-HRP for 45 min at RT

↓ aspirate and wash 4 times

Incubate 100 μl of stabilized chromogen for 30 min at RT

↓

Add 100μl of stop solution and read at 450 nm

Figure 2.2 Summary for rat TNF-α ELISA assay

2.7.6. Determination of Serum Nitrite Concentration

The amount of nitrite in the serum was measured using Griess reagent (Crespo et al., 1999). 100 μl of samples or sodium nitrite standard (0-100 μM) was added to a 96-well microplate in duplicate, then 100 μl of Griess reagent [0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride; 1% sulphanilamide in 5% H_3PO_4; 1:1] was added to each well and mixed by shaking the plates. After 20 min of incubation at room temperature, the absorbance at 540 nm was measured using a microplate reader. Nitrite concentration in samples were calculated from the standard curve and expressed as nmol/mg protein.

2.8. Effect of Melatonin on *In Vivo* Cell-mediated Immunity (Experiment 3)

2.8.1. Experimental Protocol

This study aimed to observe the beneficial effect of melatonin on suppressed cell-mediated immunity (CMI) in 30% major burn animal model. 24 male SD rats (250-300 g) were equally assigned to three groups: (1) sham control (n=8); (2) burn control (n=8); (3) burn + mel treatment (n=8). Melatonin (10 mg/kg b.w., i.p.) was given immediately following burn, and the same injection was repeated daily at 6 p.m. (2 hours before the onset of darkness) for a total of seven days (burn day as Day 1). Animals in sham control and burn control received vehicle (10% ethanol in 0.9% saline) in the same way as melatonin treatment.

The general immune response was estimated by the changes in white blood cell (WBC) counts, and the contact hypersensitivity reaction (CHR) was used as an in vivo measurement for depressed cellular response (Cetinkale et al., 1999).

2.8.2. WBC Counting

About 0.5 ml of blood was taken by tail incision at seven time-points: 0 h (pre-burn), 6 h, 12 h, 24 h, 48 h, 72 h and 120 h postburn. 20 μl of whole blood was twenty-fold diluted in a diluting solution (2% acetic acid). The visual counting of WBC was performed with Neubauer hemacytometer, and the results were expressed in $\times 10^9$/L (Peter et al., 1999).

2.8.3. Contact Hypersensitivity Reaction (CHR)

CHR to 2, 4-dinitrofluorobenzene (DNFB) was initiated on Day 7 immediately following melatonin (or vehicle) treatment. All animals were sensitized by application of 100 μl of 0.5 % DNFB in mixture of acetone: olive oil (4:1, v/v) on the shaved abdomen. The sensitization of DNFB lasted for two consecutive days. Five days later,

the left ear was painted with 50 ml of 0.2 % DNFB immediately after measuring the thickness of two ears with an engineer's micrometer (accuracy: 0.01 mm). The same measurement was repeated again 24 h later. The left ear swelling was estimated by the difference in thickness (Δ thickness, mm) before and after DNFB challenging. Right ear swelling was also estimated and served as an internal control for each animal.

CHR was expressed as a percentage of ear swelling in all groups. The sham control value was always presented as 100%, and CHR in other two groups were calculated by the following formula:

Ear swelling (%) = [(Δthickness in burn or mel group)/Δthickness in sham group] × 100

2.9. Data Analysis

All data were expressed as mean ± SEM. One-way analysis of variance (ANOVA), followed by Newman-Keuls multiple range test, was used to compare means between groups. $P<0.05$ was considered as statistically significant.

Table 2.1 Chemicals and reagents used in experiments

Chemicals	**Sources**
1,1,3,3-tetraethoxypropane	Sigma, U.S.A.
1-butanol	Labscan, Ireland
2,4-dinitrofluorobenzene	Sigma, U.S.A.
2-thiobarbituric acid	Sigma, U.S.A.
2-vinylpyridine	Fluka, Switzerland
5,5'-dithio-bis(2-nitrobenzoic acid)	Sigma, U.S.A.
bovine serum albumin	Sigma, U.S.A.
cytochrome C	Sigma, U.S.A.
eosin	Sigma, U.S.A.
ethylenediamine-tetraacetic acid disodium salt	Sigma, U.S.A.
Folin & Ciocalteu's phenol reagent	BDH, U.K.
formaldehyde	Fluka, Switzerland
glutathione reductase	Fluka, Switzerland
hematoxylin	Sigma, U.S.A.
hexadecyltrimethyl-ammonium bromide	Sigma, U.S.A.
hydrogen peroxide	Sigma, U.S.A.
melatonin	Sigma, U.S.A.
N- (1-Naphthyl) ethylenediamine dihydrochloride	Sigma, U.S.A.
N, N-dimethyl-formamide	Sigma, U.S.A.
o-dianisidine	Sigma, U.S.A.
olive oil	Sigma, U.S.A.
oxidized glutathione	Sigma, U.S.A.

Table 2.1 Chemicals and reagents used in the experiments (continued)

Chemicals	**Sources**
oxidized glutathione	Sigma, U.S.A.
pentobarbital sodium salt	Sigma, U.S.A.
phosphotungstic acid	Sigma, U.S.A.
picric acid	Sigma, U.S.A.
pyridine	BDH, U.K.
rat TNF-α ELISA kit	BioSource, U.S.A.
reagents for Cobas Fara II clinical analyzer	BioSystem, Spain
reduced gluthathione	Fluka, Switzerland
reduced nicotinamide adenine dinucleotide phosphate	Sigma, U.S.A.
sodium azide	BDH, U.K.
sodium dodecyl sulphate	BDH, U.K.
sodium sulphate	Sigma, U.S.A.
sodium sulphite	Sigma, U.S.A.
sulphanilamide	Sigma, U.S.A.
superoxide dismutase	Sigma, U.S.A.
xanthine	Sigma, U.S.A.
xanthine oxidase	Sigma, U.S.A.

Table 2.2 Equipment and other materials used in experiments

Equipment and materials	**Sources**
Cobas Fara II analyzer	Roche Instruments
electronic oven	Memmer, Germany
high speed centrifuge (J2-M1)	Beckman Ltd.
homogenizer (Ultra Turrax T25)	IKA-Labortechnik
HP845 UV-Visible system	Tegent Technology Ltd.
Julabo water bath	Julabo Labortechnic
Leica micrometer	Sybron
low speed centrifuge (Jouan MR22)	Line Analytics Ltd.
microhematocrit centrifuge and reader	Hawksley
microplate reader (Tecan)	Tecan Austria GmbH
microscope (CH-2)	Olympus
pH meter (model 420A, digital)	Orion Research
spectrophotometer (Genesys 2)	Milton Roy
tissue processor (Hypercenter XP)	Shandon
transsonic T460	Elma Instruments
water bath (JB4)	Grant Instruments (Cambridge) Ltd

Chapter 3

RESULTS

3.1. Confirmation of Burn Depth

The depth of burn wound was estimated by the combination of wound appearance and histological examination (H-E staining). At 6 h postburn, burn eschar was evident with a charred, leathery appearance and significant tissue oedema. Thrombosed blood vessels can be seen on the inside of the burned skin. Under the microscope, normal rat skin showed a clear structure of epidermis and dermis, which was characterized by large quantities of hair follicle and no sweat glands. However, the integrity and architecture of the normal skin were destroyed following burn. Pronounced disruption was found in the epidermal layer, while the dermis lost its normal architecture and was stained in pink colour due to heat-induced tissue necrosis (Figure 3.1).

3.2. Estimation of Biochemical Parameters in Plasma and Tissue Oedema

3.2.1. Determination of Hematocrit, Plasma TP Concentrations and MDA Levels

Table 3.1 lists the determination of hematocrit, TP concentrations and MDA levels in the plasma. The hematocrit markedly increased in burn group (54.4 ± 1.2 %, $P < 0.01$) and burn + mel group (52.3 ± 1.8 %, $P < 0.01$) at 6 h post burn when compared with the basal level in the sham control (46.3 ± 1.1 %). No significant difference was observed between these two groups (burn group and burn + mel group).

The plasma TP concentration following burn was significantly lower than that of sham control (5.19 ± 0.22 *vs* 6.01 ± 0.16 g/100ml plasma, $P<0.05$). Melatonin treatment (10 mg/kg, i.p. immediately postburn) did not prevent the loss of plasma TP (5.56 ± 0.28 g/100 ml plasma, $P>0.05$, compared with burn control).

The MDA level in the plasma showed a significant rise at 6 h postburn from the level of sham control (6.97 ± 0.45 nmol/ml) to 9.28 ± 0.42 nmol/ml ($P<0.01$). This rise was partially reduced by melatonin treatment (7.75 ± 0.41 nmol/ml, $P<0.05$, compared with burn control).

3.2.2. Effect of Melatonin on Tissue Oedema

The Wet / Dry (W/D) weight ratio represents water contents of different tissues and was used to estimate the tissue oedema in this study. The W/D ratios in the liver and kidney were not different between each group (sham control, burn control and burn + mel group), while the W/D values following burn were elevated markedly in the lung (4.42 ± 0.16 *vs* 3.74 ± 0.28 of sham control, $P<0.05$) and in the burned skin (3.39 ± 0.09 *vs* 2.64 ± 0.16 of sham control, $P<0.001$). The increased water contents were reduced by melatonin treatment at these two sites (lung: 3.90 ± 0.07, $P<0.05$; burned skin: 2.98 ± 0.06, $P<0.05$; compared with the respective burn controls) (Figure 3.2).

3.3. Oxidative Stress in Organs and Protective Effects of Melatonin

The oxidative stress in remote organs (liver, kidney, lung and heart) and the protective effect of melatonin were estimated within 72 h postburn by measuring the tissue levels of MDA and GSH. The protective effect of melatonin (single dose) was

found only at 6 h postburn in this study, thus further studies on enzyme activities were carried out at the same time-point.

3.3.1. Malondialdehyde Levels

Malondialdehyde (MDA) is an indicator of lipid peroxidation, and the tissue levels of MDA were tested up to 72 h following burn. The liver MDA in the sham control was 1.04 ± 0.14 nmol/mg protein, and 30% burn did not induce any significant increase in the MDA levels. The liver MDA levels were also not affected by single or repeated melatonin treatment (Figure 3.3).

The MDA levels of kidney were increased significantly in three burn controls (Burn-6 h: 4.54 ± 0.23 nmol/mg protein, $P<0.001$; Burn-24 h: 3.68 ± 0.21 nmol/mg protein, $P<0.001$; Burn-72 h: 2.94 ± 0.11 nmol/mg protein, $P<0.05$), compared with the sham control (2.24 ± 0.17 nmol/mg protein). The maximal elevation (about 2-fold) was found at 6 h postburn, then the MDA levels dropped with time. Single dose of melatonin (10 mg/kg, i.p.) reduced the increased MDA by 27.8 % (3.28 ± 0.09 *vs* 4.54 ± 0.23 nmol/mg protein, $P<0.001$), while repeated administration (10 mg/kg, i.p. two and six doses for 24 h and 72 h respectively) had no effect (Figure 3.4).

The lung MDA levels were also found to be much higher both at 6 h postburn (2.02 ± 0.17 nmol/mg protein, $P<0.01$) and 24 h postburn (1.93 ± 0.10 nmol/mg protein, $P< 0.05$) than the basal level (1.39 ± 0.12 nmol/mg protein). After a single injection of melatonin, the lung MDA level was reduced by 45.5 % (1.11 ± 0.09 nmol/mg protein, $P <0.001$, compared with burn-6h control), and was restored completely to the basal level. However, repeated melatonin treatment failed to exert the same effect (Figure 3.5).

The rise of heart MDA level was found only at 6 h postburn (1.55 ± 0.17 *vs* 0.93 ± 0.05 nmol/mg protein of sham control, $P<0.05$). Melatonin treatment (single dose) reversed completely the rise in the heart MDA level (0.89 ± 0.08 nmol/mg protein, $P< 0.01$ *vs* burn-6 h control) (Figure 3.6).

3.3.2. Reduced Glutathione Levels

Reduced glutathione (GSH) was one of the most important endogenous antioxidants, and the tissue levels were measured within 72h postburn as another indicator for burn-induced oxidative stress. The liver GSH levels showed a significant decrease at 6 h and 24 h postburn (Burn-6 h: 8.5 ± 0.6 μmol/mg protein, $P<0.001$; Burn-24 h: 10.3 ± 0.8 μmol/mg protein, $P<0.05$), compared with the sham control (14.7 ± 0.8 μmol/mg protein). However, this decrease was not found at 72 h postburn. Single dose of melatonin reversed the decrease in the liver GSH level (12.6 ± 0.5 μmol/mg protein, $P<0.05$ *vs* burn-6 h control), while repeated administrations of melatonin had no effect (Figure 3.7).

The GSH level of kidney was much lower than that of sham control (15.1 ± 0.7 μmol/mg protein) at all postburn time-points, especially at 6 h postburn (Burn-6 h: 8.1 ± 0.4 μmol/mg protein $P<0.001$; Burn-24 h: 10.3 ± 0.8 μmol/mg protein, $P<0.01$; Burn-72 h: 13.7 ± 0.9 μmol/mg protein, $P<0.05$). The decrease at 6 h postburn was partially improved by the treatment of melatonin (11.5 ± 0.3 μmol/mg protein, $P<0.05$), but was not completely restored ($P<0.05$ *vs* sham control) (Figure 3.8).

The lung GSH level was also found to decrease significantly within 24 h postburn (Burn-6 h: 7.4 ± 0.3 μmol/mg protein, $P<0.01$; Burn-24 h: 8.4 ± 0.8 μmol/mg protein, P

<0.05) before it returned to the basal level (12.7 ± 0.6 μmol/mg) at 72 h postburn. Melatonin therapy increased the lung GSH level significantly at 6 h postuburn (11.1 ± 0.5 μmol/mg protein, P<0.05), but was not effective at 24 h and 72 h postburn (Figure 3.9).

The heart GSH level was observed to be lower only at 6 h postburn (13.6 ± 0.3, μmol/mg protein, P<0.01) than the sham control (18.8 ± 0.6 μmol/mg protein), and no significant differences were found at the subsequent postburn time-points. Single dose of melatonin reversed the decreased GSH level at 6 h postburn (17.2 ± 0.5 μmol/mg protein, P<0.05, compared with burn-6 h control) close to the level found in the sham control (Figure 3.10).

3.3.3. Glutathione Peroxidase Activity

Glutathione Peroxidase (GSH-Px) is an enzyme, which can remove H_2O_2 and organic peroxides effectively, thus preventing the subsequent formation of hydroxyl radicals and oxidative damages. Figure 3.11 illustrates the alterations in the organ GSH-Px activities at 6 h postburn.

The liver was found to have the highest basal level of GSH-Px (258.2 ± 20.3 nmol NDAPH oxidized/min/mg protein) compared with other three organs (kidney, lung and heart). Six hours following burn, the liver GSH-Px activity was markedly decreased by 23.6 % to 197.7 ± 10.2 nmol NDAPH oxidized/min/mg protein (P<0.05). Melatonin treatment counteracted the loss of GSH-Px activity (318.6 ± 17.5 nmol NDAPH oxidized/min/mg protein, P<0.001), even enhanced it (23.4 %) above the basal level (P <0.05).

Burn injury also resulted in decreased GSH-Px activities in the kidney, lung and heart. The decrease was 25.7 % in the kidney (71.8 ± 3.0 *vs* 96.7 ± 8.6 nmol NDAPH oxidized/min/mg protein of sham control, P<0.01), 15.1 % in the lung (62.9 ± 2.3 *vs* 74.1 ± 2.4 nmol NDAPH oxidized/min/mg protein of sham control, P<0.01) and 21.1 % in the heart (74.0 ± 3.4 *vs* 93.8 ± 3.8 nmol NDAPH oxidized/min/mg protein of sham control, P<0.05). Different from the liver, melatonin therapy did not stimulate the GSH-Px activities in these three organs.

3.3.4. Superoxide Dismutase Activity

Superoxide dismutase (SOD) can catalyze the dismutation of $O_2^{\bullet -}$ to H_2O_2, and is considered as an important enzyme in the body's antioxidant defence. Figure 3.12 demonstrates the changes of SOD activity at 6 h postburn.

The liver SOD showed a trend to increase following burn (84.8 ± 6.0 *vs* 73.9 ± 4.9 IU/mg protein of sham control, P>0.05), while melatonin treatment caused a further increase (59.2%) in the SOD activity (135.0 ± 6.6 IU/mg protein *vs* burn control, P<0.001).

The SOD activities following burn was elevated significantly in the kidney (71.9 ± 3.1 *vs* 58.0 ± 3.5 IU/mg protein of sham control, P<0.05) and in the lung (82.0 ± 5.7 *vs* 63.3 ± 4.3 IU/mg protein of sham control, P<0.05), but not in the heart (53.2 ± 3.3 *vs* 47.1 ± 3.0 IU/mg protein, P>0.05). Melatonin treatment did not show any effect on the SOD activities in these three organs.

3.3.5. Myeloperoxidase Activity

The alterations in myeloperoxidase (MPO) activities were demonstrated in Figure 3.13. Burn injury caused significant increases in MPO activities in all tested organs. The MPO level was elevated 2.0-fold in the liver (8.0 ± 0.3 *vs* 15.7 ± 1.9 U/g tissue, $P< 0.01$), 3.9-fold in the kidney (3.2 ± 0.3 *vs* 12.6 ± 1.5 U/g tissue, $P<0.001$), 5.4-fold in the lung (18.2 ± 1.6 *vs* 99.1 ± 7.3 U/g tissue, $P<0.001$) and 2.8-fold in the heart (3.4 ± 0.3 *vs* 9.4 ± 1.1 U/g tissue, $P<0.001$).

Melatonin treatment (10 mg/kg, i.p.) was very effective in counteracting the rise of MPO in all tested organs. The MPO level was decreased by 23.6 % in the liver (12.6 ± 0.7 U/g tissue, $P<0.05$), 30.2 % in the kidney (8.8 ± 0.9 U/g tissue, $P<0.05$), 21.1 % in the lung (78.2 ±7.0 U/g tissue, $P<0.05$) and 26.6 % in the heart (6.9 ± 0.5 U/g tissue, $P< 0.05$), compared with their respective burn controls. However, the MPO levels following melatonin treatment were still higher than their respective sham controls ($P< 0.05$ for the liver, $P<0.001$ for the kidney and lung, $P<0.01$ for the heart).

3.4. Protective Effects of Melatonin on Multiple Organ Dysfunction

3.4.1. Liver Dysfunction

Liver dysfunction was assessed at three postburn time-points (6 h, 24 h, 72 h) by measuring the serum levels of ALT, AST and ALP. All serum samples were analyzed within 24 h using an automated analyzer.

Serum ALT level was elevated 4.6-fold at 6 h postburn (240.2 ± 22.6 IU/L *vs* 52.6 ± 3.4 IU/L of sham control, $P<0.001$), and then dropped significantly with time. The

level of ALT was still higher at 24 h postburn (144.8 ± 13.1 IU/L, P<0.001), but was not significantly different at 72 h postburn (65.7 ± 5.3 IU /L, P>0.05) from the basal level. Single dose of melatonin (10 mg/kg, i.p.) was found to reduce the ALT level by 21.0 % (189.8 ± 14.7 IU/L *vs* burn-6 h control, P<0.05), whereas repeated administrations had no effect (Figure 3.14).

Serum AST increased in a very similar way as ALT but with a higher magnitude. The level of AST was elevated 8.5-fold at 6 h postburn (835.0 ± 77.2 IU/L, P<0.001) and 5.6-fold at 24 h postburn (553.1 ± 31.4 IU/L, P<0.001) compared with the sham control (98.5 ± 9.9 IU/L). Melatonin treatment (single dose) resulted in 21.7 % decrease in the AST level (653.4 ± 64.2 IU/L *vs* burn-6 h control, P<0.05), while repeated administrations of melatonin were not effective (Figure 3.15).

In contrast, serum ALP seemed to be more resistant to burn injury and did not show any difference at all postburn time-points from the basal level (264.4 ± 19.7 IU/L). Melatonin (single or repeated injections) had no effect on the ALP levels in the serum (Figure 3.16).

3.4.2. Kidney Dysfunction

Kidney dysfunction was estimated at 6 h, 24 h and 72 h postburn by the rise in the serum levels of creatinine and urea. All samples were analyzed within 24 h after blood sample collection.

Serum creatinine was significantly increased at 6 h postburn (0.85 ± 0.07 mg /dl, P <0.001) and was still higher at 24 h postburn (0.69 ± 0.04 mg/dl, P<0.05) when

compared with the sham group (0.54 ± 0.02 mg/dl). This rise was not evident at 72 h postburn (0.59 ± 0.03 mg / dl). Single dose of melatonin (10 mg/kg, i.p.) reduced the creatinine level by 15.1 % at 6 h postburn (0.72 ± 0.03 *vs* burn-6 h control, $P<0.05$), while repeated administrations had no effect (Figure 3.17).

The rise of serum urea was also found at 6 h postburn (116.8 ± 2.9 mg/dl, $P<0.001$) and 24 h postburn (81.0 ± 6.6 mg/dl, $P<0.001$) compared with the sham group (47.2 ± 3.7 mg/dl). Serum urea showed a tendency to decrease with time, and was restored completely at 72 h postburn (47.6 ± 1.6 mg/dl). Single or repeated administrations of melatonin had no effect on the increased serum levels of urea following burn (Figure 3.18).

3.4.3. Heart Dysfuntion

The heart dysfunction was monitored by measuring the serum levels of creatine kinase (CK) and lactate dehydrogenase (LDH). The rise of serum CK was found only at 6 h postburn, (2053 ± 57 IU/L, $P<0.001$) compared with the sham group (454 ± 83 IU/L), and there was no significant difference between the sham control and burn controls at 24 h and 72 h postburn. Single dose of melatonin reduced the increased CK by 32.9 % (1377 ± 135 *vs* burn-6 h control, $P<0.01$). Repeated melatonin treatment was not as effective as single injection (Figure 3.19).

The rise of serum LDH (also a nonspecific marker for hepatic injury) lasted up to 24 h following burn. Serum LDH was elevated 7.3-fold at 6 h postburn (2503 ± 183 IU/L, $P<0.001$) and 2.0-fold at 24 h postburn (701 ± 66 IU/L, $P<0.05$) from the basal level (344 ± 39 IU/L). Similar to serum CK, LDH level was also decreased by 13.8 %

(2158 ± 165 IU/L *vs* burn-6 h control, P<0.05) after single injection of melatonin (10 mg/kg, i.p.), but was not affected by repeated administrations (Figure 3.20).

3.5. Estimation of Serum TNF-α and Nitrite Levels

3.5.1. Serum TNF-α Level

Serum levels of TNF-α in seven groups (8 samples/each group) were determined using specific rat TNF-α ELISA kits (sensitivity < 4 pg/ml). The concentration of low and high serum controls supplied with the kits were estimated from the standard curve (TNF-α standards: 0-500 pg/ml), and the values were measured as 14.2 pg/ml and 342.2 pg/ml respectively. However, the TNF-α levels in most serum samples were undetectable except two samples in Burn-6 h + Mel group. The values of these two samples were 45.7 pg/ml and 71.3 pg/ml respectively.

3.5.2. Serum Nitrite Level

The serum level of nitrite (an indicator of NO formation) at 6 h, 24 h and 72 h postburn was measured by the Griess reaction. The nitrite levels showed a trend to increase in three burn controls (Burn-6 h: 8.47 ± 1.37 μM; Burn-24 h: 10.7 ± 1.98 μM; Burn-72 h: 11.4 ± 2.98 μM) when compared with the baseline seen in the sham control (5.28 ± 1.09 μM). The nitrite levels of melatonin-treated animals were lower (Burn-6 h + Mel: 6.77 ± 1.09 μM; Burn-24 h + Mel: 7.03 ± 1.55 μM; Burn-72 h + Mel: 9.58 ± 3.20 μM) than their respective burn controls. However, these alterations did not reach any statistical significance due to large standard deviation (Figure 3.21).

3.6. Effect of Melatonin on Depressed Cell-Mediated Immunity

3.6.1. Changes of Body Weight and Mortality

The body weights (b.w.) of all animals were recorded immediately postburn or sham operation (PBD0), and then at an interval of every two or three days at 4 p.m. until seven days postburn (PBD7). The animals in the sham group showed a constant increase in b.w. at 2.6 g per day (PBD0: 277 ± 4 g; PBD7: 295 ± 4 g, $P<0.05$). In contrast, the b.w of burned animals decreased by 5.8 % compared with the baseline (PBD0: 278 ± 5 g; PBD7: 262 ± 5 g). The burn-induced loss in b.w was not alleviated by melatonin treatment (Table 3.2).

The overall mortality rate of animals receiving 30% TBSA burn was 16% (3 deaths in a total of 19 burned rats) during 12-day observation. Most deaths occurred within the first 24 hours following burn due to burn stress and /or burn shock.

3.6.2. Changes of Total White Blood Cell Counts

Total white blood cells (WBC) were counted at seven postburn time-points: 0 h (pre-burn, basal level), 6 h (PB6h), 12 h (PB12h), 24 h (PBD1), 48 h (PBD2), 72 h (PBD3) and 120 h (PBD5). In the sham group, WBC counts was found to increase slightly at 6 h following sham operation from $15.4 \pm 0.6 \times 10^9$/L to $18.8 \pm 0.4 \times 10^9$/L ($P<0.05$), and there was no difference from the baseline at the other time-points. The changes of WBC counts were more complex in burn control and burn + mel group. All animals in the burn control experienced an initial (first 12 h) rise in WBC numbers from the baseline $15.8 \pm 0.9 \times 10^9$/L to $22.0 \pm 1.1 \times 10^9$/L at PB6h ($P<0.05$) and $22.9 \pm 1.3 \times 10^9$/L at PB12h ($P<0.05$). Afterwards, the WBC counts dropped significantly at PBD1

and PBD2 to the baseline. Melatonin-treated animals also showed similar changes within 12 h postburn (PB6h: 21.1 ± 1.2×10^{9}/L, $P<0.05$; PB12h: 20.9 ± 1.2×10^{9}/L, $P<0.05$, compared with the baseline 16.3 ± 0.8×10^{9}/L). From PBD3 to PBD5, WBC counts in burn control rose again (PBD3: 24.0 ± 2.6×10^{9}/L, $P<0.05$; PBD5: 24.9 ± 1.9 $\times10^{9}$/L, $P<0.05$), but this rise was not significant in burn + mel group (PBD3: 18.1 ± 1.7×10^{9}/L; PBD5: 16.2 ± 0.5×10^{9}/L) (Figure 3.22).

3.6.3. Effect of Melatonin on Contact Hypersensitivity Reaction

The ear swelling in contact hypersensitivity reaction (CHR) was found to be a highly sensitive *in vivo* method to evaluate cell-mediated immunity in burn injury. The percentage change of ear swelling in the sham group was always presented as 100%. 30% major burn induced significant inhibition of ear swelling from 100 % ± 7.6 in the sham control to 41.2 ± 8.2 % in the burn control ($P<0.001$), suggesting severely suppressed cellular immune response. Melatonin treatment (10 mg/kg, daily for 7 days) resulted in a significant improvement of ear swelling (73.0 ± 10.1 % *vs* 41.2 ± 8.2 %, $P<0.01$). However, it did not restore CHR completely when compared with that of the sham control ($P< 0.01$) (Figure 3.23).

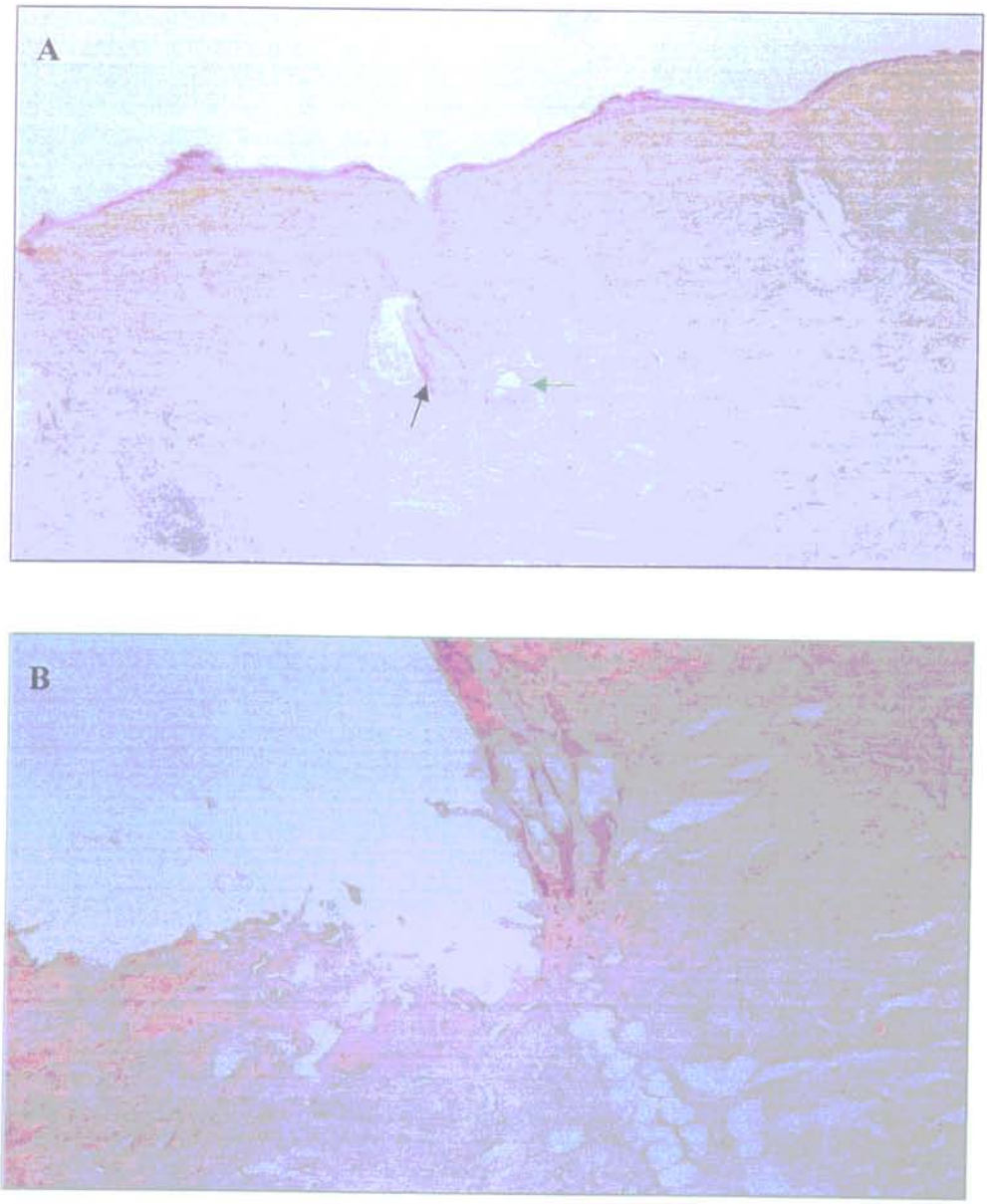


Figure 3.1 Histological examination of normal (A) and burned (B) skin with hematoxylin-eosin staining.

A: Normal skin shows continuous epidermis layer and clear structures of hair follicle (black arrow) and sebaceous gland (green arrow). B: Pronounced disruption is found in the epidermal layer of the burned skin, and the dermis loses its normal architecture and is stained in pink colour. Magnification: ×10 for both A and B.

Table 3.1 Determinations of hematocrit, plasma total protein concentrations and malondialdehyde levels at 6 h postburn.

Groups	No. of animal	Hematocrit (%)	Plasma TP (g /100 ml)	Plasma MDA (nmol/ml)
Sham	9	46.3 ± 1.1	6.01 ± 0.16	6.97 ± 0.45
Burn	10	54.4 ± 1.2**	5.19 ± 0.22*	9.28 ± 0.42**
Burn + Mel	11	52.3 ± 1.8**	5.56 ± 0.28	7.75 ± 0.41+

Data are mean ± SEM. TP = Total Protein; MDA = Malondialdehyde;

Mel = Melatonin (10 mg/kg, given i.p. immediately postburn).

* $P<0.05$, ** $P<0.01$ *vs* sham control; + $P<0.05$ *vs* burn control.

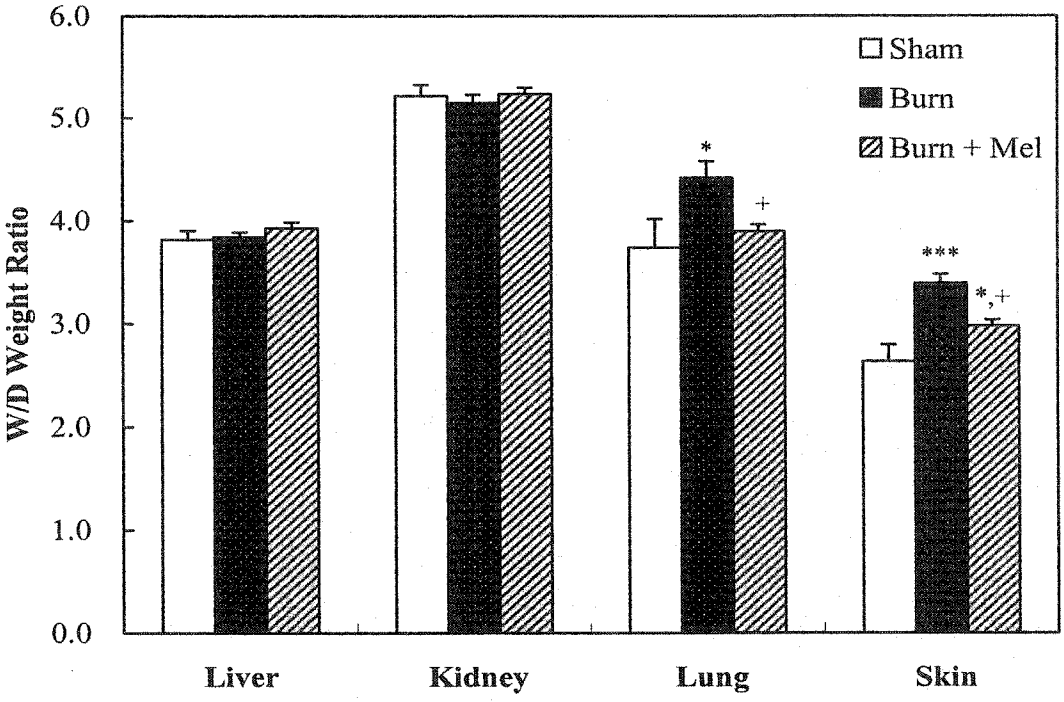


Figure 3.2 Effect of melatonin on wet /dry (W/D) weight ratios of different tissues at 6 h postburn.

Sham control: n=9; Burn control: n=10; Burn + Mel group: n=11.

Melatonin (10 mg/kg) was administered i.p. immediately postburn.

* P<0.05, *** P<0.001 *vs* sham control; $^{+}$ P<0.05 *vs* burn control.

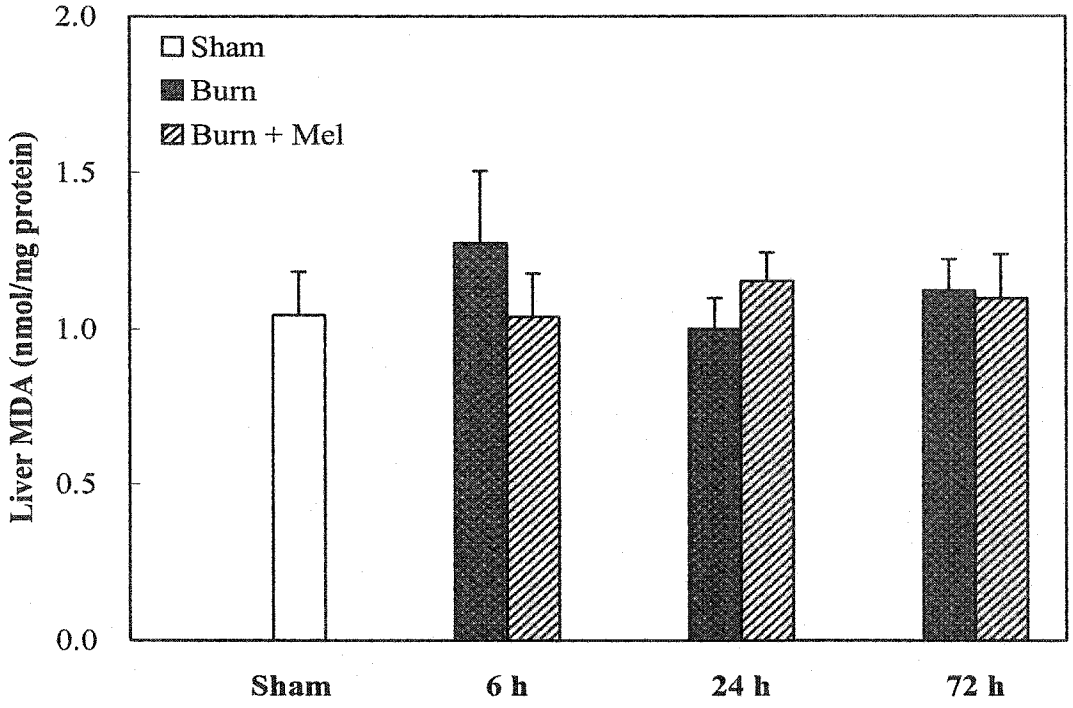


Figure 3.3 Liver levels of malondialdehyde (MDA) at 6 h, 24 h and 72 h after burn and burn plus melatonin treatment.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately after burn, and the same dose was given every 12 hours up to 72 h postburn. P>0.05, burn controls or burn plus melatonin groups *vs* sham control.

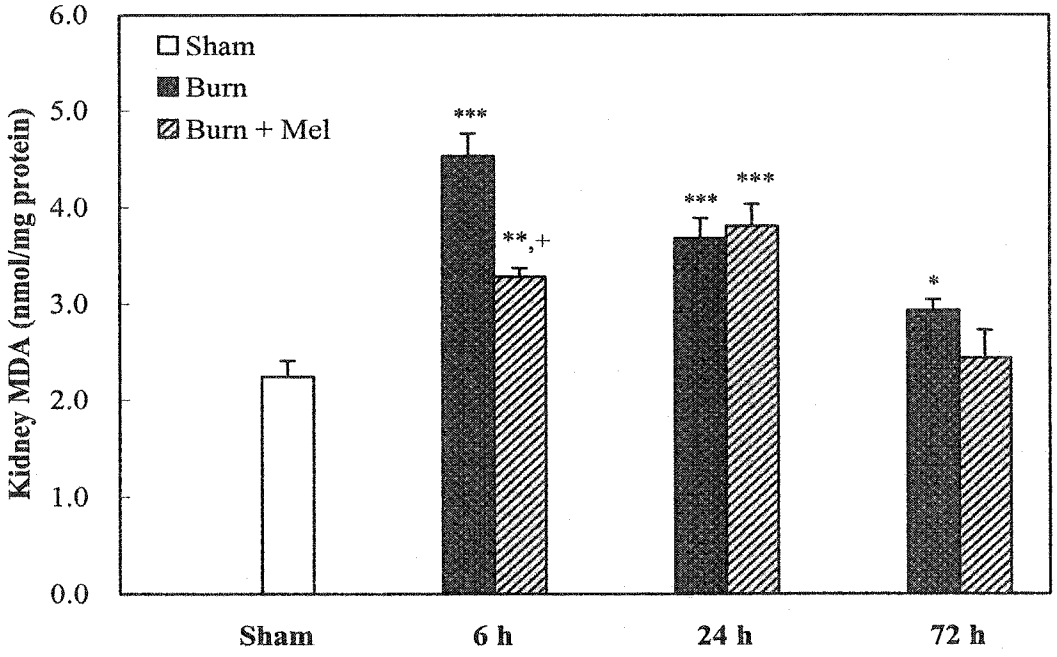


Figure 3.4 Kidney levels of malondialdehyde (MDA) at 6 h, 24 h and 72 h after burn and burn plus melatonin treatment.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately after burn, and the same dose was given every 12 hours up to 72 h postburn.

* P<0.05, ** P<0.01, *** P<0.001 *vs* sham control; $^{+}$ P<0.001 *vs* burn-6 h control.

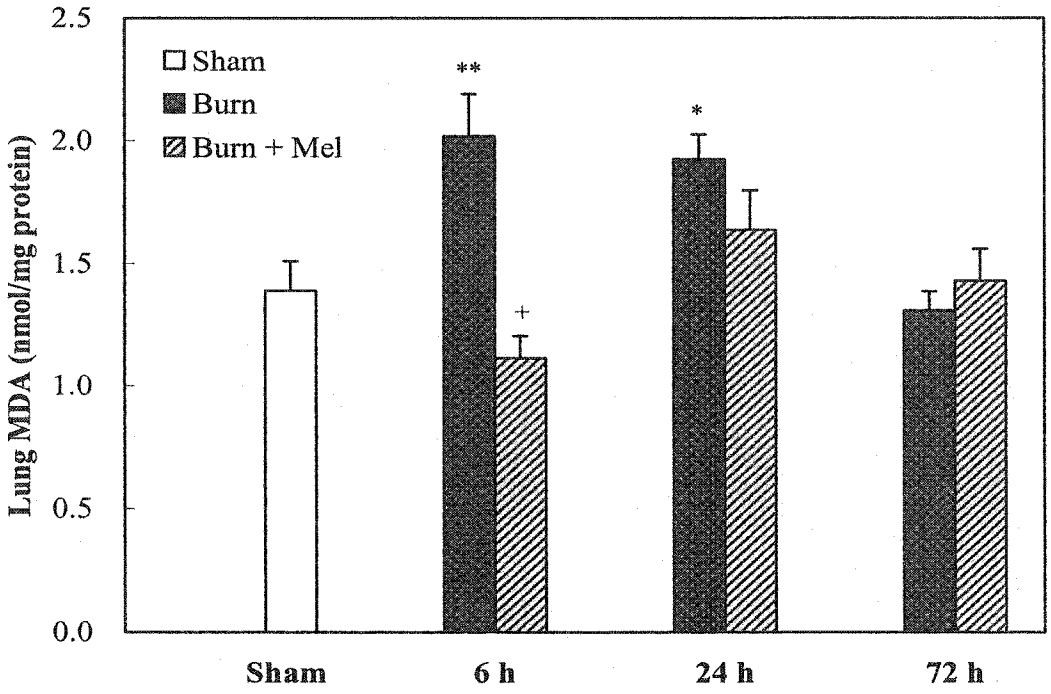


Figure 3.5 Lung levels of malondialdehyde (MDA) at 6 h, 24 h and 72 h after burn and burn plus melatonin treatment.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately after burn, and the same dose was given every 12 hours up to 72 h postburn.

* $P<0.05$, ** $P<0.01$ *vs* sham control; $^{+}$ $P<0.001$ *vs* burn-6 h control.

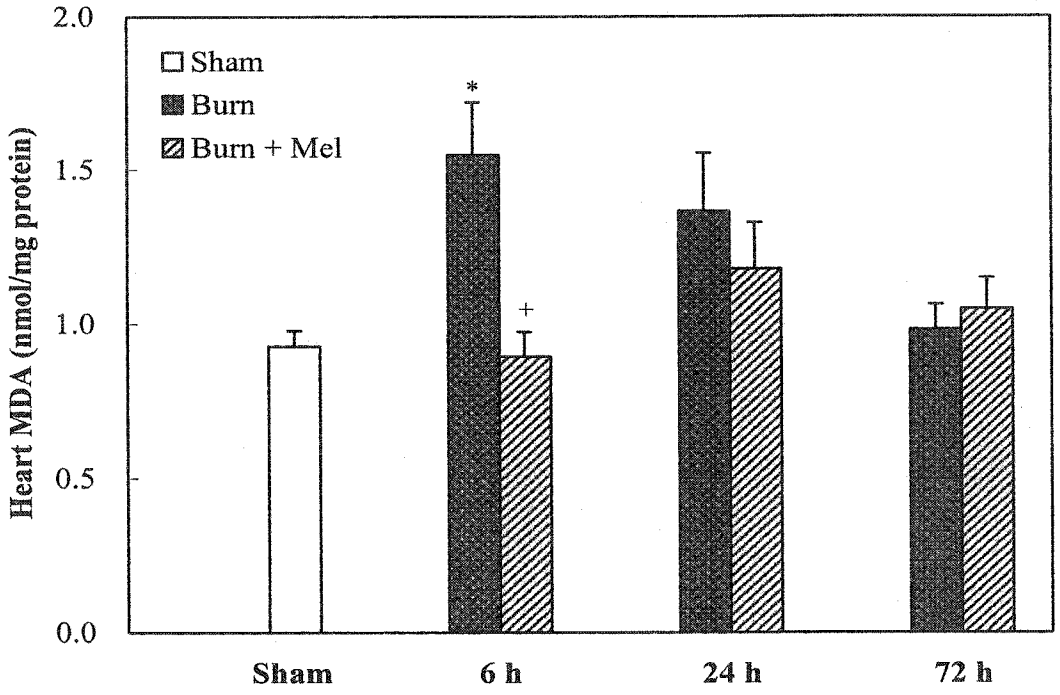


Figure 3.6 Heart levels of malondialdehyde (MDA) at 6 h, 24 h and 72 h after burn and burn plus melatonin treatment.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately after burn, and the same dose was given every 12 hours up to 72 h postburn.

* $P<0.05$ *vs* sham control; + $P<0.01$ *vs* burn-6 h control.

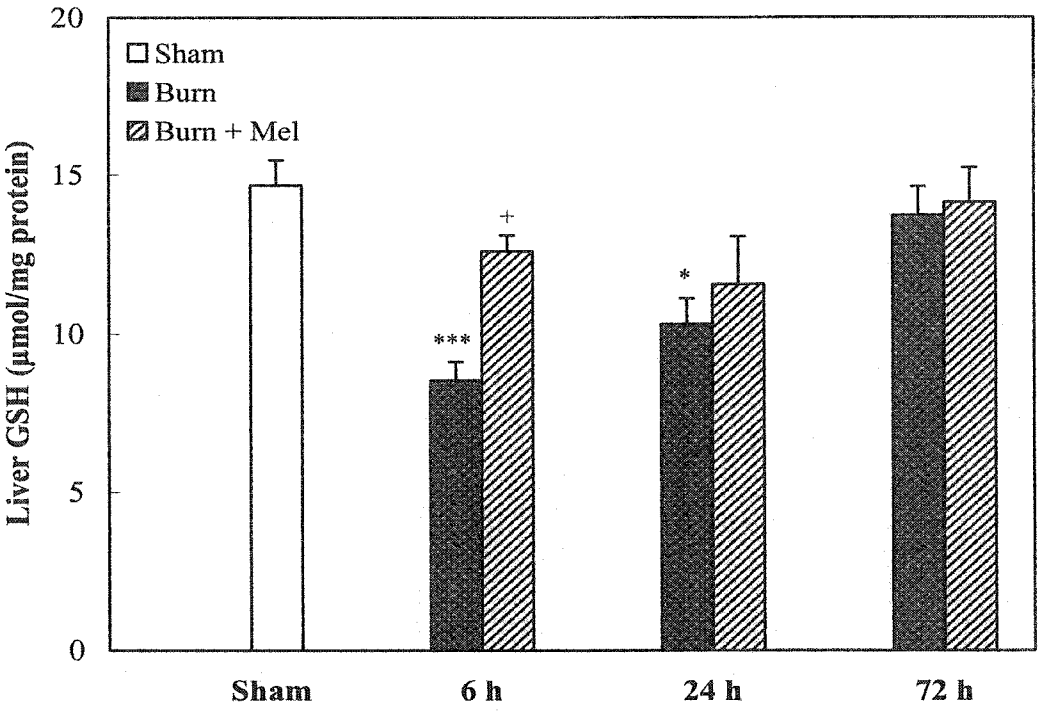


Figure 3.7 Liver levels of reduced glutathione (GSH) at 6 h, 24 h and 72 h after burn and burn plus melatonin treatment.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately after burn, and the same dose was given every 12 hours up to 72 h postburn.

* P<0.05, *** P<0.001 *vs* sham control; $^{+}$ P<0.05 *vs* burn-6 h control.

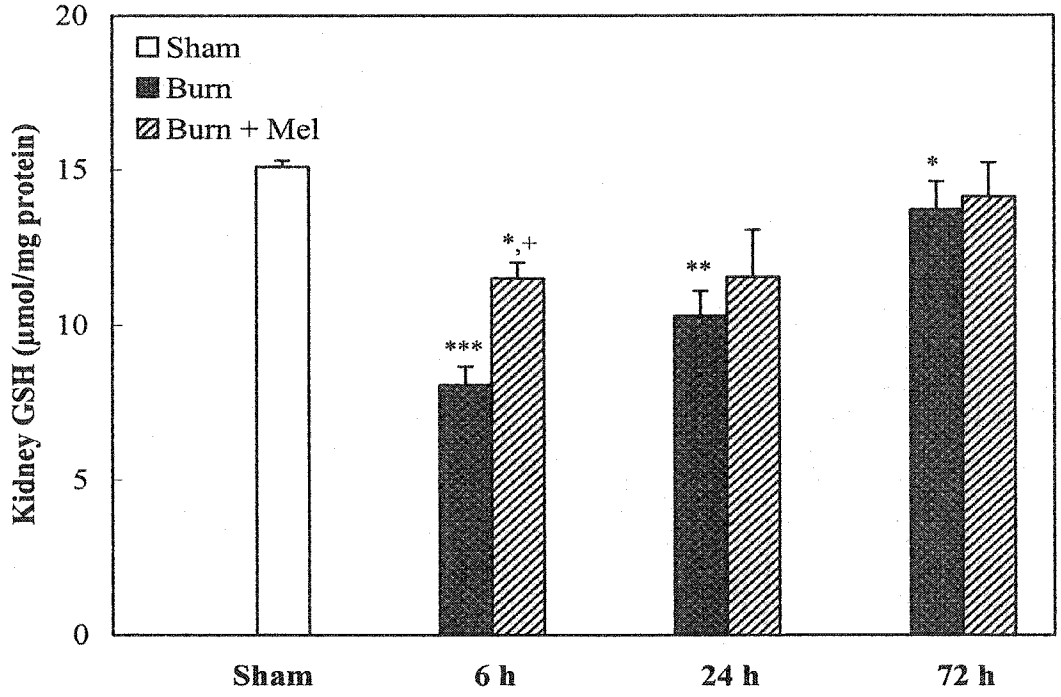


Figure 3.8 Kidney levels of reduced glutathione (GSH) at 6 h, 24 h and 72 h after burn and burn plus melatonin treatment.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately after burn, and the same dose was given every 12 hours up to 72 h postburn.

* P<0.05, ** P<0.01, *** P<0.001 *vs* sham control; $^{+}$ P<0.05 *vs* burn-6 h control.

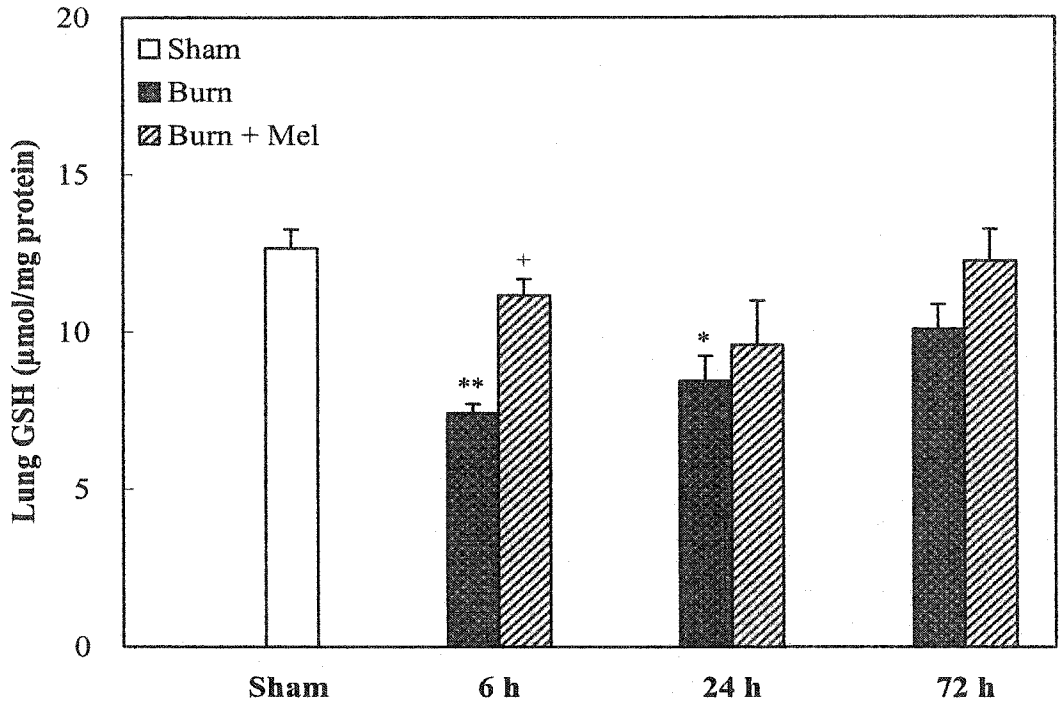


Figure 3.9 Lung levels of reduced glutathione (GSH) at 6 h, 24 h and 72 h after burn and burn plus melatonin treatment.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately after burn, and the same dose was given every 12 hours up to 72 h postburn.

* P<0.05, ** P<0.01 *vs* sham control; $^{+}$ P<0.05 *vs* burn-6 h control.

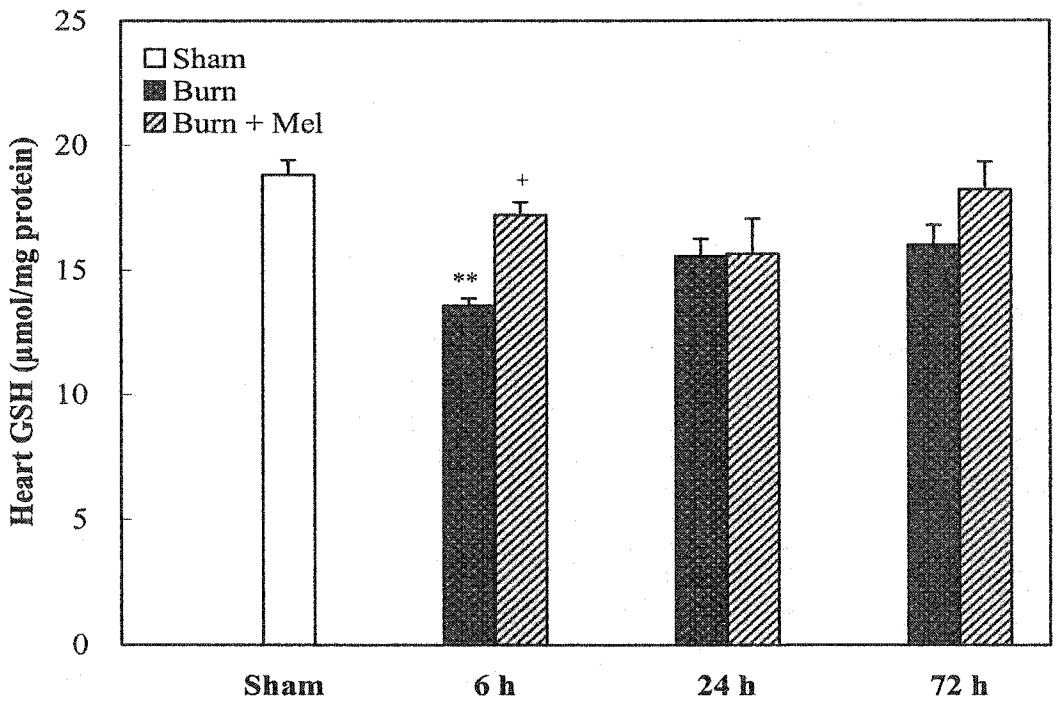


Figure 3.10 Heart levels of reduced glutathione (GSH) at 6 h, 24 h and 72 h after burn and burn plus melatonin treatment.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately after burn, and the same dose was given every 12 hours up to 72 h postburn.

* $P<0.05$, ** $P<0.01$ *vs* sham control; $^{+}$ $P<0.05$ *vs* burn-6 h control.

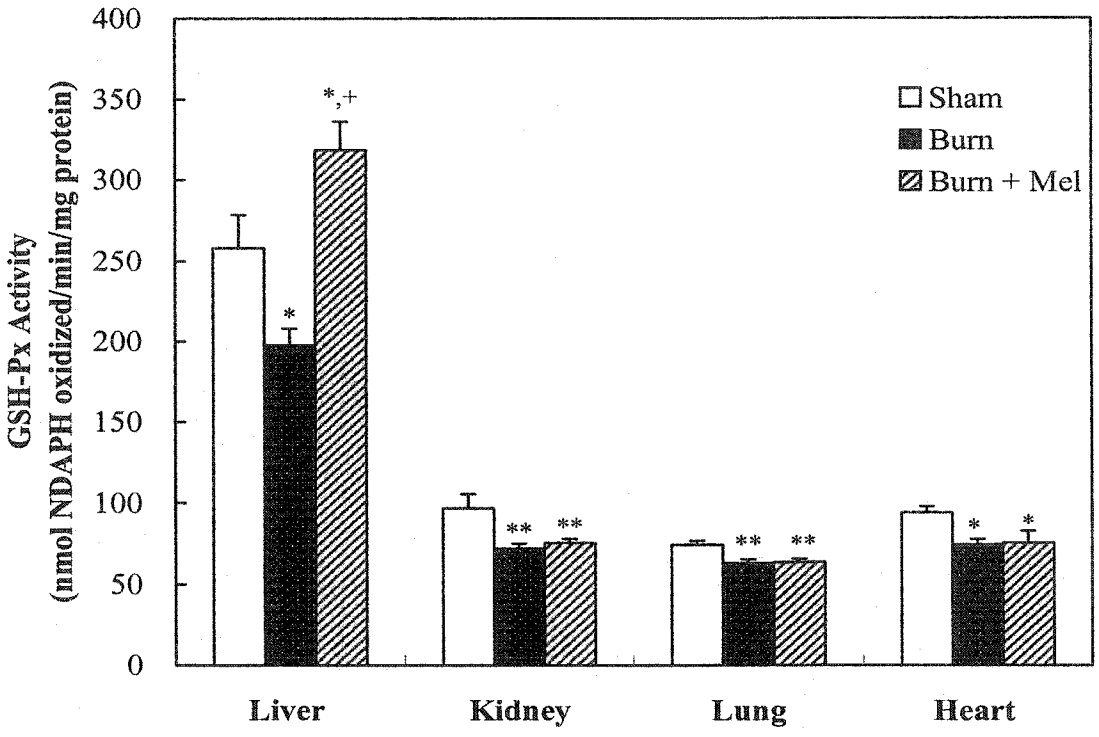


Figure 3.11 Effect of melatonin on glutathione peroxidase (GSH-Px) activities in remote organs at 6 h postburn.

Sham control: n = 9; Burn control: n = 10; Burn + Mel group: n =10. One dose of melatonin (10 mg/kg, i.p.) was administered immediately following burn. * $P<0.05$, ** $P<0.01$ *vs* sham control; $^{+}$ $P<0.001$ *vs* burn control.

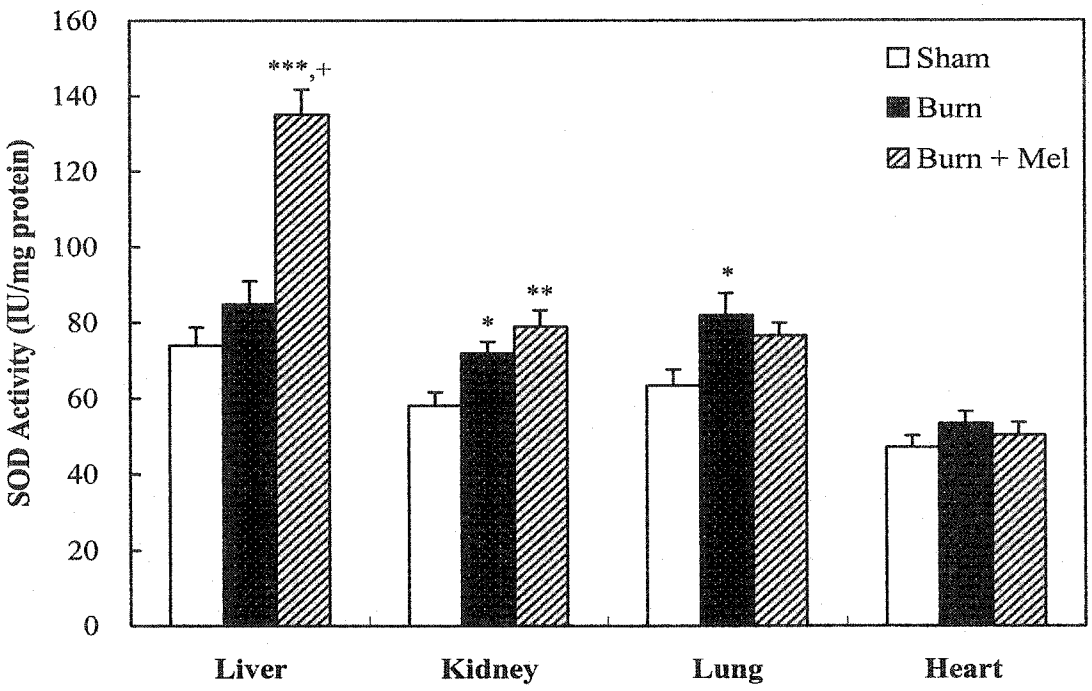


Figure 3.12 Effect of melatonin on superoxide dismutase (SOD) activities in remote organs at 6 h postburn.

Sham control: n = 9; Burn control: n = 10; Burn + Mel group: n =10. One dose of melatonin (10 mg/kg, i.p.) was administered immediately following burn. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* sham control; $^{+}$ $P< 0.001$ *vs* burn control.

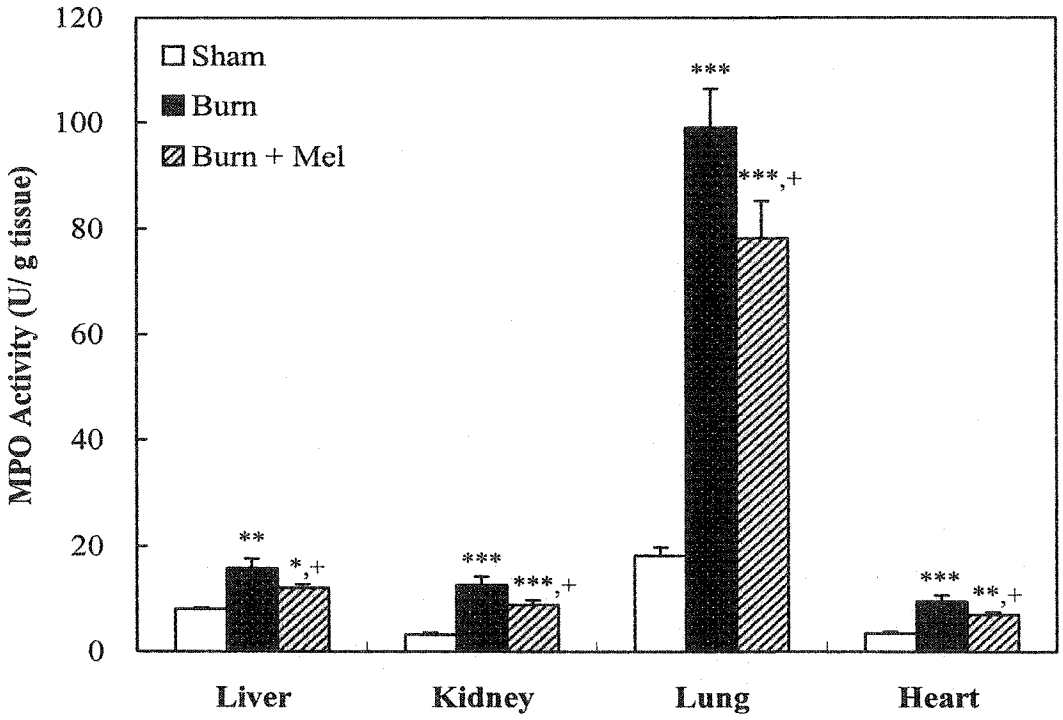


Figure 3.13 Effect of melatonin on myeloperoxidase (MPO) activities in remote organs at 6 h postburn.

Sham control: n = 9; Burn control: n = 10; Burn + Mel group: n =10. One dose of melatonin (10 mg/kg, i.p.) was administered immediately following burn. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* sham control; $^{+}$ $P<0.05$ *vs* burn control.

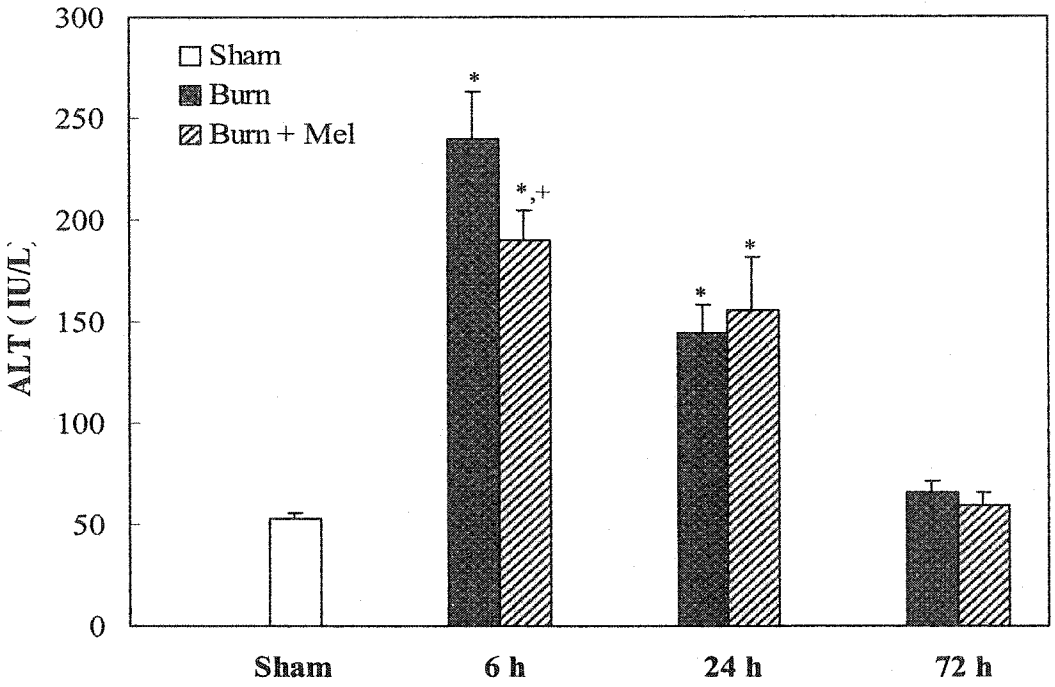


Figure 3.14 Effect of melatonin on serum level of alanine aminotransferase (ALT) at 6 h, 24 h and 72 h postburn.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately following burn, and the same dose was given every 12 hours within 72 h postburn. * $P<0.001$ *vs* sham control; + $P<0.05$ *vs* burn control.

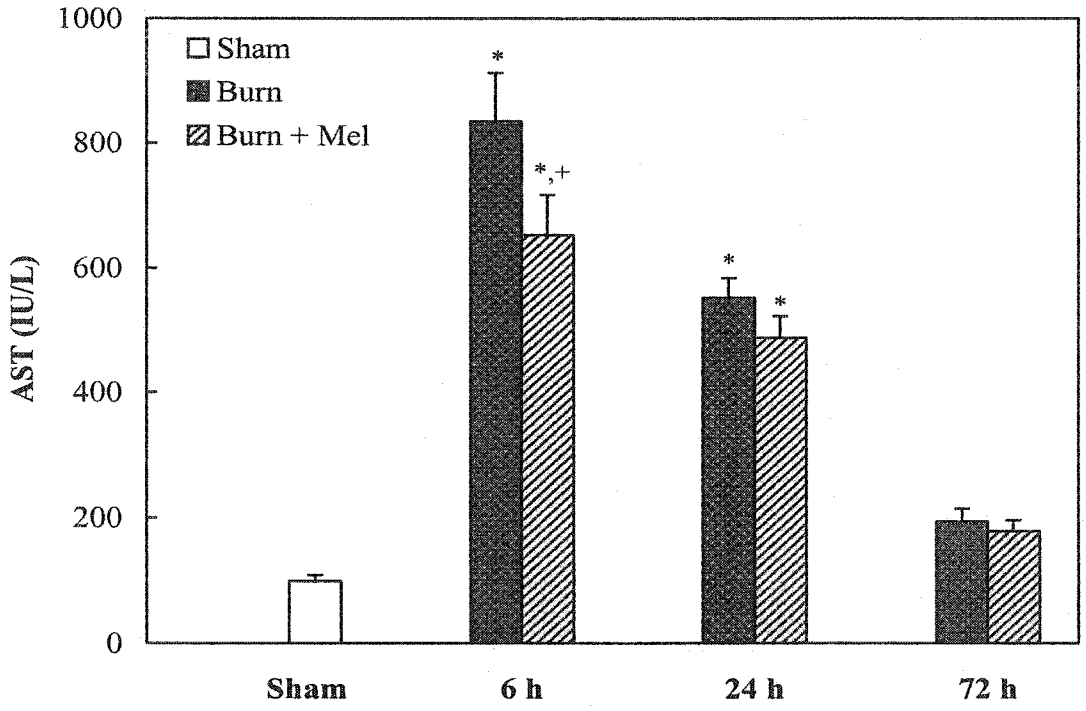


Figure 3.15 Effect of melatonin on serum level of aspartate aminotransferase (AST) at 6 h, 24 h and 72 h postburn.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately following burn, and the same dose was given every 12 hours within 72 h postburn. * $P<0.001$ *vs* sham control; $^{+}$ $P<0.05$ *vs* burn control.

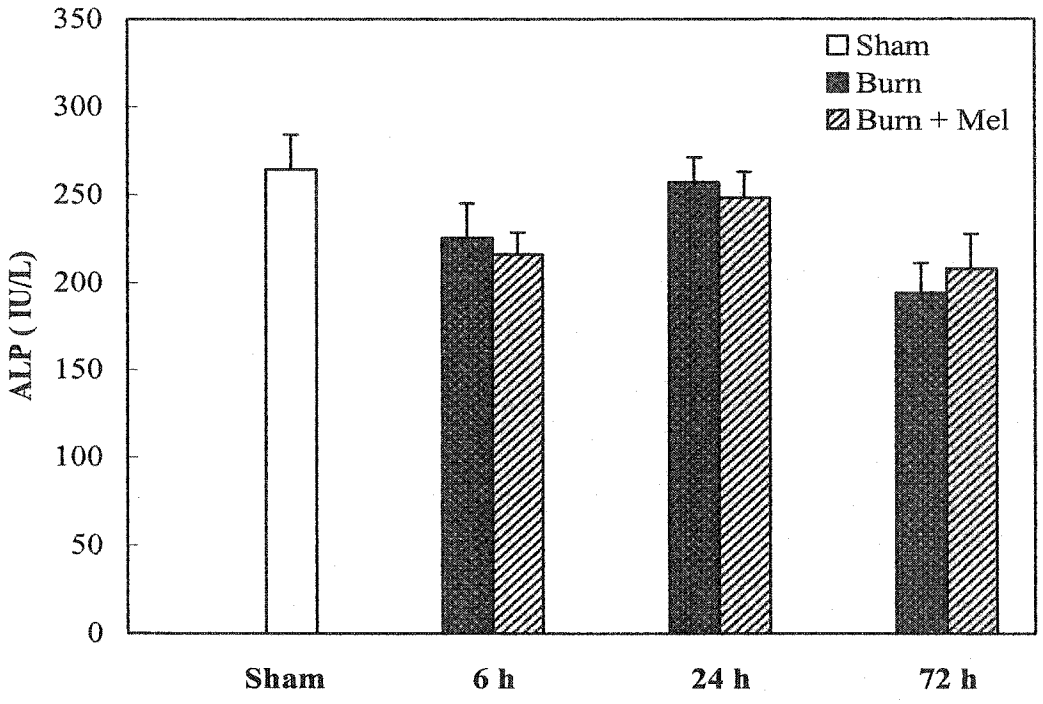


Figure 3.16 Effect of melatonin on serum level of alkaline Phosphatase (ALP) at 6 h, 24 h and 72 h postburn.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + alkaline phosphatase (ALP).Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately following burn, and the same dose was given every 12 hours until 72 h postburn. P>0.05, burn controls or burn plus melatonin groups *vs* sham control.

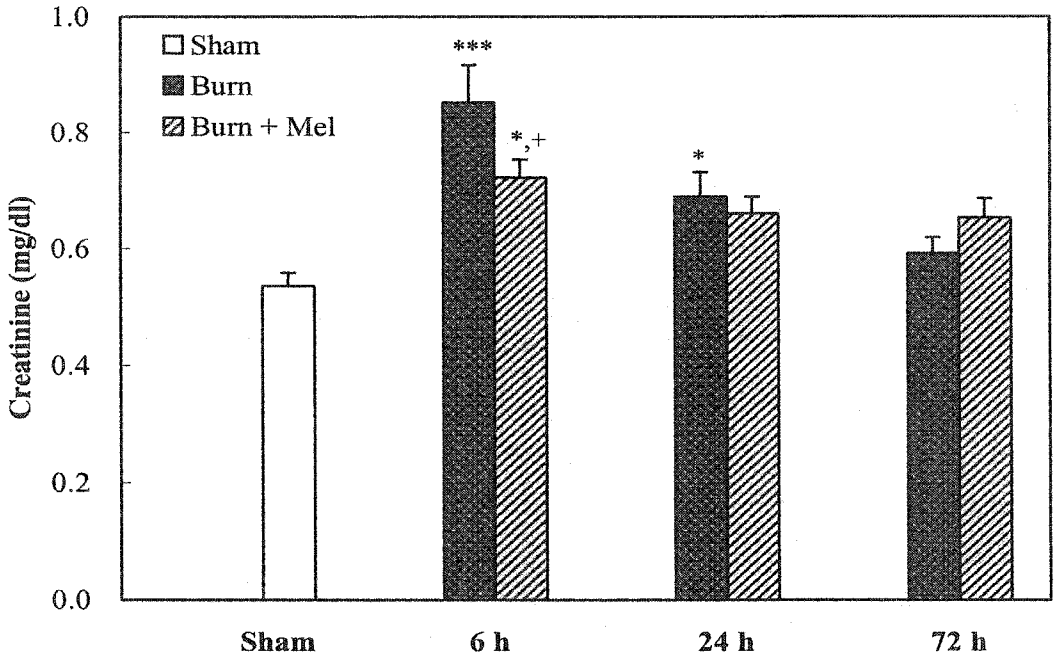


Figure 3.17 Effect of melatonin on serum level of creatinine at 6 h, 24h and 72 h postburn. .

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately following burn, and the same dose was given every 12 hours within 72 h postburn. * $P<0.05$, *** $P<0.001$ *vs* sham control; $^{+}$ $P<0.05$ *vs* burn control.

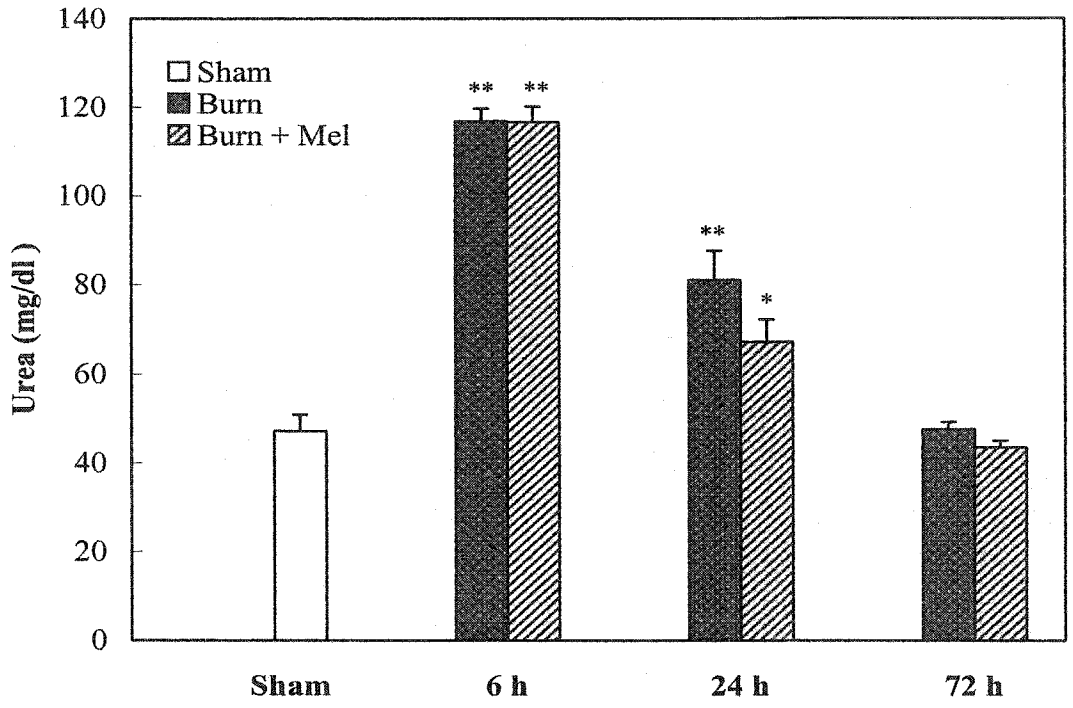


Figure 3.18 Effect of melatonin on serum level of urea at 6 h, 24 h and 72 h postburn. Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately following burn, and the same dose was given every 12 hours within 72 h postburn. * $P<0.01$, ** $P<0.001$ *vs* sham control.

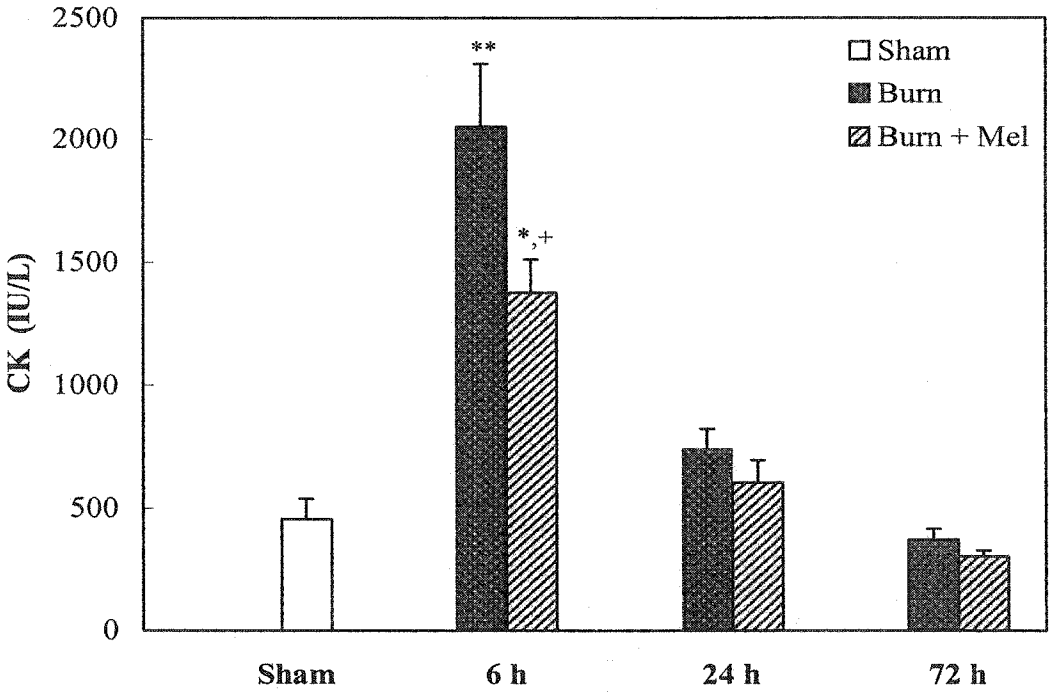


Figure 3.19 Effect of melatonin on serum level of creatine kinase (CK) at 6 h, 24 h and 72 h postburn.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately following burn, and the same dose was given every 12 hours within 72 h postburn. $^{*}P<0.01$, $^{**}P<0.001$ *vs* sham control; $^{+}P<0.01$ *vs* burn control.

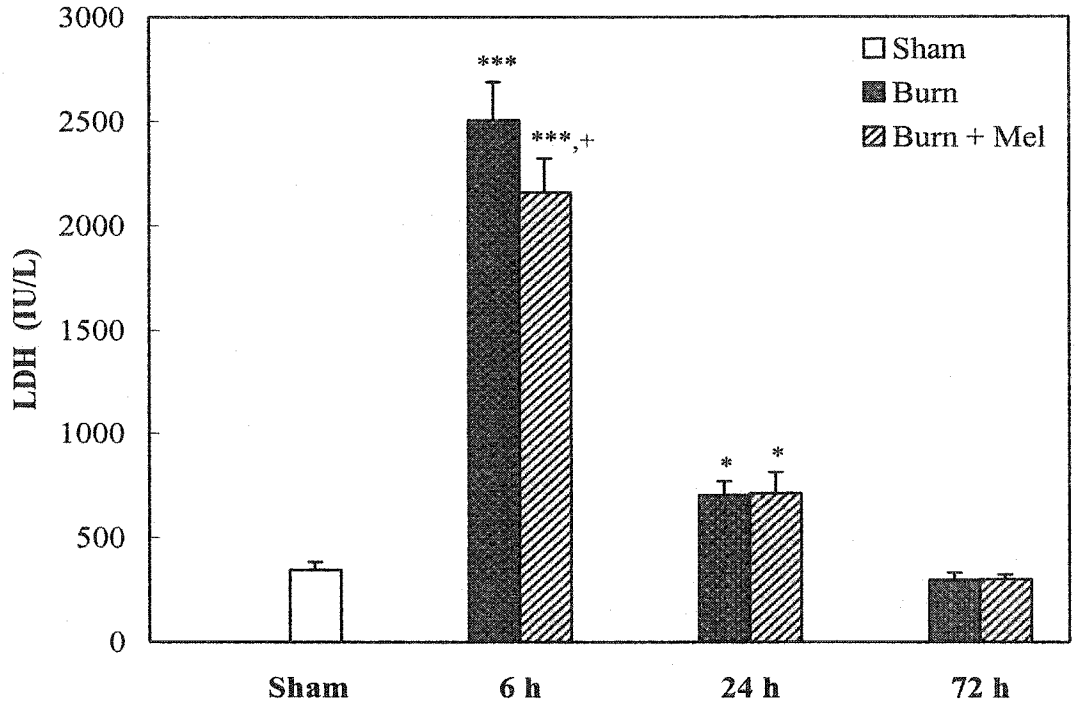


Figure 3.20 Effect of melatonin on serum level of lactate dehydrogenase (LDH) at 6 h, 24 h and 72 h postburn.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately following burn, and the same dose was given every 12 hours within 72 h postburn. * $P<0.05$, *** $P<0.001$ *vs* sham control; $^{+}$ $P<0.05$ *vs* burn control.

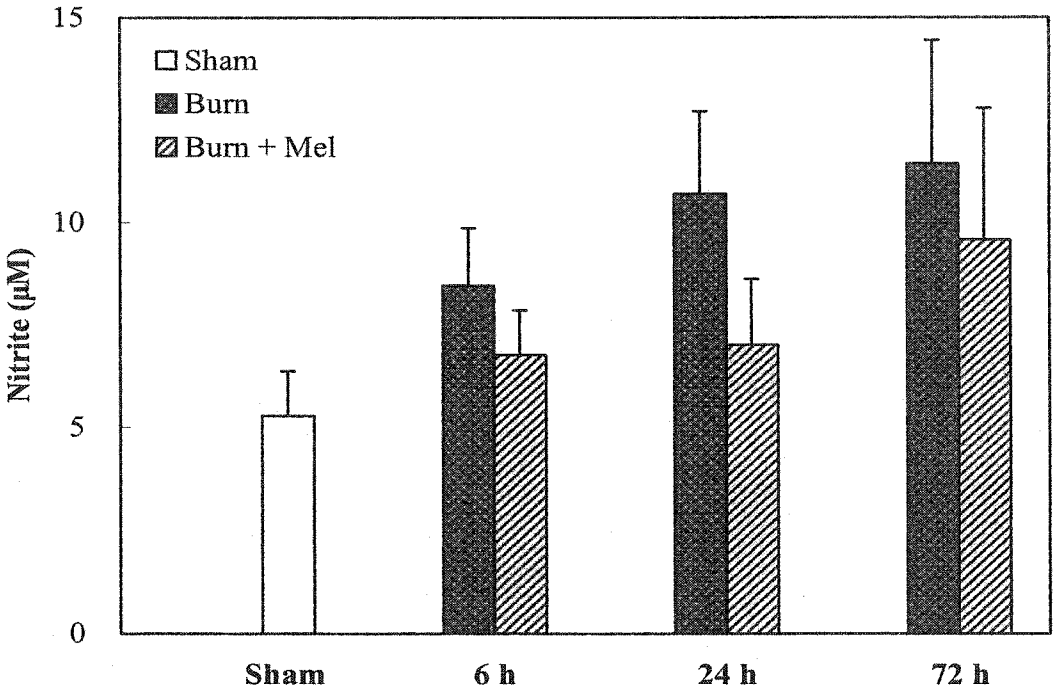


Figure 3.21 Serum nitrite levels at 6 h, 24 h and 72 h after burn and burn plus melatonin treatment.

Sham control: n = 9; Burn controls: n = 10 for each; Burn + Mel groups: n =10 for each. Melatonin (10 mg/kg, i.p.) was administered immediately following burn, and the same dose was given every 12 hours until 72 h postburn. P>0.05, burn controls or burn plus melatonin groups *vs* sham control.

Table 3.2 Changes of body weights in rats treated with burn and burn plus melatonin Treatment

Groups	PBD0	PBD2	PBD5	PBD7
Sham	277 ± 4	281 ± 4	290 ± 4 *	295± 4 *
Burn	278 ± 5	263 ± 5	263± 4+	262 ± 5+
Burn + Mel	279± 5	272± 5	264±54+	265 ± 4+

Mel = Melatonin; PBD = Postburn Day.

Sham group: n=8; Burn group: n=8; Burn + Mel group: n=8.

Melatonin treatment (10 mg/kg, i.p.) was initiated immediately following burn and repeated daily at 6 p.m. (2 hours before darkness onset) for seven days.

* $P<0.05$ *vs* PBD0; + $P<0.05$ *vs* sham control at the same time-point.

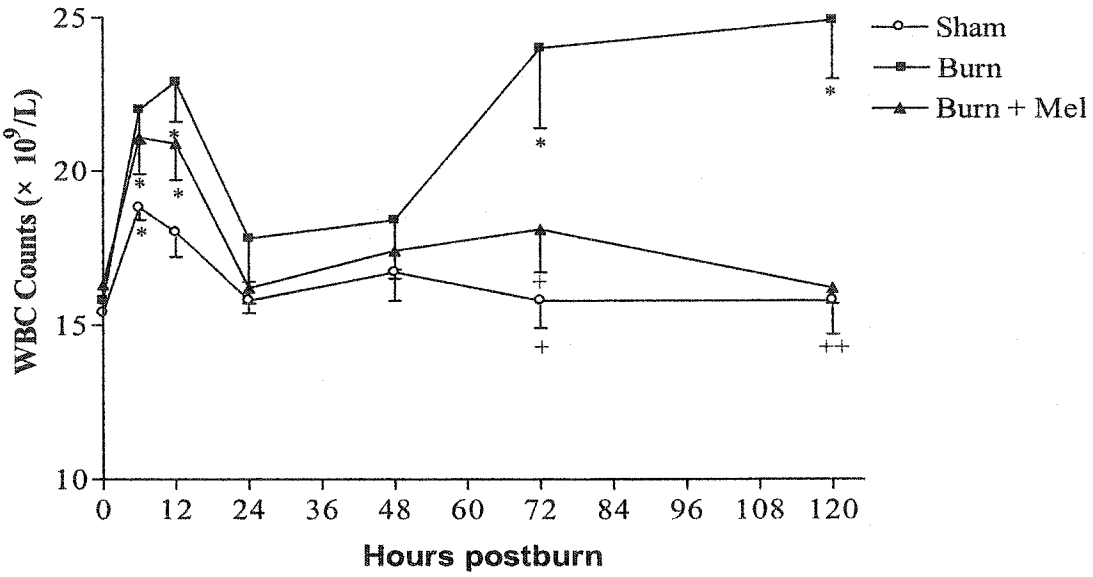


Figure 3.22 Changes of total white blood cell (WBC) counts after burn and melatonin treatment.

Sham group: n=8; Burn group: n=8; Burn + Mel group: n=8.

Melatonin (10 mg/kg, i.p.) was given immediately following burn and was repeated daily at 6 p.m. (2 hours before darkness onset) for seven days. * $P < 0.05$ vs respective preburn (0h) values, $^{+}$ $P < 0.05$, $^{++}$ $P < 0.001$ vs burn control at 72 h and 120 h postburn respectively.

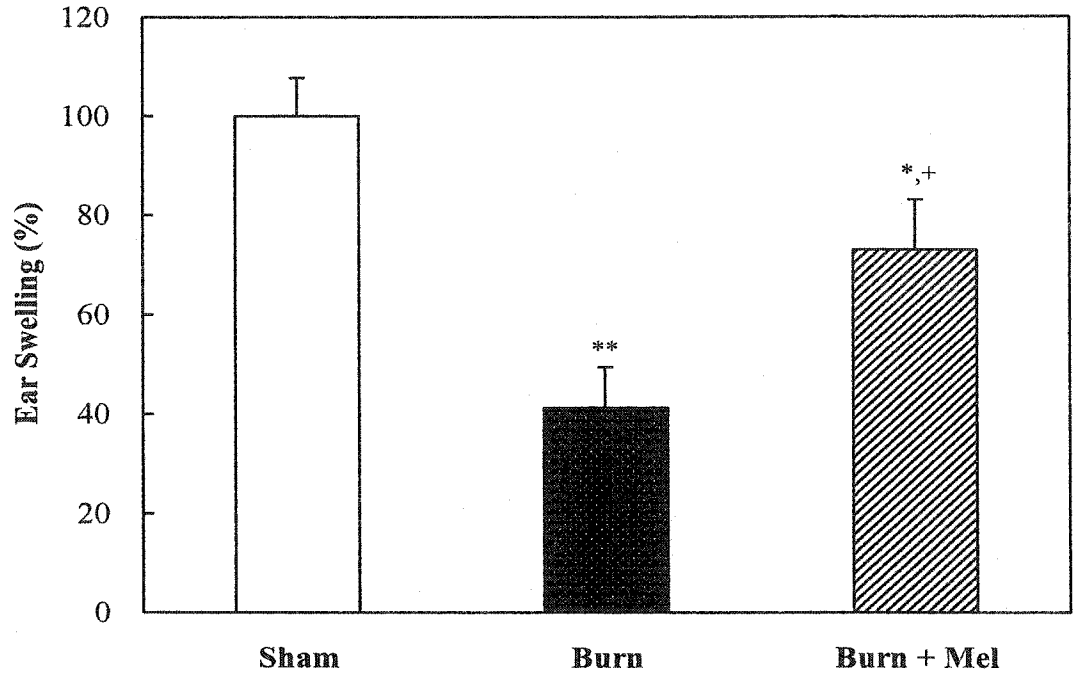


Figure 3.23 Effect of melatonin on percentage changes of ear swelling in contact hypersensitivity reaction (CHR) initiated at Day 7 postburn.

Sham group: n=8; Burn group: n=8; Burn + Mel group: n=8, melatonin (10 mg/kg, i.p.) was given immediately following burn and was repeated daily at 6 p.m. (2 hours before darkness onset) for seven days.

* P<0.01, ** P<0.001 *vs* sham group; $^{+}$ P<0.01 *vs* burn group.

Chapter 4

DISCUSSION

4.1. Burn Model Used in the Study

A 30% TBSA, full-thickness burn model in rats has been widely used in burn research due to its easy induction, low mortality rate and a variety of abnormality in the defence system of the animals. Firstly, 30% is almost the maximal burn area which can be induced on the dorsum, thus 30% burn model is more convenient to be conducted compared with those with greater burn size (Walker and Mason, 1968). Secondly, low mortality rate ranging from 0% to 20% is another benefit for this animal model, especially in long-term studies. The overall mortality rate in our study was about 16% during the 12-day observation, similar to that reported by Cetinkale et al. (1999). In addition, 30% major burn is sufficient to induce a variety of abnormality such as oxidative stress, MOD as well as immunosuppression. Therefore, this animal model has been used throughout the study.

4.2. Hypovolemia and Tissue Oedema Following 30% Major Burn

The immediate response of the body to burn injury is the markedly increased capillary permeability, which results in extensive loss of fluid and plasma proteins from the circulation into the extravascular spaces, leading to hemoconcentration, hypoproteinemia, hypovolemia as well as severe tissue oedema (Demling et al., 1984).

In severe burn injury with no or insufficient fluid resuscitation, these hemodynamic changes together with other alterations such as the sustained vasoconstriction, decreased cardiac output and increased blood viscosity eventually caused the reduced blood flow to vital organs, leading to a very serious state called burn shock (Lund and Reed, 1986). It has been widely agreed that burn shock is the main cause for MOD occurring in the early stage of burn.

Fluid resuscitation is the main strategy for burn shock management. In the present study, all burned animals received an immediate intraperitoneal injection of saline (4 ml/kg/%burn) as fluid resuscitation (a commonly used resuscitation protocol in animal model). However, hemoconcentration and hypovolemia were still evident at 6 h postburn. This can be explained by the relatively slow fluid absorption in the peritoneal cavity in contrast to the very rapid fluid loss during the very early stage of burn. Nevertheless, this animal model still shows some similarity to the clinical situations, in which the insufficient or delayed fluid resuscitation is very common.

The tissue oedema, estimated from the wet/dry (W/D) weight ratios in our study, was found both at the burn site and in the lung, similar to previous studies (Xia et al., 1991; Saitoh et al., 1994; Lindblom et al., 2000). The burn wound oedema is mainly secondary to the increased capillary permeability, whereas, more complex mechanisms are involved in the lung oedema formation, including hypoproteinemia, oxidative damages and the transient increase in the capillary permeability. Although the tissue oedema in the liver and kidney was not detected, our study have shown that 30% major burn still resulted in severe tissue damages and organ dysfunctions in these two organs.

4.3. Burn-induced Oxidative Stress and its Mechanisms

Oxidative stress may result from increased free radical generation and/or lowered antioxidative defence, thus causing serious damage to cells and tissues by lipid peroxidation, protein oxidation and DNA damage (Sies, 1993). MDA, an end product of lipid peroxidation, is the most commonly used marker for the oxidative stress due to its easy measurement and high sensitivity. However, many studies have shown that the measurement of MDA, based on its reaction with thiobarbituric acid (TBA), can be interfered by a number of substances (Conti et al., 1991). For this reason, the MDA data should be interpreted with caution, and is often used in combination with other indicators (e.g. GSH, protein oxidation) in estimating the oxidative stress.

GSH is a key component of the cellular defence system against the oxidative damages. The GSH redox cycle catalyzed by GSH-Px reduces H_2O_2 or lipid peroxide, thus breaking the chain reaction from $O_2^{\bullet -}$ to the highly toxic $^{\bullet}OH$. The oxidized glutathione (GSSG) is subsequently reduced to GSH by GSH-Rd. The stable GSH content is essential for keeping GSH-Px activities (Reed, 1986). Additionally, GSH also scavenges $O_2^{\bullet -}$, protects protein thiol groups from oxidation and restores other antioxidants such as Vitamin E and C to their reduced states (Ross, 1988). Therefore, the oxidative stress is often accompanied by the decreased GSH due to its over-consumption against free radical-mediated tissue damage.

Major burns induce significant oxidative damage both at the burn wound and in remote organs. It has been widely accepted that burn-induced oxidative stress is evident within 24 h postburn (Till et al., 1985; Saitoh et al., 1994; Konukoglu et al., 1997; Horton et al., 1998), but it may exist up to one week following burn (Yang et al., 1995

and 1997). In the present study, the oxidative stress induced by 30% major burn was estimated within 72 h postburn by measuring the tissue levels of MDA and GSH in four remote organs (liver, kidney, lung and heart). Our results showed markedly increased MDA levels (liver excluded) and decreased GSH levels in all tested organs within 24 h postburn, especially at 6 h postburn. These results are consistent with previous observations. Furthermore, the kidney seems to be especially susceptible to free radical attack, as assessed from the sharp (two-fold increase at 6 h postburn) and longer (up to 72 h postburn) alterations in the MDA and GSH levels. These findings suggest that antioxidant therapy in burn injury should be initiated as early as possible, and persist a longer time (e.g. at least three days). The liver MDA level did not change during the entire observations, a finding similar to that reported by Saitoh et al. (1994), in which even 40% major burn did not elevate the liver MDA level. They suggested that the abundant antioxidant defence system in the liver, especially very high GSH-Px activity, might protect the cell membrane against the free radical attack, as such there was no elevation in the liver MDA level. However, in our study, the remarkable decrease in the liver GSH levels implied the overproduction of OFR in this organ following burn, suggesting that the oxidative damages may be the most important cause for the liver dysfunction.

There are several mechanisms responsible for the burn-induced oxidative stress, amongst which the most important ones are: (1) activation of xanthine-oxidase (XO) system; (2) neutrophil accumulation and activation; (3) lipid peroxide produced at the burn wound and then transferred into the remote organs; (4) lowered antioxidative defence (both enzymatic and non-enzymatic). Other pathways may also be involved, such as pro-inflammatory cytokines and reactive nitrogen species (RNS), and these will

be discussed in 4.6.

Organ hypoperfusion and ischemia increase the degradation of ATP and subsequent accumulation of its derivative hypoxanthine, they also activate the XO system which oxidizes hypoxanthine to xanthine and then to uric acid with the production of $O_2^{\bullet -}$ and H_2O_2 (Parks and Granger, 1986; Chen et al., 1995). In the present study, despite all burned animals were resuscitated with large quantities of saline, the organ hypoperfusion and tissue ischemia still occurred, and served as a very important source for free radical generation following burn.

Neutrophil accumulation and activation is another important mechanism for burn-induced oxidative stress. Burn stress and burn wound initiate the complex local and systemic inflammatory responses, during which a number of mediators such as TNF-α, LTB_4, C3a and C5a promote neutrophils to emigrate into the burn wound and the remote organs (Nuytinc et al., 1988; Mileski et al., 1993; Stengle et al., 1996). Neutrophil may release both toxic oxygen species and lysosomal enzymes, propagating the initial tissue damages (Till et al., 1989; De La Ossa et al., 1992; Cetinkale et al., 1996). Our results showed the presence of increased MPO levels in all tested organs, confirming the involvement of neutrophils in burn-induced oxidative damages.

Although the half-lives of OFR are extremely short, some of their reaction products, such as lipid peroxides, are fairly stable and may be released from the burned skin into the circulation and then reach the remote organs (Nishigaki et al., 1980). The lipid peroxide may propagate the free radical reactions, leading to further oxidative damages and organ dysfunctions far from the burned site.

The burn-induced oxidative stress results not only from the overproduction of ROS, but also from the reduced antioxidative defence. The contents of GSH, Vitamin E and C, the main endogenous non-enzymic antioxidants, have been found to decrease significantly following burn (Nguyen, et al., 1993; Rock et al., 1997), and these decreases may be due to their over-consumptions against the burn-induced oxidative damages. Only the tissue levels of GSH were estimated in our study, and the results were consistent with other reports (Konukoglu et al., 1997; Sener et al; 2002 a, b).

The enzymatic antioxidant responses to burn injury are more complex, both the increased and decreased enzyme activities have been reported (Leff et al., 1993; Yang et al., 1995; Saitoh et al., 2001; Ding et al., 2002). GSH-Px and GSH-Rd usually act in a co-operated manner to remove H_2O_2 and lipid peroxide, preventing the subsequent $^{\bullet}OH$ formation, however, their activities both in the plasma and tissues are often inhibited following major burns (Meng 1991; Sabeh et al., 1995; Yang et al., 1995). Recently, Ding et al. (2002) found that the activities of GSH-Px and GSH-Rd in the liver increased at 24 h and then decreased at 48 h postburn. Our results demonstrated the decreased GSH-Px activities in four tested organs at 6 h postburn, a finding that is consistent with most previous studies. As the stable GSH levels are crucial for the normal GSH-Px activities, the over-consumption of GSH following burn may partially account for the decreased GSH-Px activity. Moreover, the burn-induced tissue ischemia and toxic OFR products may directly inhibit the enzyme activities.

Catalase is another important enzyme in the decomposition of H_2O_2, especially in some pathological conditions with high H_2O_2 production. Burn injury is often accompanied with large quantities of H_2O_2 generation, however, the decreased catalase

activities in the plasma as well as in the liver and kidney have been found in several studies (Yang et al., 1995; Lalonde et al., 1997, Youn et al., 1998). In contrast, Leff et al. (1993) reported the increased catalase in the serum of rats subjected to 25% major burns. The changes in catalase activities were not measured in our study.

The responses of SOD to burn injury are also conflicting. In the observations of Saitoh et al. (2001), the plasma Mn-SOD and Cu/Zn-SOD concentrations were found to increase significantly at 6 h postburn, and the kidney Mn-SOD and lung Cu/Zn-SOD concentrations were significantly higher at 24 h in the animals with 30% burns than those with either sham or 50% burns. Kawai et al. (1988) also reported the slightly increased SOD levels in the plasma at 4 h postburn and in the liver from 4 h to 24 h postburn in mice receiving 25% scald burn. On the contrary, a number of studies showed the inhibited SOD activities in the plasma (Cui et al., 1994; Qin et al., 2002) and tissues (Saitoh et al., 1994; Gao et al., 1997, Yang et al., 1997). In the present study, the increased SOD activities were found both in the lung and kidney at 6 h postburn, but no significant alterations were found in the liver and heart. This finding is similar to that reported by Saitoh et al. (2001).

The discrepancy in the different responses of main antioxidative enzymes, i.e. GSH-Px, GSH-Rd, catalase, SOD, may be explained by the variations in burn severity (or burn model), tested species, observed postburn time-points, enzyme assay methods, fluid resuscitation and other burn management. In general, the increased enzyme activities are often considered as an adaptive response against burn-induced oxidative stress. However, in severely burned patients or animals (e.g. 50% burns), these enzymes may be inhibited significantly as a result of tissue ischemia, ROS-mediated damages or

some unknown pathways.

In summary, major burns result in severe oxidative damages in the plasma especially in the remote organs. Both the increased free radical generation and lowered antioxidative defence account for its occurrence.

4.4. Antioxidant Action of Melatonin in Burn Injury

Melatonin (*N*-acetyl-5-methoxytryptamine) is the primary pineal hormone synthesized from the amino acid tryptophan, and its biological functions have been widely studied since its first identification in 1958 (Lerner et al. 1958). In recent decade, melatonin has been proved as a potent antioxidant, and may protect cells, tissues and organs against oxidative damages induced by a variety of free radical generating agents and processes (Tan et al., 1993 a, b; Reiter et al., 1997a, b; Vijayalaxmi et al., 2002).

The present study showed that a single dose of melatonin (10 mg/kg, given i.p. immediately postburn) resulted in the reduced MDA levels as well as the elevated GSH levels in most tested organs at 6 h postburn, suggesting melatonin acting as an antioxidant in burn injury. Our findings were partially in agreement with the observations of Sener et al. (2002 a, b), who also demonstrated the antioxidant role of melatonin in a 30% major burn model, as assessed by the reduced MDA levels and protein oxidation as well as the restored GSH levels in four remote organs (liver, kidney, lung and intestine).

The antioxidant action of melatonin is more likely to be attributed to its very high scavenging capacity on very toxic $^{\bullet}OH$ radicals. Tan et al. (1993a) found melatonin

scavenged $^{\bullet}OH$ in a cell free system with greater efficacy than GSH (a main intracellular antioxidant) and mannitol (an antioxidant found in plants). Burn injury is often accompanied by the overproduction of H_2O_2 as well as the increased iron contents. Therefore, $^{\bullet}OH$ may be extensively produced via the Fenton reaction following burn. Because of the very high reactivity, the generation of $^{\bullet}OH$ and its damage to the cells are site-specific, thus the removal of $^{\bullet}OH$ requires high concentrations of antioxidant at its production site. The effectiveness of melatonin as an antioxidant may also be assisted by its high lipid-soluble property, with which melatonin crosses morphophysiological barriers, e.g. the blood-brain barrier, and enters cells and subcellular compartments easily (Reiter et al., 1997).

When applied *in vivo*, melatonin may interact with other endogenous antioxidants such as GSH, Vitamins E and C to improve the total antioxidant capacity (Reiter et al., 1997; Gitto et al., 2001). In our study, melatonin was found to remarkably restore the decreased GSH levels at 6 h postburn, similar to the observations using other animal models, e.g. excessive exercise (Hara et al., 1997), the liver ischemia-reperfusion injury (Sewerynek et al., 1996) and LPS-induced oxidative damages (Sewerynek et al., 1995). Since GSH plays a major role in cellular antioxidative defence, the restoration of GSH levels by melatonin may prevent the subsequent oxidative damages.

It has been suggested that the stimulation on GSH-Px, GSH-Rd and SOD as well as the inhibition on NOS enhance the antioxidant action of melatonin (Reiter et al., 1997). However, our study found that melatonin stimulated the GSH-Px and SOD activities only in the liver but not in other organs. The liver is the main site for melatonin distribution and catabolism (Kopin et al., 1961; Lane and Moss, 1985; Young

et al., 1985), the unique influences of melatonin on liver enzymes may depend on its relatively high concentration at this site. Furthermore, melatonin was also found to reduce the MPO levels in all tested organs, thus alleviating the neutrophil-mediated tissue damages.

Unfortunately, repeated melatonin therapy in our study did not exert any protective effect compared to the single injection. This failure is most likely due to the long injection interval (every 12 hours) of melatonin relative to its very short half-life. As discussed above, the antioxidant action of melatonin in burn injury depends primarily on its direct scavenging on free radicals, and this requires high melatonin levels in the remote organs where free radicals are usually over-produced following burn. Melatonin can be absorbed quickly via multiple routes, distributed widely in different tissues but also metabolized promptly in the liver. Therefore, the repeated melatonin treatment (every 12 hours) was used in our study in order to keep the high tissue levels of melatonin to protect against burn-induced oxidative damages within 72 h postburn. However, this injection interval is significantly longer that those used in other animal models ranging from every 1 hour to 6 hours (Sewerynek et al., 1995 and 1996; Crespo et al., 1999), thus the very low tissue levels of melatonin may lead to the failure of repeated melatonin treatment in our study. Our hypothesis is also supported by the finding of Sener et al. (2002 a, b), in which repeated melatonin treatment at an 8 hours-interval partially inhibited the burn-induced oxidative damages.

4.5. Protection of Melatonin against Burn-induced MOD

Multiple organ dysfunction or failure (MOD or MOF) is a very serious complication and accounts for the high mortality in severely burned patients. Burn shock is closely

related to MOD occurring in the early stage of burn, while serious infections often lead to its late occurrence (Cipolle et al., 1993; Jarrar et al., 1999).

In the present study, the liver, kidney and heart dysfunctions estimated by the rise in serum levels of organ-specific markers were found as early as 6 h postburn, and tended to recover but were still evident at 24 h postburn. This finding is similar to the previous studies on burn-induced organ dysfunctions (Lu et al., 1995; Yang et al., 1995 and 1997; Horton et al., 1998). Blood gas analysis was not carried out to estimate the lung dysfunction due to the limitation of assay facilities. However, the obvious lung oedema found in our study suggested that the lung function had been impaired following a 30% major burn.

There are two mechanisms responsible for the early occurrence of MOD. Firstly, burn stress and burned skin may induce a strong systemic inflammatory response, which is mediated by a number of inflammatory mediators (e.g. cytokines, OFR, arachidonic acid metabolites, PAF, NO and complement). These mediators interact with each other and finally result in cell and tissue damages. Secondly, all tissues are subjected to ischemia very shortly after burn injury due to the very rapid fluid loss from the circulation as well as the strong vasoconstriction and consequently, during fluid resuscitation, reperfusion occurs (Kaufman et al., 1989; Ward and Till, 1990; Lalonde et al, 1992). Thereby, the ischemia-reperfusion damage is a very important mechanism for burn-induced oxidative stress and MOD. In our animal model, the fluid resuscitation was not sufficient to counteract the rapid fluid loss, as evidenced from the significantly increased heamatocrit at 6 h postburn, so the tissue ischemia could not be avoided at this time-point. However, the increased saline absorption as well as a variety of adaptive

responses enabled the organ perfusion markedly improved at 24 h postburn or completely restored at 72 h postburn. This may be partially responsible for our observations showing both the burn-induced oxidative stress and MOD tended to recover with time.

Melatonin has been shown to be a potent antioxidant in many studies (Rieter et al., 2000). Considering the important role of OFR in both burn-induced oxidative stress and MOD, the protective effect of melatonin on MOD was further investigated. Our results showed that only a single injection of melatonin was useful in improving the liver, kidney and heart dysfunctions and also alleviated the lung oedema at 6 h postburn, a time-point at which melatonin significantly inhibited the burn-induced oxidative damages in all tested organs. Thus the protection of melatonin against MOD is most likely due to its antioxidant action in burn injury.

The early organ support is a very important strategy for burn management. The improved organ functions by melatonin may enhance the general defence capacity, preventing the subsequent infections. Although melatonin showed some promising effects on burn-induced MOD in animal model, further studies and clinical trials are necessary to explore the underlying mechanisms and optimal therapy for melatonin.

4.6. Involvement of TNF-α and NO in Burn-induced MOD

TNF-α is the most important pro-inflammatory cytokine in burn injury, and is closely associated with burn-induced oxidative stress and MOD (see 1.2.2.2). Under normal conditions, the serum level of TNF-α is very low and even undetectable. However, burn injury induces the rapid over-production of TNF-α, and the elevated serum levels of

TNF-α have been found (Peter et al., 1999; Deveci et al., 2000). On the other hand, melatonin has been shown to exert an inhibitory effect on the LPS-induced TNF-α production both *in vivo and in vitro* (Di Stefano and Paulesu, 1994; Sacco et al., 1998). So, it is possible that melatonin may also inhibit the over-production of TNF-α following burn, alleviating the burn-induced MOD. To verify this hypothesis, the serum levels of TNF-α were measured at three postburn time-points using a sensitive rat TNF-α ELISA kit. Unfortunately, we did not find any rise in the serum TNF-α as reported by Peter et al. (1999) and Deveci et al. (2000). Instead, the serum TNF-α in most of samples were not detectable. Demling et al. (1993) has suggested that the circulating cytokines including TNF-α were usually rapidly cleared from the circulation. This may be a possible explanation for our result. It seems that the measurement of serum TNF-α is not reliable, and further studies (e.g. TNF-α mRNA expression) are necessary to confirm or reject our hypothesis.

NO is a simple and unstable radical which is converted to stable nitrite and nitrate ions in aqueous solution very shortly after its production (Erik, 1994). Similar to TNF-α, NO also acts as an important inflammatory mediator and may be involved in the burn-induced MOD. Excessive amount of NO was produced after burn injury, as evidenced by the elevated nitrite or nitrite + nitrate levels in the serum, urine or lymphocyte supernatant (Becker et al., 1993; Masson et al., 1998; Chen et al., 2001). Most of the excessive NO further reacts with $O_2^{\bullet -}$ to form very toxic peroxynitrite anion, leading to RNS-mediated tissue damages and organ dysfunction (Chen et al., 1998 and 2001). Melatonin has been found to inhibit the NO production (Battahi et al., 1998), and also to scavenge the very toxic peroxynitrite anion via a direct interaction (Gilad et al., 1997; Blanchard et al., 2000). The protection of melatonin against

burn-induced MOD may be partially due to the reduced NO production. Our data showed that serum nitrite concentrations tended to increase following burn, but did not reach a statistical significance. Because nitrite only represents a small part of the total NO production in the serum, the total nitrite + nitrate measurement may be necessary. No conclusions can be drawn from the measurement of serum TNF-α and nitrite, nevertheless, further investigations are still valuable for a better understanding of the protective effects of melatonin in burn injury in relation to the involvement of TNF-α and nitric oxide production.

4.7. Immunomodulation of Melatonin on Cell-mediated Immunity Following Burn

The cell-mediated immunity (CMI) is the main function of T lymphocyte cells, and plays a central part in the host defence mechanism. It is generally believed that the activation of macrophages by Th 1 cells and the killing of intracellular pathogens or tumour cells by cytotoxic T cells make up the CMI (Roitt et al., 2001). The delayed-type hypersensitivity (DTH) is a specific cellular immune response, and is mediated by Th 1 cells. These cells enter the site of antigen injection, recognize the complexes of peptide: MHC II on antigen-presenting cells, and release inflammatory cytokines that increase the local blood vessel permeability and recruit accessory cells, finally causing the obvious swelling at the site of antigen injection. Each of these phases takes several hours and so the mature response appears only 24-48 hours after challenge (Janeway, et al., 1999). The contact hypersensitivity reaction (CHR), a form of DTH, has been used in many studies as a reliable, sensitive and *in vivo* measurement in evaluating the status of CMI (Shelby and Merrell, 1987; Cetinkale et al, 1993 and 1999).

Major burns predominantly impair the cellular immune response, increasing the susceptibility to infections (Mannick, 1993). Shelby and Merrell (1987) found that the depressed CMI occurred only when burn injury exceeded 20% TBSA full-thickness burn. Furthermore, both the *in vivo and in vitro* studies have shown the impaired CMI becoming progressively severe in the postburn period, with the maximal suppression occurring at 7–10 days after injury (Renk et al, 1982; Singh et al., 1986; Cetinkale et al., 1993; O' Sullivan and O' Connor, 1997). So in our study, the depressed CMI was estimated at the 7th day of burn injury in rats receiving a 30% TBSA full-thickness burn.

Melatonin has been found to stimulate the immune response in most conditions tested till now, especially in the immunodeficiency states such as aging, trauma-hemorrhage, viral diseases and cancer (Maestroni et al., 1993; Vijayalaxmi et al., 2002). The immunomodulating effect of melatonin can be affected by several factors such as species, dosage, administration time and the pre-existing immune status. For example, Maestroni et al. (1986 and 1988) found that melatonin administration (0.1-10 mg/kg, s.c.) in the late afternoon counteracted the depressed CMI induced by acute stress or corticosterone treatment in mice, but melatonin injection in the morning did not produce the similar effect. This phenomenon has not been well understood. With reference to their findings, the daily administration of melatonin in this study was only performed in the later afternoon (2 hours before the onset of darkness) for a total of seven days.

Our results showed that CHR was severely suppressed on 7th day after 30% major burn, and melatonin treatment improved CHR significantly. This is the first evidence of

melatonin acting as an immunoenchancing agent in burn injury. No further studies were carried out to reveal the underlying mechanisms, but there are at least two explanations for our finding. Firstly, melatonin can affect a number of important steps in CMI, e.g. improving the antigen presentation (Pioli et al., 1993), stimulating the T cell proliferation and IL-2 production (Maestroni, 1993) as well as enchancing the ADCC (Giordano and Palermo, 1991). With the increasing evidence indicating the wide existence of melatonin receptors in a variety of immune cells, it is very likely that the immunomodulating effect of melatonin in burn injury is receptor-dependent. The binding of melatonin with specific receptors of membrane, cytoplasm or nucleus can modulate the immune cell functions directly or indirectly by the release of immunoregulatory mediators such as IL-2 (Maestroni, 1993).

Secondly, it has been suggested that the increased OFR productions following burn may impair the immune cells in the same way as the tissue cells via lipid peroxidation, protein oxidation and DNA damages (Cetinkale et al, 1999). The circulating immune cells such as lymphocytes, neutrophils and macrophages serve as the first line to be affected. The antioxidant therapy always showed some beneficial effects on depressed immune response, suggesting that the free radicals are an important cause for the immunosuppression following burn (Demling and Lalonde, 1990; O'Sullivan and O'Connor, 1997; Cetinkale et al., 1999). According to these studies, it could be hypothesized that the improvement of depressed CMI by melatonin was partially due to its antioxidant property in burn injury.

4.8. Suggested Future Work

The present study suggests that melatonin applied to burn injury may exert some beneficial effects by acting as an antioxidant and immunomodulator. The antioxidant action of melatonin and its protection against burn-induced MOD are our specific concerns, while the immunomodulating effect of melatonin in burn injury is only a start. More experiments should be carried out to elucidate the following issues.

1. In our study, the repeated melatonin treatment did not show any protective effects on burn-induced oxidative stress and MOD. Although it has been hypothesized that this failure is very likely due to the long injection interval of melatonin, however, the optimal therapy protocol (e.g. dosage, administration time and interval) has to be further investigated. In addition, our findings were obtained in the animal burn model, and this needs to be tested and confirmed in the clinical trials.

2. To better understand the protection of melatonin against burn-induced MOD, the involvement of TNF-α and NO needs to be further studied. Compared to the rapidly degraded TNF-α in the plasma, the tissue TNF-α mRNA expression is more stable and can be measured by RT-PCR or more sensitive methods. Moreover, the total nitrite + nitrate concentrations will be measured once again before further studies are performed to estimate the putative protection of melatonin on NRS-mediated tissue damages in burn injury.

3. Much work can be done to investigate the immunomodulating effect of melatonin in burn injury. In our study, only CHR (a simple *in vivo* method) was used to observe the suppressed cellular immune response as well as the protective effect of melatonin. In subsequent studies, the *in vitro* cellular immune response estimated by the

lymphocyte proliferation, cytokine and NO production, the ratio of Th to Ts cells can provide more information. Additionally, the putative protective effects of melatonin on macrophage dysfunction as well as thymus apoptosis following burn should be very interesting and rewarding research topics.

4.9. Conclusions

(1) 30% major burn induces significant oxidative stress, as evidenced by the increased MDA levels in the plasma (at 6 h postburn) and in the tissues of most tested organs (liver excluded) as well as the reduced tissue levels of GSH in all tested organs (liver, kidney, lung and heart). The burn-induced oxidative stress is evident within 24 h postburn, especially at 6 h postburn. In the kidney, the oxidative damage even lasts up to 72 h postburn.

(2) Burn-induced oxidative stress may result from both the over-produced free radicals and the lowered antioxidative defence capacity. Our study has shown that the large quantities of saline resuscitation (15 ml, i.p.) did not counteract the very rapid fluid loss from the circulation at 6 h postburn, and the resulting tissue ischemia might cause the overproduction of $O_2^{\bullet -}$ and H_2O_2 by activating the xanthine oxidase (XO) system. The increased MPO activities were observed in all tested organs in the present study, suggesting that neutrophil is another important source for free radical generation. In addition, the reduced GSH levels and inhibited GSH-Px activities lowered the total antioxidative defence capacity of the body, and contribute to the burn-induced oxidative stress.

(4) Melatonin has been proved to be a potent antioxidant both *in vivo* and *in vitro*

experiments. Our study investigated the antioxidant action of melatonin in burn injury. Single injection of melatonin (10 mg/kg, given i.p. immediately postburn) markedly inhibited the MDA levels and elevated the GSH level at 6 h postburn, enhanced the activities of GSH-Px and SOD in the liver, and decreased the MPO levels in all tested organs. These findings imply that melatonin may act as an effective antioxidant in burn injury. The antioxidant property of melatonin is most likely due to its very strong free radical scavenging capacity, and in the liver, also due to its stimulation on the antioxidative enzymes. Additionally, the decreased neutrophil infiltration by melatonin treatment may prevent the free radical formation and the ongoing tissue damage in burn injury. Unfortunately, repeated melatonin therapy (10 mg/kg, given i.p. immediately postburn and then supplemented every 12 hours until 72 h postburn) did not exert similar effect as single dose of melatonin. This may be explained by the long interval of melatonin injection relative to its very short half-life.

(4) 30% major burn also induces multiple organ dysfunctions (MOD), estimated by the rises in the serum levels of ALT & AST (for liver dysfunction), creatinine & urea (for kidney dysfunction) and CK & LDH (for heart dysfunction). Similar to burn-induced oxidative stress, these changes are also evident within 24 h postburn, especially at 6 h postburn, but return to the basal level at 72 h postburn. Single dose of melatonin partially inhibited the serum levels of these organ-specific markers, but repeated melatonin treatment had no effect. Considering free radicals play a very important role in the occurrence of MOD, the protection of melatonin against burn-induced MOD may be partially mediated by its antioxidant property. Our study also tried to investigate the possible involvement of TNF-α and NO in the protective

effect of melatonin on burn-induced oxidative stress and MOD, but no conclusions can be drawn, and further studies need to be carried out.

(5) The present study has confirmed the severe immunosuppression after 30% major burn using the contact hypersensitivity reaction (CHR) as a sensitive and *in vivo* method to estimate the immune status. Melatonin treatment (10 mg/kg, given i.p. immediately postburn then repeated daily for a total of seven days) markedly improved the CHR but did not restore it to the level of the sham control. This result suggests another beneficial effect of melatonin as an immuno-enhancing agent in burn injuries.

In conclusion, melatonin may protect against burn-induced oxidative stress and MOD by acting as an effective antioxidant. Melatonin may also enhance the suppressed cellular immune response in major burns. These beneficial effects together with the very low toxicity may enable melatonin to become a promising therapeutic agent of clinical significance in burn injuries.

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