INVESTIGATION OF MACROPHAGE MEDIATED HOMEOSTASIS DURING MALARIA

A Thesis
Submitted in Partial
Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

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December 2013

Dedicated to
My Family and Guruji

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STATEMENT

I hereby declare that the matter embodied in this thesis entitled "**Investigation of Macrophage Mediated Homeostasis during Malaria**" is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India under the joint supervision of **Dr. Vishal Trivedi** and **Dr. Vikash Kumar Dubey**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Investigation of Macrophage Mediated Homeostasis during Malaria"** by **Mr. Rohitas Deshmukh** (Roll No: 09610624), submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under our supervision at the Department of Biotechnology, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

Dr. Vishal Trivedi
(Supervisor)

Dr. Vikash Kumar Dubey
(Co-supervisor)

Acknowledgements

I take the opportunity to express my indebtness to all those who have made this thesis possible. I would first of all like to pay obeisance to the almighty for giving me ability, courage & strength to face and cope up with the barriers & hurdles that came by my way. It is moment of great pleasure and immense satisfaction for me to express my reverence, deep gratitude and respect to my esteemed guide, **Dr. Vishal Trivedi**, who was continuous source of inspiration throughout the course of my work. I do not have words to express my feelings about his dedication, hard work and quest for the research. His deep involvement, logical and analytical thinking has made a big difference towards the research. I consider myself to be fortunate to work under his guidance and I am indebted for shaping my research life. I am grateful to him for seeking support to improve by all means. I hope it will be continued.

I express my sincere thanks to my research co-supervisor **Dr. Vikash Kumar Dubey** for his valuable guidance throughout my research work. His continuous suggestions gave me a lot of inspiration to carry out my work.

I am thankful to the other members of my Doctoral Committee, **Dr. K. Pakshirajan, Dr. Sanjukta Patra, and Dr. Santabrata Das** for assessment of my work and giving valuable suggestions and advices which enabled me to improve my work.

I am grateful to the successive Heads of Department of Biotechnology, Indian Institute of Technology Guwahati, **Prof. Arun Goyal** and **Prof. V.V. Dasu**, for providing me the departmental facilities to carry out my research work.

I would also like to thank the technical and non-technical staffs of the department for their help and assistance. A very special thanks to Mr. **Nurul Islam** for his help and cooperation and technical assistance throughout my PhD work.

I am thankful to my lab members **Kimjolly, S.N.Balaji, Suman, Arnish, Sushant, Sourav,** and **Anish** as well as ex-lab members **Ananya, Ankita, Rakesh, Anupriya,** and **KMN Prasad** for their help, cooperation and also for providing a healthy working environment in the lab.

I am also thankful to all the members of **Dr. Dubey's** group for their friendly behavior and kind cooperation. A special thanks to **Shalini Singh** for critically reviewing my thesis.

I am also thankful to **Himanshu Sharma** for helping me in operation of Acta Purifier and Gel filtration columns and **Aditya Ayer** for helping me with AFM studies.

I owe my special thanks to my friend; **Dr. Abhay Narayan Singh, Rishikesh Shukla, Dr. Anil Shukla, Manjeet Kumar, Himangshu Sonowal, Sushant Singh, Devendra Maravi** and **Sahil Batra** for providing valuable suggestions, help and motivation. My other friends at IIT Guwahati and Art of Living family members deserve special mention for making my stay in the campus lively and enjoyable. Their love and affection has been a constant source of motivation that helped me sail through difficult times.

I am thankful to Indian Institute of Technology Guwahati, India and its Department of Biotechnology for providing me the best facilities to carry out my doctoral research. I am also thankful to Indian Institute of Technology Guwahati for research fellowship.

Last but not the least; I owe personal gratitude to my parents, family members and my beloved wife **Madhuri** for their endless love, support and encouragement.

I have tried all my best to remember & thanks all those who helped me in my thesis work but in case I miss out on any of the names this is my special thanks to them.

Rohitas Deshmukh

December, 2013

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ABBREVIATIONS

BBB	Blood Brain Barrier	**MMPs**	Matrix Metalloproteases
BSA	Bovine Serum Albumin	**Mϕ**	Macrophage
CLT	Clotrimazole	**MTT**	3-(4,5-dimethyl thiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide
CM	Cerebral Malaria	**NAC**	N-Acetyl Cysteine
DMEM	Dulbecco's Modified Eagle Medium	**PBN**	Phenyl N-tert-butylnitrone
DNPH	Dinitrophenyl Hydrazine	**PBS**	Phosphate Buffer Saline
E.coli	*Escherichia coli*	**PRBC**	Parasited Red Blood Cell
EDTA	Ethylene Diamine Tetra Acetic Acid	**PS**	Phosphatidylserine
Et-Br	Ethidium Bromide	**RBC**	Red Blood Cells
FBS	Fetal Bovine Serum	**ROS**	Reactive Oxygen Species
FITC	Fluorescein Isothiocyanate	**SD**	Standard deviation
GPIs	Glycosylphosphatidylinositols	**TBARS**	Thiobarbituric Acid Reactive Substances
GSH	Glutathione	**TCA**	Trichloro Acetic Acid
HO-1	Heme Oxygenase-1	**TEMPO**	2,2,6,6-Tetramethylpiperidine 1-oxyl
HP	Heme Polymer	**TLR**	Toll like Receptors
Hz	Haemozoin	**TNF-α**	Tumor necrosis factor-α
IL	Interleukin	**UV**	Ultra Violet
LPS	Lipopolysaccharides	**βH**	β-hematin
MDA	Malondialdehyde	**β-ME**	β-mercaptoethanol
MetHb	Methemoglobin	**LPS**	Lipopolysaccharides

UNITS

µg	Microgram	**h**	Hour
µl	Microlitre	**mg**	Milligram
µg/ml	Microgram per Milliliter	**mg/ml**	Milligram per Milliliter
µm	Micrometer	**min**	Minute
µM	Micromolar	**mM**	Millimolar
°C	Degree Celsius	**nm**	Nanometer
gm	Gram	**rpm**	Revolution per Minute
gm/l	Gram/Liter	**w/v**	Weight/Volume
gm/dl	Gram/Deciliter	**v/v**	Volume/Volume

SYNOPSIS

Cell of body perform various metabolic process and chemical reaction perfectly to maintain homeostasis. But homeostasis of body is constantly threatened by many factors. One such factor is malaria where RBC is infected by *Plasmodium* species. Malaria parasites during their life cycle in RBC, causes the rupture of RBC to release daughter merozoite along with various pro-oxidants such as Hb, MetHb, hemin and haemozoin. These released pro-oxidants can affect the various cells of immune system to disturb body homeostasis. Therefore in order to understand the mechanism for immuno-depression during malaria we have studied the effect of various pro-oxidants on macrophages which are important cells of immune system. Based on the present knowledge and results of the experiments, the thesis **"Investigation of Macrophage Mediated Homeostasis during Malaria"** is divided in following chapters:

Chapter I. Introduction and Literature Review

This chapters deals with the insight on macrophages biology (origin, distribution and functions), and role of various pro-oxidants molecules in disturbing homeostasis released from ruptured malaria infected RBC. An updated literature on effects of methemoglobin, hemin, haemozoin and glycosylphosphatidylinositol on the various cells, tissue and organ system has been given. Hemin in free form is highly toxic, therefore a detail literature search has also been done on hemin detoxification systems present in the malaria parasite, and in-*vitro* operating bio-mineralization process.

Chapter II. Experimental Procedures

This chapter deals with the experimental procedures in details so that research work described can be reproduced. In this chapter more focus has been given to describe experimental procedures used throughout the thesis whereas procedures related to the specific experiment are given in the respective relevant chapters.

Chapter III: Methemoglobin Exhibits Toxicological Effects in Macrophages due to Multiple ROS Spike Induced Apoptosis

This chapter highlights the effects of methemoglobin exposure on a macrophage J774A.1 viability, cellular integrity and underlying molecular mechanism of its toxic response. It is observed that MetHb reduces viability of macrophage cells in a dose dependent manner and death of macrophages was due to the apoptosis. MetHb treatment generates ROS

spikes periodically inside the macrophage cytoplasm to develop oxidative stress which was responsible cytotoxicity and apoptosis. Work in this chapter indicates that death of macrophage is one of the reasons for immune-depression and tissue damage observed during malaria.

Chapter IV: Mutual Cross-Talk Modulates Immuno-toxic Potentials of Pro-oxidant Molecules towards Macrophages

This chapter focused on the cytotoxic effects of β-hematin in presence of non-toxic concentration of methemoglobin and underlying molecular events in mouse macrophage cell line J774A.1. Results show that β-hematin in combination of non-toxic concentration of methemoglobin dose dependently has reduced macrophage viability. Combination of β-hematin with methemoglobin treated cells shows a significant amount of ROS to cause oxidative stress and death via apoptosis. MetHb causes the generation of β-hematin radical by one electron transfer mechanism which is cytotoxic to the macrophages. The chapter highlights the contribution of MetHb in potentiating the β-hematin toxicity towards macrophage and its role in immune depression during malaria.

Chapter V: Methemoglobin Mediated Homeostatic Disturbance through Hemin Oxidation and Polymerization

This chapter aimed to identify and characterize the role of major pro-oxidant factor present in the malaria culture supernatant contributing inflammation during malaria. Methemoglobin is the pro-inflammatory factor present in the culture supernatant which can polymerize hemin to heme polymer by its peroxidase activity. Methemoglobin accepts hemin as a substrate and oxidizes it through a single electron transfer mechanism to form heme polymer. The heme polymer is pro-inflammatory in nature and produces ROS inside the macrophage, and methemoglobin further amplifies the potential of heme polymer to produce several folds high ROS from macrophages. This chapter illustrates the pro-inflammatory effect of MetHb, the underlying novel mechanism by which this occurs and possible clinical interventions.

Chapter VI: Role of LPS Stimulated Macrophages in Maintaining Homeostasis during Hemin Toxicity

In this chapter we explored whether lipopolysaccharide (LPS) stimulated macrophages can be able to reduce the level of free hemin present in the microenvironment during malaria. It was observed that LPS stimulated macrophages culture supernatant shows a heme polymerization activity. The secretion product was specific in response to amount

of bacterial (LPS) stimulation. ROS was generated inside the macrophages in response to stimulation and was responsible for the secretion of activity in the macrophage supernatant. The secretion product is proteinous in nature and require hydrogen peroxide for its optimal activity. In an *in-vitro* condition the secretion product protects the macrophages from free hemin toxicity by converting it into less toxic heme polymer. This chapter highlights new mechanism for the hemin detoxification which is being operated in LPS stimulated macrophages.

Introduction and Literature Review

1.1 Introduction

Cell is the basic structural and functional unit of life. It forms tissues, organs, and organ systems in the body. A normal human individual consists of more than 50 trillion cells working together to perform diverse functions. For the growth, development and survival of individual cell and whole body, it is important to maintain a constant internal environment. Thus the various processes by which the cell or body maintain and regulates its inner environment is collectively termed as **"Homeostasis".** It may be defined as ***"the condition of equilibrium (balance) in the body's internal environment due to the consistent interaction of the body's main regulatory processes"*** (Tortora G. T 2009). The homeostatic condition disturbed by pathogenic microbes is restored by concerted action of various immune cells. During the protective or restoration process, cells of immune system release various factors (pro-inflammatory cytokines) which may leads to homeostasis imbalance. Macrophages of the immune system are main player responsible for maintaining homeostasis of the body.

1.2 Macrophage biology

The term 'macrophage' was first used by Elie Metchnikoff about 100 years ago to describe the large mononuclear phagocytic cells he observed in tissues (Karnovsky 1981). In 1924, Aschoff named these cells to the reticuloendothelial system (RES), a broad system of cells which included reticular cells, endothelial cells, fibroblasts, histiocytes and monocytes. Macrophages are the major differentiated cells of the mononuclear phagocyte system (MPS) which includes bone marrow monoblasts, promonocytes and peripheral blood monocytes. Macrophages originates from human bone marrow which compises of resident macrophages, as well as their precursors; monoblasts, promonocytes and monocytes. Monoblast gives rise to two promonocytes and each dividing promonocyte gives rise to two monocytes. Newly formed monocytes remain in the bone marrow for less than 24h before entering to the peripheral blood (van Furth and Sluiter 1986). The migration of peripheral blood monocytes into extravascular tissues to become resident tissue macrophages involved in various processes like adherence to the endothelium, diapedesis between endothelial cells and migration through subendothelial structures.

1.2.1 Distribution of macrophages in host body

Macrophages are the important cells of host immune system which are widely distributed throughout the body. It displays structural and functional heterogeneity to participate in wide range of physiological and pathological processes. They are found in lymphoid organs, liver, lungs, gastrointestinal tract, central nervous system (CNS), serous cavities, bone, synovium and skin. Various types of macrophages present in body are as follows:

Kupffer cells- Kupffer cells present in liver are the largest proportion and account for more than 50% of the total tissue macrophages present in the body. They are mainly distributed in the lumen of hepatic sinusoids and attached to the outer surface of endothelial cells. It allows them to constantly trap gut-derived bacteria, microbial debris and bacterial endotoxins. This exposure activates them which results in release of inflammatory mediators such as TNF-α, IL-1, IL-6, prostaglandins and nitric oxide (NO). They also express high levels of phagocytic receptors like Fc receptors, complement receptors, scavenger and TLRs by which they identify and remove bacteria, endotoxin from the circulation (Naito, Hasegawa et al. 2004). During malarial infection, kupffer cell recognizes circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP) of sporozoite to phagocytose and clear the malaria parasite (Murthi, Kalionis et al. 2006).

Alveolar macrophages- These macrophages work as the front line of cellular defense against the respiratory pathogens and protect the blood-alveolar interface. They are found in the pulmonary alveolus, near the pneumocytes at the interphase between air and lung tissue. They are associated with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) during malaria. Macrophages activation by malaria parasite components (haemozoin etc) lead to the secretion of various pro-inflammatory cytokines, like IL-1β and IL-18 to contribute in ALI/ARDS (Mohan, Sharma et al. 2008). Macrophages upregulate adhesion molecules on lung endothelial cells and induce sequestration of malaria infected RBCs. It is in turn increases vascular permeability and allow access of lung epithelial cells to cause local injury via inflammatory mediators (Dolinay, Kim et al. 2012).

Splenic macrophages- Spleen is divided into white pulp and red pulp separated by the marginal zone. Macrophages present in the marginal zone are sub-classified as marginal metallophilic macrophages (MM) and marginal zone macrophages (MZM) (Buckley, Smith et al. 1987).These macrophages express high level of scavenger receptor to uptake the blood-

born particles. The macrophages present in red pulp region red pulp macrophages (RPM) are actively phagocytic and remove old and damaged erythrocytes. Furthermore, malaria parasite containing infected RBC can be removed by splenic macrophages through a process called "pitting"(Angus, Chotivanich et al. 1997).

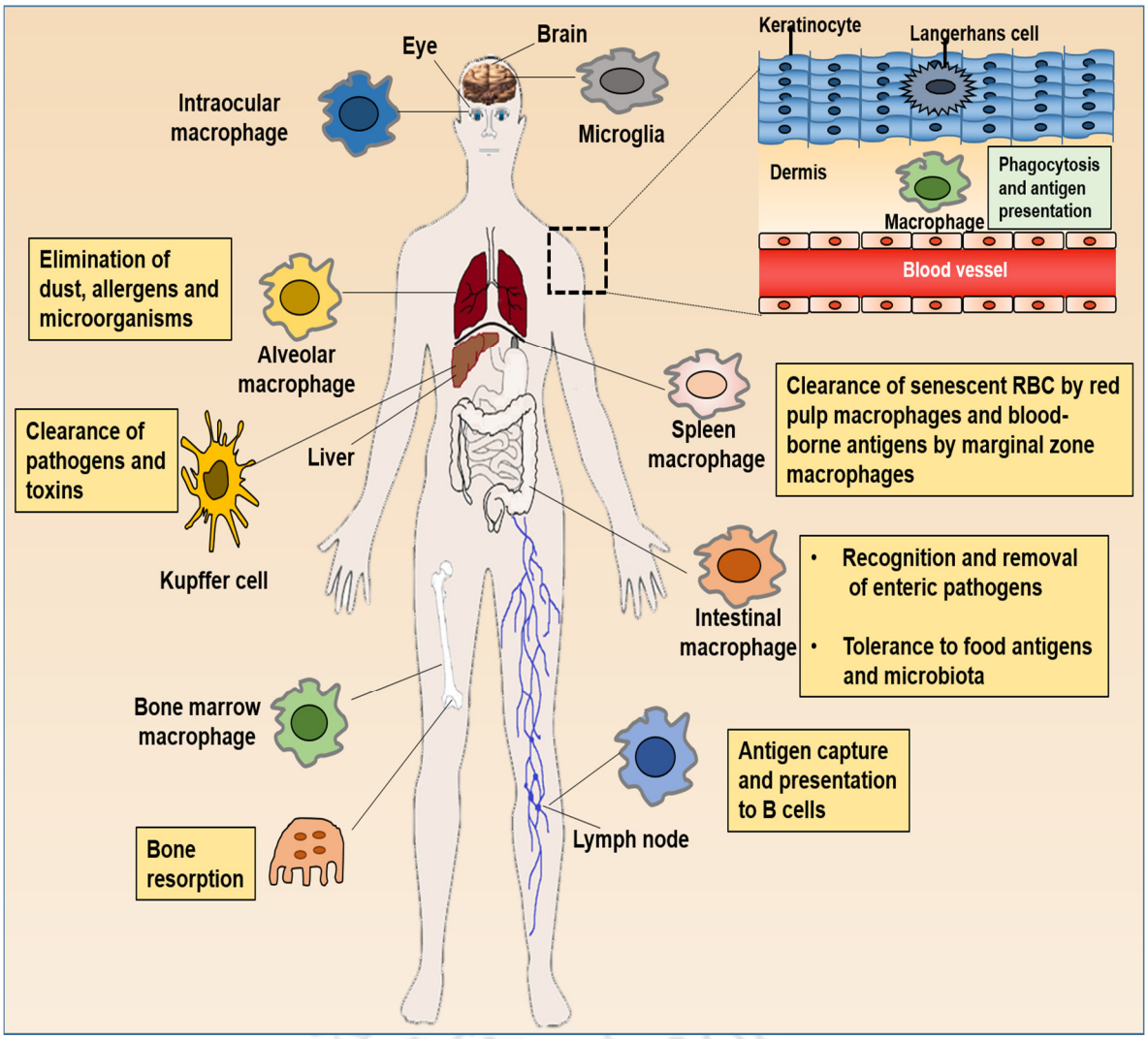

Figure 1.1: Distribution of macrophages in different parts of human body. Different tissue associated are strategically placed to all over the body to execute immune surveillance activities, including phagocytosis, antigen presentation, tissue repair, removal of dead cells and senescent RBCs.

Bone marrow macrophages- Macrophages present in bone marrow supports hemopoiesis. Bone marrow contains network of mature macrophages which ramifies through the stroma. These stromal macrophages make intimate contact with 'nest' of developing hemopoietic cells. The stromal macrophages are essential for maintaining the growth and differentiation of

hemopoietic precursors. They possess adhesion molecules for maintaining contact with developing hemopoietic cells (Hanspal 1997).

Microglia cells- They are mononuclear phagocytes of the central nervous system and distributed throughout the brain, spinal cord. Adequate and proper function of microglial cell is crucial for maintaining homeostasis of the CNS in both health and disease condition. In normal physiology, these cells remains in resting state. But activated microglia express TLRs, CD14, and mannose receptors to recognize pathogen-associated molecular patterns (such as LPS of the gram-negative bacteria, peptidoglycan and teichoic acid from gram positive bacterial cell wall). It results in phagocytosis and release of various cytokines/chemokines, MMPs and inflammatory molecules. These molecules contribute to both defense against infectious organism and neuro-pathogenesis of CNS. The release of pro-inflammatory cytokines, nitric oxide and ROS contribute to adverse and degenerative process in the brain. During cerebral malaria, activated microglia cells contribute to lesion formation by modulating the blood flow, secretion of pro-inflammatory cytokines, release of free radicals and immunological dysfunction (Deininger, Kremsner et al. 2002; Luo and Chen 2012).

1.2.2 Macrophage functions

Macrophages are important cells of the immune system responsible for maintaining homeostasis. They play central role in removal of foreign pathogens, clearance of apoptotic and necrotic debris (removal of senescent RBC), wound healing, tissue repair and remodeling. Macrophages accomplishes these primary roles through following functions:

Recognition of foreign particles- Macrophages sense the local environment through presence of different types of pattern-recognition receptors (PRRs) on their cell-surface and within intracellular endosomes (Figure 2.1A). The macrophage PRRs can identify molecular patterns common to broad range of pathogenic and non-pathogenic microbes. The receptors present on macrophages can generally be divided into two sensing groups: pathogen and danger signals (exogenous) or modified host proteins, lipids and necrotic/apoptotic cellular debris (endogenous). When the macrophages receptor detect a ligand, it generates signal to direct the immunological responses. A list of selected PRRs with their cognate ligand and localization within cell is given in Table 1.1

Table 1.1: List of selected PRRs with their ligands and localization

Receptor	Ligands	Location	References
TLR 1	Lipopeptides	Cell Surface	(Rock, Hardiman et al. 1998)
TLR 2	Peptidoglycan, lipopeptides, lipoteichoic acid, porins, lipoarabinomannan, zymosan	-do-	(Rock, Hardiman et al. 1998)
TLR 4	Lipopolysaccharide, heat shock protein, fibrinogen, viral protein (e.g. F protein, Env protein), Taxol	-do-	(Rock, Hardiman et al. 1998)
TLR 5	Flagellins	-do-	(Rock, Hardiman et al. 1998)
TLR 6	Lipoteichoic acid, zymosan	-do-	(Takeuchi, Kawai et al. 1999)
TLR 3	dsRNA, siRNA, viral RNA	Endosomal	(Rock, Hardiman et al. 1998)
TLR 7	ssRNA, synthetic agonist R848	-do-	(Du, Poltorak et al. 2000)
TLR 9	CpG-ODN, unmethylated DNA	-do-	(Du, Poltorak et al. 2000)
NOD family	MDP (MurNAc-L-Ala-D-isoGln)	Cytoplasmic	(Hugot, Laurent-Puig et al. 1996; Inohara, Koseki et al. 1999)
NALP family	Bacterial/viralRNA, poretoxins, ATP, uricacid	-do-	(Tschopp, Martinon et al. 2003)
NAIP	Flagellin	-do-	(Roy, Mahadevan et al. 1995)
RIG-I	5'-triphosphate dsRNA	-do-	(Hornung, Ellegast et al. 2006)
MDA5	dsRNA	-do-	(Kang, Gopalkrishnan et al. 2002)

Phagocytosis, antigen presentation and killing of micro-organisms- Phagocytosis is a dynamic process by which pathogens and unwanted tissue debris are scavenged from the host body. Phagocytosis starts when macrophage extend its pseudopodia around the foreign particle (such as microorganism) and entrap it into vesicular structure called as "phagosome". The phagosomal compartment subsequently fuses with lysosomes to form phago-lysosome, facilitating the destruction of ingested material into smaller peptides (Figure 1.2B). In addition, micro-organisms are killed by ROS, such as superoxide, H_2O_2 and hydroxy radicals released after respiratory burst (Klebanoff 1998). Oxidized halogens (HOCl) is known to destroy many bacterial components including nucleotides and redox enzymes at a rapid rate. Besides foreign particles, dead and damaged cells are cleared by the macrophages through phagocytosis. Aged normal RBCs bind nonspecifically large quantities of Ig recognized by the macrophage Fc

receptors and phagocytized. Once ingested, the RBCs are degraded to liberate iron from hemin and stored in the form of protein complexes to support erythropoiesis (Connor, Pak et al. 1994). The peptide fragments activate other immune cells (T-lymphocytes) to produce immunological response to further clears foreign pathogens (Janeway and Medzhitov 2002).

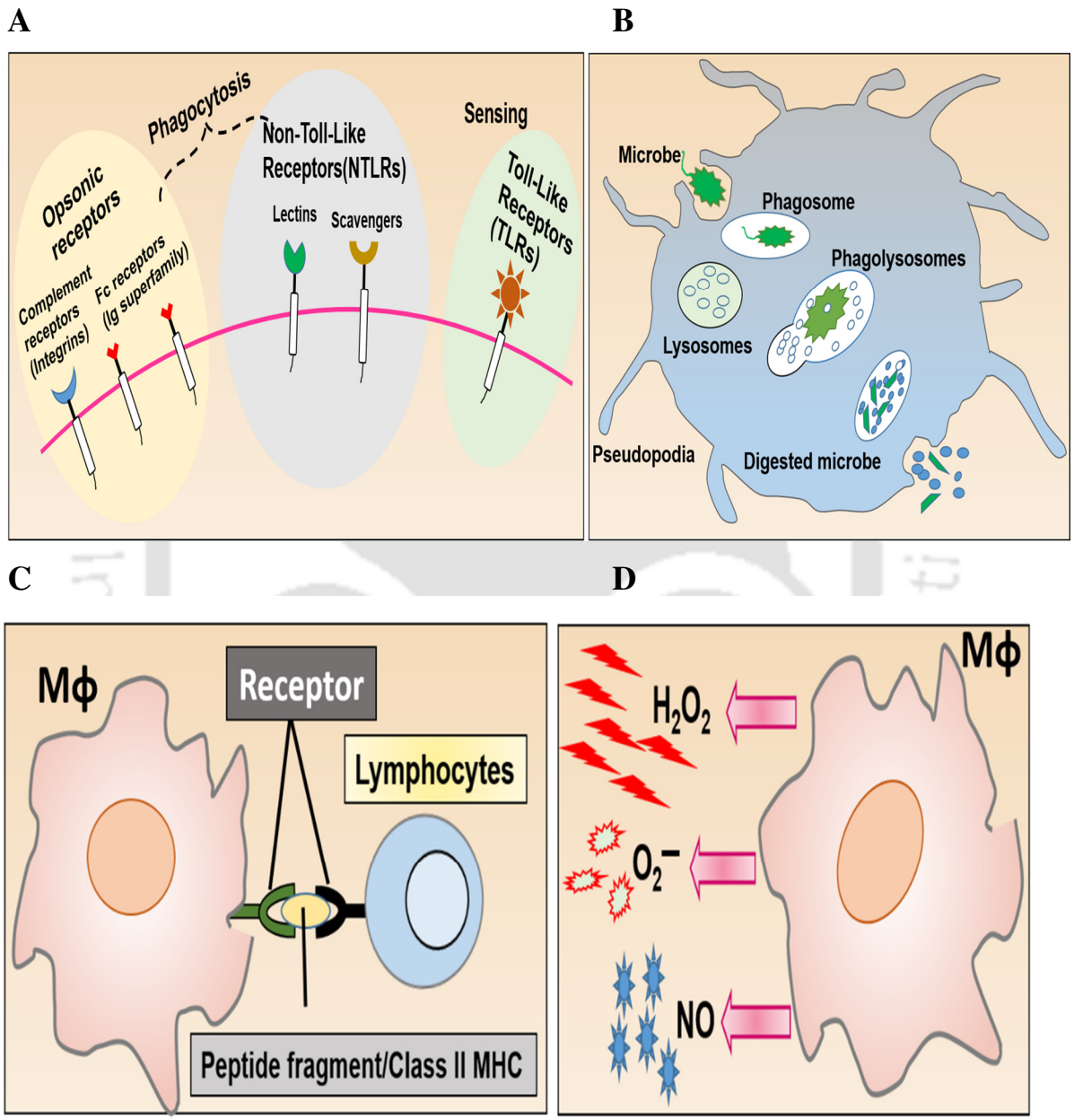

Figure 1.2: Different functions performed by macrophages to maintain homeostasis. (A) Different receptors present on macrophages surfaces for sensing foreign particles. **(B)** Phagocytosis of microbes followed by formation of phagolysosomeand discharge of waste material. **(C)** The peptide fragment of degraded microbes are presented along with the help of class-II MHC on the macrophages cell surface to perform antigen presentation to lymphocytes.. **(D)** Release of inflammatory molecules which destroy microbes present in microenvironment.

Inflammation- Macrophages exposed to bacterial components or interferon-γ (IFN-γ) activate to produce wide array of inflammatory molecules to inhibit or kill pathogens (Figure 1.2D). They produces reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide and reactive nitrogen intermediate (RNS) to oxidize pathogenic organism. The inflammatory molecules reduce the microbial burden in host but excess prodution lead to host tissue damage and disturb homeostasis.

Tissue repair and remodeling- Macrophages play an important role in wound healing, tissue repair and remodeling**.** At wound site, injury results in accumulation of dead and apoptotic cells and cellular debries which will be phagocytized by macrophages. Additionally, at wound bed macrophages secrete transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2) and insulin like growth factor-1 (IGF-1) to stimulates collagen production from fibroblast cells. Later, macrophages secrete MMPs to degrade collagen which helps fibroblast and endothelial cells to migrate at wound site for new blood vessels formation (Werner and Grose 2003).

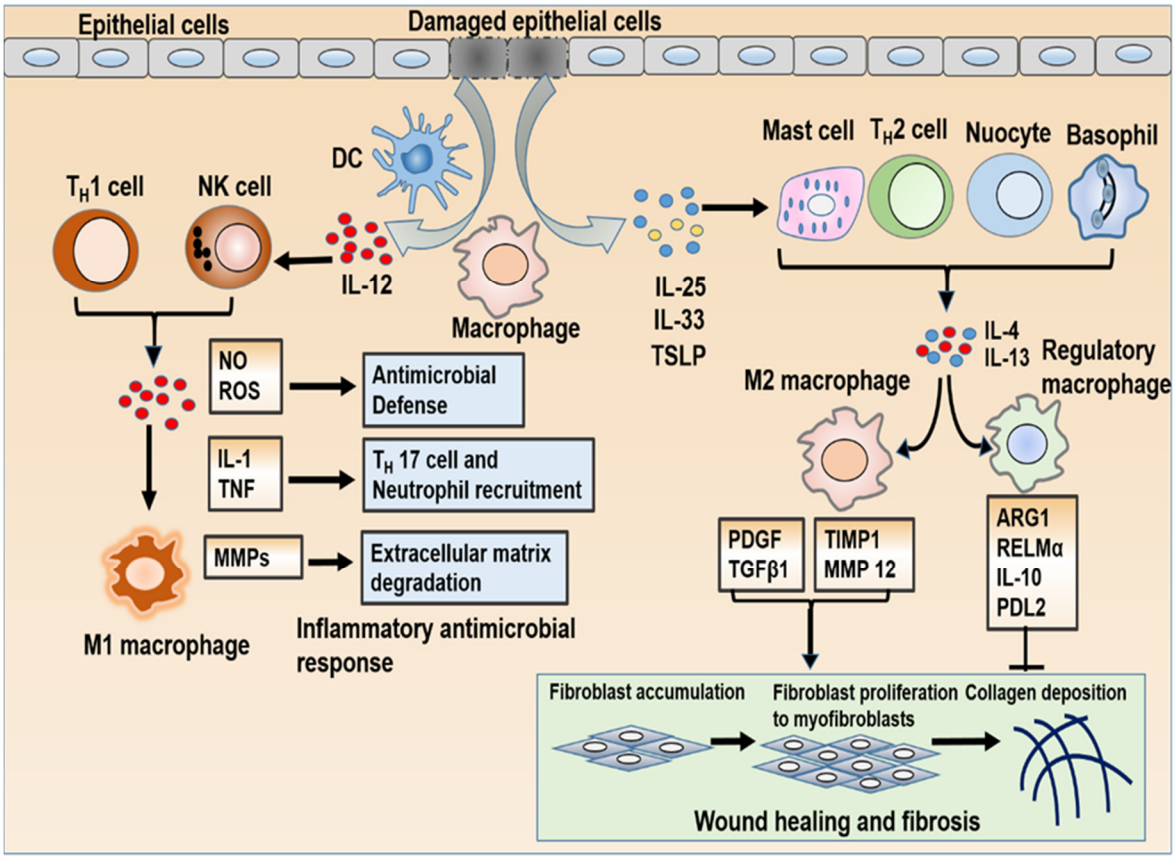

Figure 1.3: Overview of events during tissue damage to maintain the homeostasis.

Production of TNF-α and vascular endothelial growth factor (VEGF) from macrophages induce angiogenesis (Tammela, Zarkada et al. 2008). Macrophages play additional role in healing of skin injury by restoration of damages keratinocyte (re-epithelialization). TGF-β from macrophages promotes keratinocyte migration and development on the newly formed extracellular matrix. MMPs from macrophages dissolve the clot at injury site for smooth migration of keratinocyte and extracellular matrix for tissue remodeling. Figure 1.3 summarizes the role of macrophages in tissue repair and remodeling.

1.2.3 Different modes of macrophage activation

Macrophages are involved in innate and adaptives immunity to provide defense against invading pathogens and maintain homeostasis. The microenvironment (microbial or inflammatory) stimulus phenotypically activates and differentiate macrophages to perform specific functions. There are four different modes of macrophage activation known:

Classical activation- Macrophages exposed to bacterial components (TLR agonist) and interferon-γ (IFN-γ) result in classical activation of macrophages. The classical activation in macrophages express an enhanced levels of major histocompatibility complex (MHC) and CD80/86. It enables them to act as APCs with enhanced nitric oxide (NO), superoxide radicals as well as other proteolytic enzymes (Figure 1.4A).

Alternate activation- Macrophages stimulated with cytokines (IL-4 or IL-13) derived from T-cells or parasitic infection/protein antigens result in alternatively activated macrophages (Figure 1.4B). The exact *in vivo* role of the alternatively activated macrophages has not completely characterized, but it has role in response to parasitic infection, asthma, controlling inflammatory reactions and the induction of humoral response (Modolell, Corraliza et al. 1995).

Innate activation- Macrophages exposed to bacterial components (such as LPS or CpG) results in innate activation with altered phenotypic and functional properties (Figure 1.4C). Macrophages exposed to TLR agonists increase the expression of macrophage receptor with collagenous structure (MARCO) without a secondary stimulus, as well as enhance ability to produce IL-12. MARCO increases the phagocytic ability of the macrophage towards broad classes of bacteria especially *Neisseria* (Mukhopadhyay, Chen et al. 2006).

Deactivation-Once the macrophage mediated immunological reaction is over, it is necessary to downregulate the pro-inflammatory program and bring macrophages to deactivation mode (Figure 1.4D). Deactivation of macrophages is induced by exposure to anti-inflammatory cytokines (e.g., IL-10, TGF- β), receptor ligation (e.g., CD200 - CD200R), steroids, or uptake of apoptotic cells. Deactivated macrophages show high expression of CD163, reduced expression of MHC class II and decreased respiratory burst (Komohara, Hirahara et al. 2006).

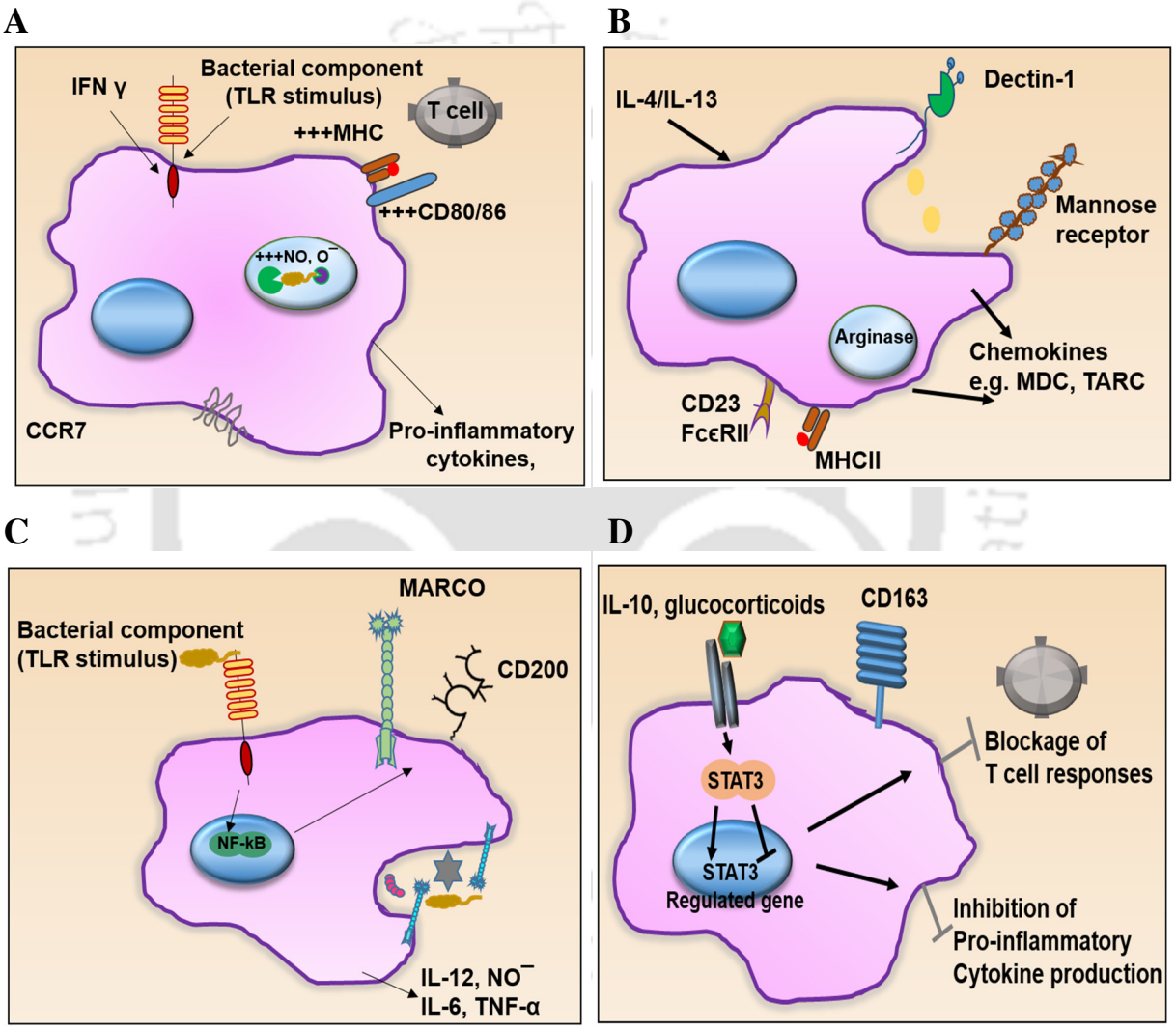

Figure 1.4: **Macrophage activation phenotypes. (A)** Classical activation **(B)** Alternative activation **(C)** Innate activation **(D)** Deactivation.

1.3 Pro-oxidant molecules and homeostasis

During erythrocytic stages of malaria and other hemolytic disorder, RBC rupture releases various pro-oxidant molecules such as hemoglobin (Hb), methemoglobin (MetHb), hemin (He) and haemozoin (Hz) (Shio, Kassa et al. 2010). Effects of these pro-oxidants in disturbing

homeostasis is summarized in Figure 1.5. In the following section discussion will be on the pro-oxidants and their effect on various cells, tissue or organ system and resulting disturbance on homeostatsis.

1.3.1 Methemoglobin

During malaria, lysis of malaria infected RBCs result in the release of hemoglobin (Hb) into the circulation. Free Hb is highly unstable and is readily oxidized by molecular oxygen to form methemoglobin (MetHb) with Fe^{3+} or oxidation by reactive oxygen species (ROS) into ferryl hemoglobin (Fe [IV] = O). But ferryl Hb is highly unstable and gets converted into the MetHb through electron transfer. In addition to direct oxidation of Hb, other extrinsic factors such as toxic drug molecules, metabolic by-products of pathogenic organisms, and pro-inflammatory cytokines cause redox imbalance and oxidized intracellular Hb to form MetHb [32]

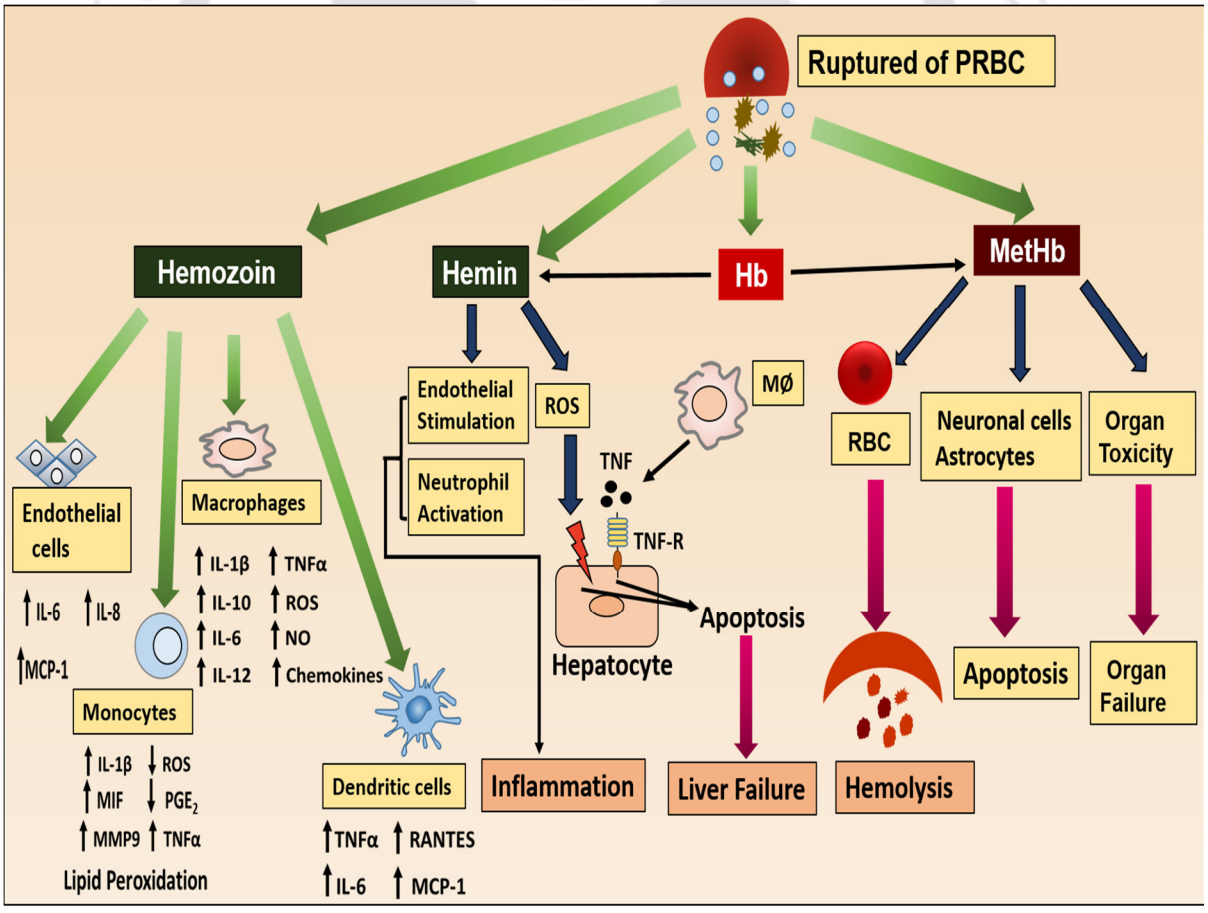

Figure 1.5: Rupture of malaria infected RBC releases various pro-oxidants molecules in circulation to disturb homeostasis

1.3.1.1 MetHb and homeostasis

In normal physiology, MetHb level remains below 2% but during severe malaria condition the concentration reaches up-to toxic levels (>4%) and it effects various cells and organ. The various ways in which it disturbs homeostasis are as follows:

MetHb and RBC physiology- MetHb is pro-oxidant in nature and exhibits toxicity towards RBCs to disturb the homeostasis. During malaria, an enhanced RBC lysis occurs, where single parasitized RBC (PRBC) causes lysis of more than ten uninfected RBCs, leading to the development of severe hemolytic anemia (Pamplona, Hanscheid et al. 2009). MetHb dose dependently makes RBC susceptible to osmotic stress and causes hemolysis. Pseudo-peroxidase activity of MetHb causes production of large amount of ROS in microenvironment. It disturbs the redox balance of RBC to develop oxidative stress as indicated by increase in lipid peroxidation, protein carbonyl and decreased GSH level. As a result of oxidative stress, cytoskeleton changes occur which results in loss of membrane fragility and hemolysis. MetHb initiates a type of chain reaction where initially released MetHb causes destruction of more RBC and amplify the lysis reaction to cause anemia as observed during severe malaria (Balaji and Trivedi 2011). Oxidative stress within RBC causes phosphatidylserine (PS) externalization to generate sticky patches to initiate aggregation. Higher degree of RBC aggregation (5-10) is accountable for vascular blockage and associated with the development of patho-physiology of malaria.

MetHb and cellular toxicity- *Plasmodium* infection is associated with oxidative stress in RBC, responsible for membrane lipids/proteins oxidation, leading to band-3 clustering as well as PS externalization and opsonization of RBC membranes. The modifications on the surface of the infected RBCs are sufficient for the recognition of the cells by macrophages and their consequent phagocytosis. Moreover, free Hb is also taken up by macrophages from circulation as a detoxification mechanism. It is shown that in an *in-vitro* system these extracellular Hb effect the normal functioning of macrophages and induces death (Cambos and Scorza 2011). MetHb has a potential to produce large amount of peroxide which accelerate oxidative-mediated cellular and tissue damage. Pro-oxidant nature of MetHb leads to changes in physiology and biochemical behavior of vascular endothelium and smooth muscles. MetHb activates endothelial cells to upregulate the production and release of IL-8, IL-6 and adhesion

molecule E-selectin. This result in inflammation and vascular occlusion in brain and explains the severe patho-physiological outcomes during cerebral malaria (Liu and Spolarics 2003).

MetHb and organ toxicity - Released MetHb gets accumulated at various tissues sites and exhibits toxicity towards various organ systems. Hemolysis of RBC leads to increased accumulation of MetHb in the cardiac tissue and increased iron load to cause cardiac toxicity. It was also believed that in certain animal species MetHb scavenge the nitric oxide (NO) to cause myocardial tissue necrosis (Buehler and D'Agnillo 2010). MetHb released after intra cerebral hemorrhage plays an important role in neurotoxicity and edema development. In an *in-vitro* culture MetHb exhibits cytotoxicity towards brain and spinal cell to cause death in an oxidative stress dependent mechanism to develop coma and death as evidenced in the cases of cerebral malaria. Moreover, exposure of MetHb to brain cells cause up-regulation of HO-I in microglia cells, implying that MetHb was taken up by microglia cells. At high concentration the uptake results in death of microglia cells to disturb homeostasis (Matz, Turner et al. 1996). MetHb exposure to the pulmonary system causes changes in the vascular compliance, elasticity, distensibility, and stiffness of pulmonary cells. Generation of ROS, inflammation and autacoid disregulation are the overall pathology of MetHb in lungs which leads to disturb homeostasis (De Castro, Jonassaint et al. 2008).

1.3.2 Hemin

Hemin is an essential molecule in living system which plays vital role in multiple biological processes like respiration, oxygen transport and signal transduction. It is present as prosthetic group in hemo-proteins that are important for cellular function and metabolism. Structurally hemin consists of an iron surrounded by four porphyrin ring to form iron-protoporphyrin complex. During patho-physiological state, hemin is being released from the protein and present in free form. In case of malaria, free hemin is released from the rupture of *plasmodium* infected RBC or from MetHb in presence of reactive oxygen species (ROS). As a result the concentration of hemin reaches more than 20µM which is cytotoxic concentration. Such high concentration is responsible for the observed malaria patho-physiology.

1.3.2.1 Hemin and homeostasis

Hemin exhibits toxic effects to cells and tissues to disturbs body homeostasis. The pro-oxidant nature of hemin is known to cause toxicity towards various cells, tissue and organ system which is as follows:

Hemin and RBC physiology- The hydrophobic nature of hemin facilitates insertion into the cell membrane and increases cell's susceptibility to oxidant-mediated killing. It is reported that hemin after getting incorporated into the intact RBC results in mechanical disruption of the RBC membrane, oxidation of sulfhydryl groups, lipid peroxidation, potassium loss and swelling.In addition, it also disturbs cation gradient and induces hemolysis by a colloid–osmotic mechanism. There are 2 phases for the hemolytic process induced by hemin. (1) in phase I, loss of potassium ion leads to the depletion of glutathione and ATP wheras in phase II, massive hemoglobin loss takes place (Chou and Fitch 1981). Hemin mediated cross-linking of cytoskeletal proteins (such as spectrin and protein 4.1) induces conformational structural abnormality in RBCs (Liu, Zhai et al. 1985). Exposure of hemin to RBC causes externalization of phosphatidylserine (PS), an early signal of apoptosis (eryptosis). PS expressing RBCs are engulfed by macrophages and are rapidly eliminated from circulation to cause anemia (Boas, Forman et al. 1998). It is also reported that eryptotic cells may adhere to the vascular wall and may compromise the microcirculation. Thus, hemin mediated eryptosis further increases the pro-oxidants burden in the circulation to severely affect host homeostasis.

Hemin and macrophages- During malaria, macrophages perform robust erythrophagocytosis. Hemin accumulation within the macrophages induce ROS and may cause lipid peroxidation, DNA damage and/or protein aggregation, leading to cell death via apoptotic pathway (Cambos and Scorza 2011). It is reported that hemin causes early macrophage death characterized by the loss of plasma membrane integrity and morphologic features resembling necrosis. Hemin induced macrophage necrosis follows TLR4/Myd88 dependent expression of TNF-α and TLR4 independent ROS generation (Fortes, Alves et al. 2012).

Hemin and inflammation- Inflammation is a defense system of body which is essential for protection against pathogens and for cleaning up of damaged cells after injury. Under normal physiology there is resolution of inflammation after elimination of pathogens to maintain homeostasis. But during malaria hemin causes inflammatory reactions for prolong period to disturb homeostasis. It causes generation of intracellular ROS to stimulates the expression of intracellular adhesion molecules (ICAM-1), vascular cell adhesion molecule1 (VCAM-1) and endothelial leukocyte adhesion molecules (E-selectin) from endothelial cells. The endothelial adhesion molecule recruits leukocytes to initiate various inflammatory responses (Figure 1.6). Moreover, hemin promotes increase in vascular permeability and the infiltration of leukocytes.

The activated immune cells release proteases and ROS result in severe tissue damage. Hemin mediated inflammation is involved in the pathology of atherosclerosis, renal failure, complications after artificial blood transfusion, peritoneal endometriosis, and heart transplant failure. The inflammation caused by hemin also damages the liver. It sensitizes the hepatocytes to undergo apoptosis in response to TNF-α mediated pro-inflammatory signals (Wagener, Volk et al. 2003).

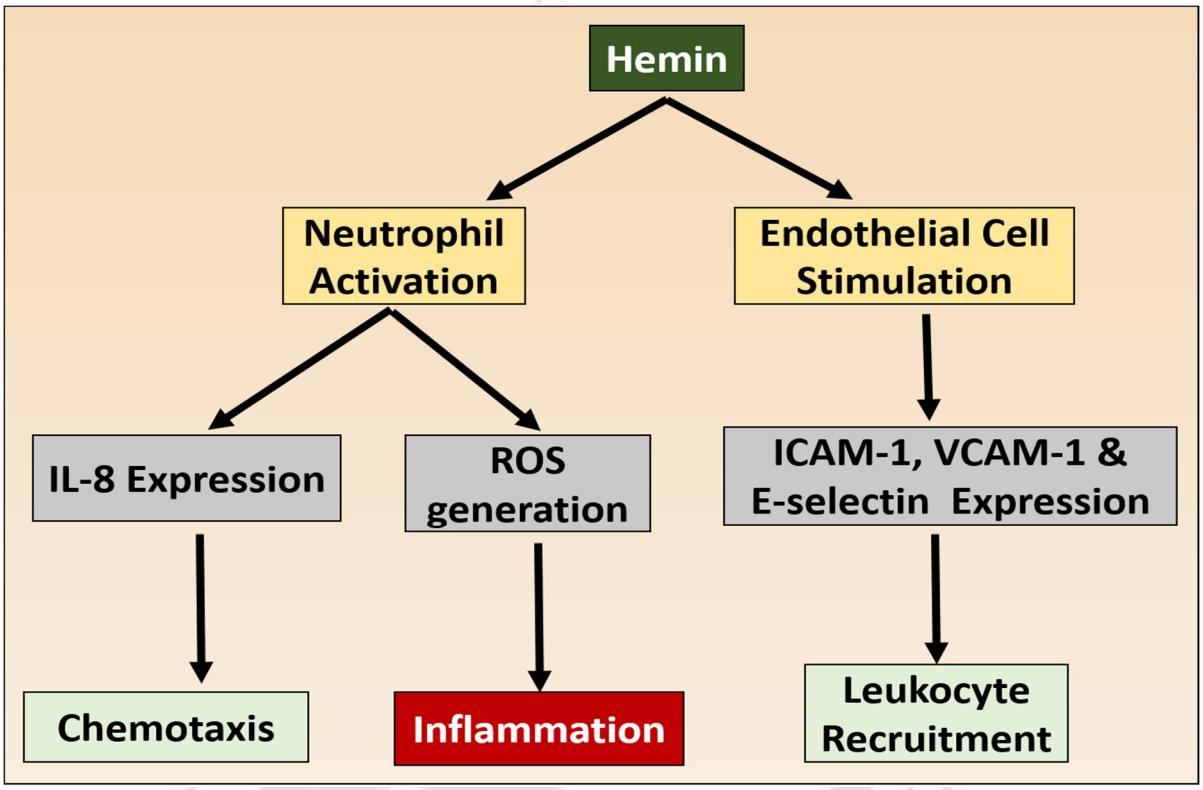

Figure 1.6: Pro-inflammatory effect of hemin

1.3.3 Haemozoin (Hz)

Malaria parasite during its intraerythrocytic stage of its life cycle digest about 80% hemoglobin of RBC into globin and hemin. Globin part is used as major source of amino acids for their growth and development whereas free hemin is converted into insoluble, inert, crystalline and less toxic haemozoin (Hz) or malaria pigment. Degradation process of hemoglobin to form Hz is summarized in Figure 1.7. Hz is heme polymer containing hematin dimers; composed of hematin molecules linked through bonding between iron of first hematin to the carboxylate of the adjacent hematin. Powder diffraction study confirms that haemozoin

from the malaria parasite is structurally identical to synthetic β-hematin (Slater, Swiggard et al. 1991).

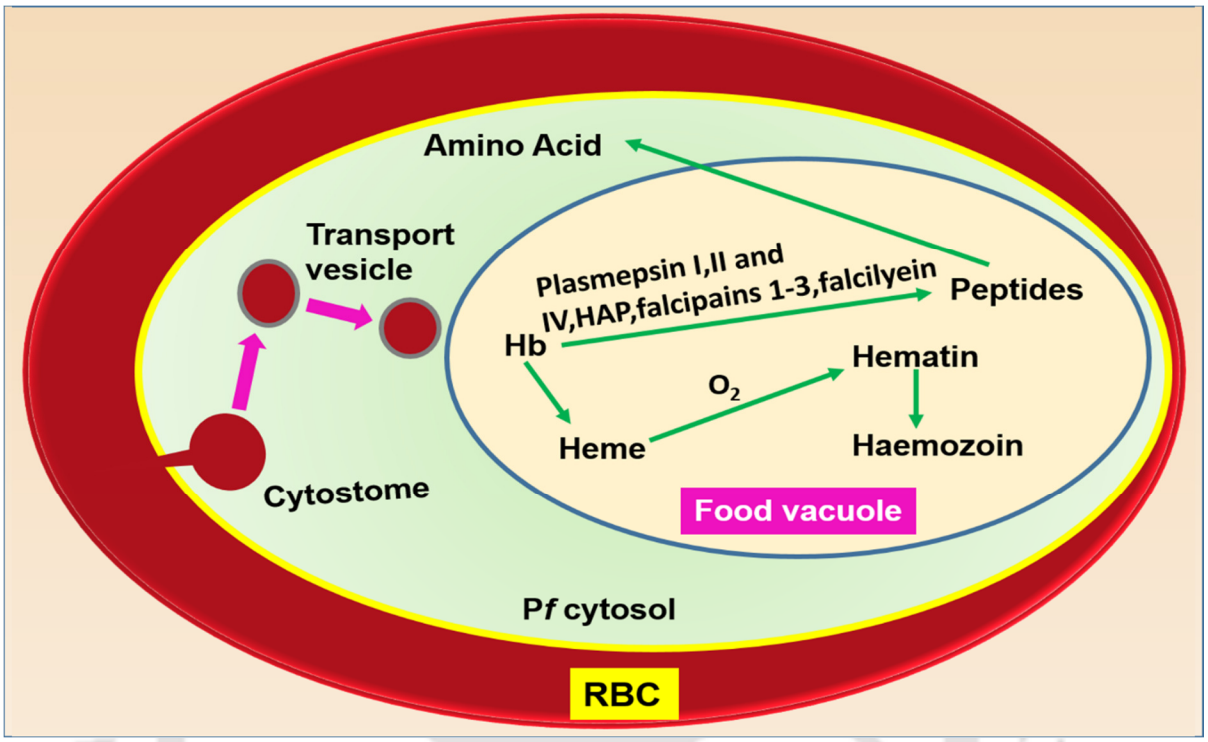

Figure 1.7: Schematic representation of processes involved in Hb ingestion, catabolism and Hz formation in the malaria parasite *P. falciparum*. Hb present in RBC cytoplasm is ingested into the parasite (Pf) via cytostome and transported to food vacuole (FV) in transport vesicles. Here Hb is digested by plasmepsins, falcipains and falcilysin to small peptides and finally degraded to aminoacids.This process releases hemin (Fe(II)PPIX) which gets oxidized by molecular dioxygen generating haematin (containing Fe[III]). Haematin is removed by incorporation into microcrystalline haemozoin.

1.3.3.1 Haemozoin and homeostasis

Haemozoin synthesis by the malaria parasite as a protective mechanism against the pro-oxidant hemin is regarded as an inert metabolic waste of the malaria parasite. But now it is well established that Hz is also responsible for malaria pathology. Macrophages, monocytes, neutrophils, endothelial cells and dendritic cells phagocytose Hz result in dysregulation of immune functions of these cells (Shio, Kassa et al. 2010). Hz-fed macrophages are viable but functionally impaired. They are unable to digest Hz or repeat phagocytosis to generate the oxidative burst upon appropriate stimulation or kill ingested bacteria, fungi, or tumor cells (Schwarzer, Turrini et al. 1992). Hz-laden macrophages stimulated with interferon is defective in induction of MHC class-II and resulting immunodepression during malaria (Schwarzer, Alessio et al. 1998). The internalized Hz remains within the phagocytic cells for prolong period

but the mechanistic details are not clear. It is suggested that Hz inhibits phagolysosome formation or inhibition of lysosomal acidification and it is accompanied with inhibition of protein kinase C (PKC) (Schwarzer, Turrini et al. 1993). It is reported that macrophages stimulated with Hz produces pro-inflammatory cytokine TNF-α. Moreover, it is als observed that severity of malaria correlates with the elevated level of TNF-α. It is reported that Hz activates the inflammasome intracellular protein complex to produce IL-1β. In addition, Hz induces the expression of chemokines such as MIP-1α/CCL3, MIP-1β/ CCL4, MIP-2/CXCL2, MCP-1/CCL2, and chemokine receptors such as CCR1, CCR2, CCR5, CXCR2 and CXCR4 (Jaramillo, Bellemare et al. 2009).

1.3.4 Malaria toxins- Glycosylphosphatidylinositol (GPI) of *Plasmodium falciparum* acts as a pathogen-associated molecular patterns (PAMPs) and is considered as toxin during malaria infection. GPI anchor increases the production of pro-inflammatory responses by the innate immune system of mammalian hosts (Arrighi and Faye 2010). GPIs have been consider as the major factors involved with the production of pro-inflammatory cytokines, TNF-α, IL-1 and IFN-γ from macrophages (Zhu, Krishnegowda et al. 2005). GPI has insulin-like activity, causing hypoglycemia, lipogenesis in adipocytes and expression of inducible nitric oxide synthase in macrophages and endothelial cells. They show increased expression of ICAM-1, VCAM-1 and E-selectin in leukocytes and endothelial cells (Schofield, Novakovic et al. 1996). It is reported that the presence of GPI anchors impair the T-cell responses and development of antibody present in sporozoite protein vaccines (Bruna-Romero, Rocha et al. 2004).

1.4 Macrophages and inflammation during malaria

During malaria, macrophage stimulation contributes to malaria pathogenesis via their expression and interaction with wide array of mediators such as reactive oxygen and nitrogen species, hydrolytic enzymes, bioactive lipids and pro-inflammatory cytokines or chemokines (Nathan 1987; Janeway and Medzhitov 2002). These mediators provide protection against pathogenic invasion but over secretion leads to the inflammation result in severe anemia, cerebral malaria, acute renal/hepatic/lung and brain injuries, osteoporosis and metabolic diseases (McGuire, Hill et al. 1994; Laskin, Heck et al. 1996). Cerebral malaria, a severe malaria is associated with high plasma levels of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 (Engwerda, Belnoue et al. 2005; Hansen 2012). Moreover, malaria toxin glycosylphosphatidylinositol (GPI) also stimulates the production of

TNF-α and lymphokine to upregulates the expression of ICAM-1 and VCAM-1 on endothelial cells. It promotes the sequestration of parasitized erythrocytes in the brain, contributing to coma (Schofield, Novakovic et al. 1996; Krishnegowda, Hajjar et al. 2005). Malaria patients with high TNF-α concentration correlates with disease severity, including hyperparasitaemia, hypoglycemia, coma, and death (Newton, Hien et al. 2000). The major macrophage-derived pro-inflammatory mediators and cytokines responsible for inflammation and tissue damage are as follows.

1.4.1 Reactive oxygen species (ROS)

Macrophages exposed to PRBC increase the uptake rate of oxygen and produce ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^*) (Halliwell and Cross 1994). Increase in ROS production disrupt redox homeostasis, resulting in pathologic condition such as malaria. Excessive ROS constantly attacks cellular structures, nucleic acids, lipids and proteins leading to severe and irreversible damage. Different cyto-toxic effects of ROS are as follows-

Lipid damage- Lipids are most susceptible to oxidative modification. ROS attacks polyunsaturated fatty acid to form peroxyl radicals. These radicals (ROO^*) undergo cyclization reaction to form endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation as malondialdehyde (MDA) or 4-hydroxy-2-nonenal (HNE) (Mao, Schnetz-Boutaud et al. 1999). MDA shows mutagenic and carcinogenic effects in human and rats respectively. HNE is weakly mutagenic but it reacts mostly with proteins to affect signaling pathways leading to cell death (Awasthi, Sharma et al. 2003).

Protein damage- ROS causes oxidation of protein in a reversible or irreversible manner, depending on the target and the form of oxidative damage. Hydroxyl/superoxide radicals are common reactive species to target proteins. ROS oxidizes all amino acids but proteins having cysteine and methionine residues of proteins are more susceptible to oxidation. Reversible oxidation of cysteine residues leads to intramolecular or intermolecular protein cross-linkages(Stadtman 2004). Severe oxidative stress can induce adduct formation between oxidized proteins and lipid peroxides or glycation products resulting in generation of aggregated bulky protein complexes which may inactivate both 26S and 20S proteosome, leading to accumulation of damaged proteins and cell death (Weber, Sircar et al. 1991).

DNA damage- In DNA the purine and pyrimidine bases and deoxyribose backbone are susceptible for the OH^*. Furthermore, OH^* also targets the double bonds within the bases. It results in the formation of oxidized product of DNA bases. Modification of DNA may induce mutations and play a role in the evolution process. But excessive DNA damage can trigger cell-cycle arrest and can induce apoptosis (David, O'Shea et al. 2007).

1.4.2 Tumor necrosis factor-α (TNF-α)

TNF-α is the first characterized cytokine produced by macrophages stimulated with malaria infected RBCs/malarial pigment or GPI. It is produced as soluble 17-kDa protein form and also in transmembrane form on the surface of macrophages. The pro-inflammatory cytokine have both beneficial and damaging functions. In low concentration, it stimulates host immune system and promotes resistance against infectious diseases whereas high level leads to toxicity and inflammation (Beutler and Grau 1993; Muniz-Junqueira, dos Santos-Neto et al. 2001). TNF-α increases the expression of Fc receptor to intensify the phagocytic capacity of macrophages. TNF-α regulates the macrophage for production and secretion of another pro-inflammatory factor interleukin-12 (Malaguarnera, Imbesi et al. 2002). TNF-α is an important co-factor for interleukin-12-induced production of interferon-γ by NK cells (Tripp, Wolf et al. 1993). TNF-α stimulates the production of IL-1, IL-6, IFN-γ, reactive oxygen intermediates (ROI) and NOI which causes inflammation and tissue damage, results in disturb homeostasis.

High TNF-α concentration is associated with severe anemia, indicates its importance in exacerbation of malaria pathology. The products of ruptured malaria-infected RBCs directly induce TNF-α production by macrophages. It is also reported that the over-production of TNF-α is also related to brain damage in pathological situations such as bacterial meningitis, multiple sclerosis and Alzheimer's disease. Pre-treatment with anti-TNF-α antibody in transgenic mice (overexpressing TNF-α) prevents brain damage pointout the potentials of TNF-α in pathology of cerebral malaria (Probert, Akassoglou et al. 1995).

1.4.3 Interleukins

Interleukin 1,6,12 and 18 are the inflammatory cytokines being released from stimulated macrophages. IL-1 induces ROS and enhances expression of adhesion molecules ICAM on endothelial cells (Berendt, Simmons et al. 1989; Dinarello 2011). Presence of IL-1 in patient blood correlates with the severity of malaria. It is reported that IL-6 level is associated with survival outcome of malaria. IL-12 is a potent immunomodulatory cytokine with its potential

role in cell-mediated and humoral immunity (Trinchieri 1995). IL-12 differntiate T-cells (CD^{4+}) into the Th-1 subset to produce IFN-γ, which in turn stimulates macrophages to perform their microbicidal functions and further production of IL-12. The increased level of IL-12 modifies macrophage activity with increased RBC destruction and bone marrow dyserythropoiesis (Malaguarnera and Musumeci 2002).

1.5 Hemin detoxification mechanism and its role in maintaining homeostasis

RBC lysis increases the hemin burden in the host which disturbs the homeostasis. Several mechanisms have been proposed for the hemin detoxification in biological system which are as follow:

1.5.1 Free hemin biomineralization (heme polymer formation)

Hemin is highly toxic in nature and malaria parasite cannot survive in this toxic environment. But the free hemin toxicity can be modulated by its conversion into the polymeric hemin through biomineralization. Several factors are known to perform this process inside the malaria parasite as well as in cell free *in-vitro* system. Few of the factors and their mechanism are as follows.

Protein-mediated formation- Initially, the presence of heme polymerase enzyme in the parasite is proposed which can facilitates the conversion of hemin to heme polymer, but it is not conclusive (Slater and Cerami 1992). Histidine rich protein (HRP) and heme detoxification protein (HDP) are the two proteins that have been identified in *Plasmodium* spp as potential factor for Hz formation. HRP-II binds to 15–18 molecules of hemin with binding affinity in the range of 340–940 nM, by bis-histidyl heme iron coordination (Schneider and Marletta 2005). In contrast, HDP has extremely high Hz production activity compared to other known factors. HDP along with falcipain-2 is present as a protein complex within food vacuole of the parasite where it catalyzes Hz formation (Wang, Kayman et al. 1993). In an *in-vitro* condition, horse raddish peroxidase can be able to catalyze conversion of hemin to heme polymer through the intermediate formation of one electron oxidation product, hemin free radical (Trivedi, Chand et al. 2005).

Lipid mediated formation-The acetonitrile extract of malaria parasites show conversion of hemin to haemozoin. The factors present in the acetonitrile extract of malaria parasite have been identified as lipids like methyl esters of oleic, palmitic and stearic acids (Bendrat, Berger et al. 1995). Moreover in an *in-vitro* condition commercially available lipids such as

arachidonic, linoleic, oleic, palmitoleic acids and di-oleoylglycerol have potentials to promote the conversion of hemin to haemozoin (Bendrat, Berger et al. 1995).

1.5.2 Hemin degradation via heme oxygenase-I (HO-I) system

In human host, heme oxygenase (HO) plays significant role in hemin degradation and protection of body against hemin toxicity and maintaining homeostasis. HO enzyme exists in 3 different iso forms; HO-1, HO-2, and HO-3. HO-1 is important for hemin degradation and it catabolizes free hemin into biliverdin, carbon monoxide (CO) and iron. HO-1 cleaves the protoporphyrin-IX ring to form biliverdin through hemin-binding pocket using one O_2 molecule and electron donated by NADPH/CytP450 reductase. Biliverdin is then converted into bilirubin by biliverdin reductase, which uses NADPH as electron donors. Bilirubin has potent anti-oxidant property towards scavenging peroxyl radicals and inhibits lipid peroxidation. it also provides protection against oxidative injury, inflammation and help in maintaining homeostasis. On the other hand, carbon monoxide inhibits the expression of pro-inflammatory cytokines TNF-α, IL-1β, and MIP-1β. CO regulates muscle tone and causes vasodilation to regulate blood pressure (Kumar and Bandyopadhyay 2005; Ferreira, Balla et al. 2008).

1.5.3 Scavenging of free hemin

Chelation of hemin from the circulation is an important mechanism to reduce the toxicity of free hemin. The circulatory system contains proteins which can lower down the free hemin and maintain homeostasis. Few of the protein which can efficiently scavenge hemin to maintain a steady state condition of the body. These factors are as follows:

Hemopexin-Hemopexin (Hx), a serum glycoprotein represents the primary line of defense against hemin toxicity. Hx binds free hemin present in vascular fluids with high affinity and function as hemin specific carrier from the bloodstream to the liver for degradation in the reticuloendothelial system. Inside the liver, heme–Hx complex binds to receptor and hemin is translocated inside the liver cell where as Hx is recycled back into the circulation. The transport is active process and it requires cellular energy, Ca^{2+} and certain temperature for hemin uptake. Hx inhibits hemin mediated lipid peroxidation and prevents cell from death to maintain homeostasis (Kumar and Bandyopadhyay 2005).

Albumin-Albumin efficiently forms complex with hemin and prevent the toxic effects of extracellular free hemin present in plasma. Human serum albumin reacts with porphyrin ring

of hemin to form albumin–hemin complexes in the molar ratios of 1:1 and 1:2 and inhibits the toxic effects of free hemin. Infusion of hemin to normal human subjects bind primarily to albumin. It is also suggested that albumin can extract hemin trapped in RBC membranes(Shaklai, Shviro et al. 1985). Unlike hemin-Hx complex, the hemin-albumin complex acts as a hemin depository and there is no evidences that hemin–albumin has transport function (Kumar and Bandyopadhyay 2005).

1.6 Scope and aim of the work

Malaria is one of the pathological conditions in which the homeostasis of the body gets disturbed. Malaria causes lysis of RBC to release Hb, He and Hz into circulation. Free Hb is highly unstable and is readily been oxidized into MetHb. Released MetHb exhibits toxicity towards cells, tissue and organ system. But no reports are available about the effect of MetHb exposure on macrophage viability, cellular integrity and underlying molecular mechanism of its toxic effects. Moreover, combitorial cytotoxic effect of MetHb and haemozoin on the macrophages viability is not studied so far. Hence our study will highlight the contributions of MetHb in exhibiting toxicity towards macrophages as well as its role in enhancing haemozoin toxicity towards macrophages.

MetHb has a pseudo peroxidase activity but it is not clear if it has the potential to polymerize hemin to heme polymer following a peroxidation mechanism. If such formation takes place then, whether it can activates macrophage to secrete ROS into the external micro-environment. Our study highlights pro-stimulatory role of MetHb to enhance production of pro-inflammatory molecules (such as ROS) through hemin oxidation and heme polymer formation. Study will also help to design therapeutic molecules or approaches to target MetHb to overcome the commencement of pathologic conditions.

Free hemin also shows enormous toxic effect in tissue and organ level. One of possible mechanisms of detoxification of hemin is the conversion into less toxic haemozoin (heme polymer); the mechanism predominately operates within malaria parasite. Hemin released from infected RBC is present outside macrophage and if macrophages need to survive in such an environment then it has to detoxify hemin present in the extra cellular environment. This study will help to discover a new pathway for hemin detoxification.

Hence, the work presented in this thesis are divided into following specific objectives.

1. **To study the cyto-toxicity of methemoglobin towards macrophages.**
2. **Exploring the role of methemoglobin mediated immune-toxicity modulation of pro-oxidant molecules towards macrophage.**
3. **Contribution of methemoglobin in pro-stimulatory effect by hemin oxidation and polymerization during malaria.**
4. **Exploring novel hemin detoxicifcation mechanism from LPS stimulated macrophages to control hemin toxicity in the microenvironment.**

Over all goal of these objectives are to understand the presence of methemoglobin in the tissue fluids and their multiple ways to disturb various processes govering homeostasis. Macrophages used as the model cells to understand these processes and their long term effects on patho-physiology in the condition of altered homeostasis.

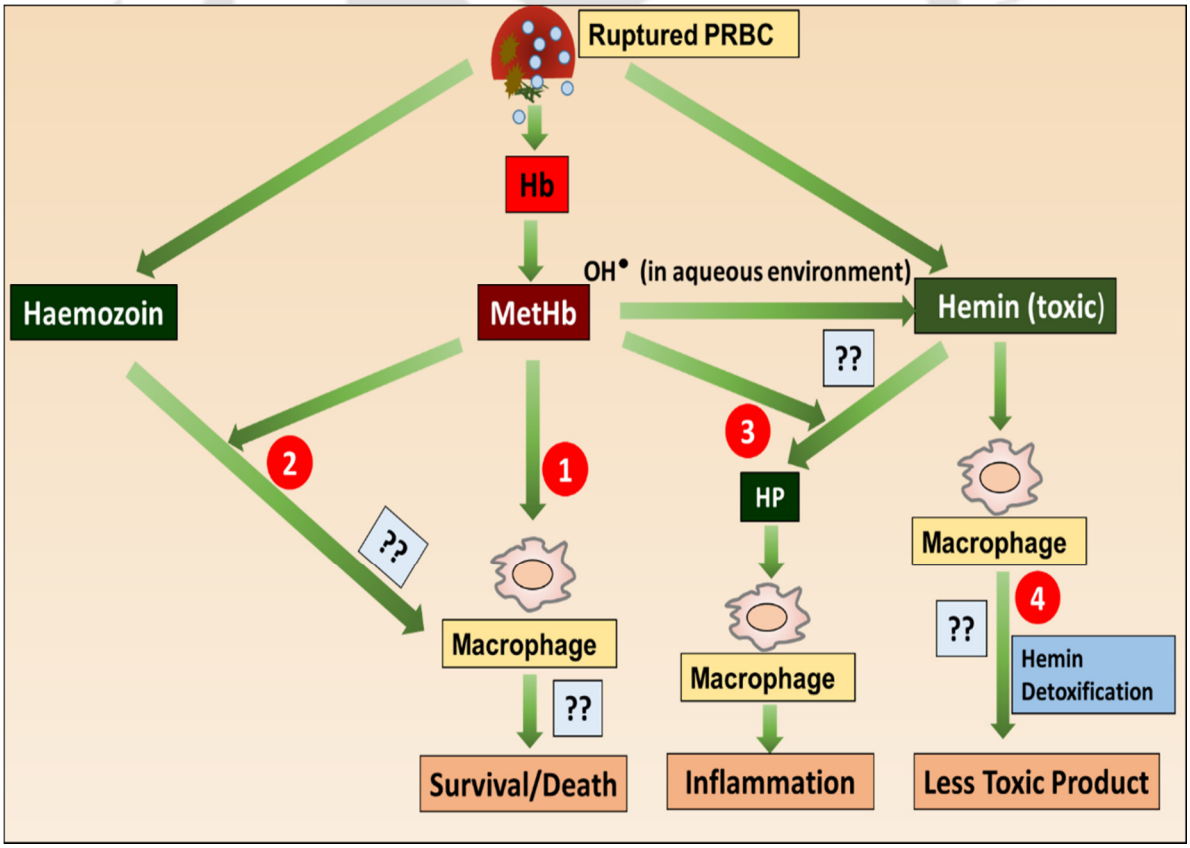

Figure 1.8: Working hypothesis to test the role of macrophages in hemostasis during malaria. The number indicates in red circle represents the objectives for the thesis work.

Experimental Procedures

2.1 Introduction

This chapter deals with the experimental procedures in details so that research work described can be reproduced. In this chapter more focus has been given to describe experimental procedures used throughout the thesis whereas procedures related to the specific experiment are given in the respective relevant chapters.

2.2 Macrophage cell culture and treatments- Macrophage J774A.1 cells were cultured in dulbecco's modified eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic solution (100units/ml penicillin and 100µg/ml streptomycin sulfate) and grown at 37°C in a humidified 5% CO_2 incubator. On the day of the experiment, cells were washed twice with cell culture grade phosphate buffer saline (PBS) and subjected to various treatments in serum free medium as described in the future chapters. In order to test the effect of various antioxidants or spin trap, cells were pre-incubated with antioxidants or spin trapper for 1hr before treatment.

2.3 Cell viability assay- MTT assay is used to test the cyto-toxicity of pro-oxidant molecules on macrophage viability as described with slight modifications (Arkusz, Stepnik et al. 2006; van Meerloo, Kaspers et al. 2011). Ten thousand macrophage J774A.1 cells were seeded overnight in 96 well plate in total volume of 0.2ml DMEM complete medium. On the day of experiment, cells were subjected to different treatments in 0.2ml of serum free medium (incomplete medium). Post treatment, cells were washed twice with PBS and incubated with 100µl of MTT (0.5 mg/ml) for 4h at 37°C with 5% CO_2. Then, MTT solution was removed and formazan crystal were dissolved in 100µl cell culture grade DMSO. The optical density was determined using a spectrophotometer at 570nm and 660nm (to subtract scattering effects of crystals). Cells treated with incomplete medium were considered as 100% viable and result of treatment is expressed as the percent survival in comparison to the control.

2.4 Light microscopy- The morphological changes in macrophage cells were observed with Nikon eclipse TS-100F inverted microscope using 20x and 40x objectives and images of cells were taken with high resolution Nikon L22 camera.

2.5 Scanning electron microscopy (SEM) - SEM analysis was done to study the morphological changes. SEM samples were prepared as mentioned earlier with slight

modification (Hortola 1992; Sanpui, Chattopadhyay et al. 2011). Untreated or different treatments cells ($1x10^5$) were incubated for stipulated time period in $35mm^2$ cell culture plate. Post treatment cells were washed with ice cold cell culture grade PBS followed by fixation in 4% glutaraldehyde containing 1% paraformaldehyde solution for 24h at 4°C. Fixed cells were washed twice with PBS and kept in humid atmosphere for 1hr at 37°C. The humid cells were dehydrated with graded ethanol from 50% to 100% in a vacuum environment. Dehydrated samples were coated with gold film in a polaron sputter coater and 10 fields were identified randomly to examine in LEO 1430VP scanning electron microscope.

2.6 Annexin V-FITC and Propidium Iodide (PI) staining of apoptotic and dead cells- The identification and differential count of healthy, apoptotic and dead/necrotic cells were done by flow cytometery with Annexin V-FITC/PI staining (Koopman, Reutelingsperger et al. 1994; Vermes, Haanen et al. 1995). About 10^5 cells were grown in 24 well cell culture dish and subjected to treatment with pro-oxidants for various time period. Post treatment cells were washed with cell culture grade PBS and removed from culture dish with PBS containing 0.6% EDTA. Apoptosis or death was detected with Annexin-V FITC/PI apoptosis detection kit according to instruction given by the manufacturer (Sigma, St. Louis, MO, USA). Ten thousand cells loaded with Annexin V-FITC/PI dye were acquired and analyzed with BD FACS caliber using cell quest pro analysis software. Annexin V-FITC was detected as a green fluorescence (FL-1) and propidium iodide was detected as a red fluorescence (FL-3). In order to determine the percentage of healthy, apoptotic and necrotic cell in the total acquired cell population quadrant statistics of dot plot was done. Macrophage treated with serum free medium was considered as control.

2.7 Acridine orange and Ethidium bromide (AO-EtBr) staining- An alternate method to identify apoptotic or dead cells can be done by staining the cells with acridine orange and ethidium bromide as described (Liegler, Hyun et al. 1995; Kasibhatla, Amarante-Mendes et al. 2006). One Lakh treated cells were detached from 24 well culture dish with PBS containing 0.6% EDTA. Cells were centrifuged and re-suspended in 200µl of PBS. Two µl of 100x stock of AO-EtBr [AO (10µM) and EtBr (25µM)] was added to cells so that the final concentration of AO and Et-Br were 0.1µM and 0.25µM respectively. Stained cells were analyzed immediately at room temperature with FACS Caliber, using Cell Quest pro

software (BD Biosciences, USA). Sample was excited at 488nm and emission was collected at 525/20-nm filter (FL-1) and 635/20-nm filter (FL-3) respectively. Quadratant analysis was performed to identify and determine healthy, early apoptotic, late apoptotic and dead cells from total population of the cells. Macrophage treated with serum free medium was considered as control and used to draw initial quadrant.

2.8 Detection of DNA fragmentation on agarose gel- Five lakh treated cells (from individual 6 well dish) were lysed in 20µl of lysis buffer (100mM Tris-Cl pH 8 containing 2mM EDTA and 0.8% w/v SDS). Lysate is incubated with 2µl of DNase free RNase A (50mg/ml) at 37°C for 30min to degrade RNA. Samples were then supplied with 10µl of proteinase K (20mg/ml) for 2h at 50°C, mixed with 6x loading buffer (NEB, USA) and resolved onto 1.8% agarose gel containing ethidium bromide (0.3µg/ml) at 60mA for 4h at 4°C. DNA fragments were visualized under UV-light and images were captured with a Kodak Gel Logic 1500 imaging system.

2.9 Intracellular ROS measurement- The intracellular ROS generated was measured with the help of 2′, 7′- dichlorofluorescein diacetate (DCFH-DA), a ROS sensitive fluorescent probe. (Eruslanov and Kusmartsev 2010; Cambos and Scorza 2011; Balaji and Trivedi 2012). The DCFH-DA inside the cell is rapidly hydrolyzed by esterases to non-fluorescent DCFH which further in presence of ROS molecule get converted to highly fluorescent 2′, 7′-dichlorofluorescein (DCF). About 10^5 treated cells were incubated with 10µM DCFH-DA for 30min to load the cells with ROS sensitive fluorescent probe. Cells were removed from culture dish with PBS containing 0.6% EDTA and 50,000 cells were analyzed in FACS Caliber, using Cell Quest pro software (BD Biosciences, USA). Macrophages treated with serum free medium were considered as control.

2.10 Heme polymerization Assay- The assay mixture in total volume of 1ml consist of 100mM sodium acetate buffer pH 5.2, hemin 100µM, H_2O_2 5mM and active fraction (malaria culture supernatant or methemoglobin or macrophage culture supernatant). The polymerization reaction was started by adding H_2O_2 and then incubated for 12h at 37°C. The reaction was terminated by centrifugation at 12000xrpm for 10min at room temperature. The pellet obtained was purified by washing twice with Tris-HCl 100mM pH 7.8 containing 2.5% SDS and then twice with bicarbonate buffer, 100mM pH 9.2. The pellet was then dissolved in 50µl of 2N NaOH and heated at 60°C for 30min to dissolve the pellet completely and was

finally diluted to 1ml with distilled water. The absorbance of the solution was measured at 400nm and an extinction coefficient of $91mM^{-1}cm^{-1}$ was used to quantify the heme polymer (Pandey, Singh et al. 1999).

2.11 Clotrimazole modified methemoglobin preparation- Clotrimazole modified MetHb was prepared as described (Balaji and Trivedi 2012). MetHb (10mg/ml), clotrimazole (20mM) and hydrogen peroxide (1mM) solutions were mixed respectively as the following proportions: - 120:6:1. After incubation at 25°C for 30min, sample was subjected to dialysis against PBS pH 7.4 for 12hrs against three changes of PBS.

2.12 Preparation of heat killed bacteria- Ten ml of bacterial culture (OD_{600} =1) was heat killed at 80°C for 30min in water bath. The heat killed bacteria was pelleted at 5000xrpm for 5min at 4°C and washed twice with PBS in aseptic condition. The washed pellet was then resuspended in 10ml of serum free medium containing penicillin-streptomycin antibiotic solution (100units/ml penicillin and 100µg/ml streptomycin sulfate).

2.13 Macrophage stimulation protocol and collection of secretion product- Preparation of macrophage culture supernatant containing heme polymerization activity consists of two phase namely stimulation and secretion phase. In stimulation phase, a confluent dish ($10cm^2$) of macrophages were stimulated with 10ml of heat killed of bacteria (OD_{600} =1) for 2h at 37°C. This was followed by secretion phase where the stimulated macrophages were gently washed once with cell culture grade PBS and 10ml of incomplete medium was added and incubate for next 12h at 37°C to secrete. The macrophage culture supernatant collected after 12h was centrifuged at 10,000xrpm for 10min at 4°C to remove any dead cells or debris. The obtained supernatant was further clarified with 0.22µm filter and stored at -20°C until further use.

2.14 Optical spectra studies- Optical spectra were recorded in a total volume of 1ml containing MetHb in 100mM Tris-HCl buffer pH 7.2, in a Cary 100 UV/VIS spectrophotometer at 25°C with quartz cells of 1cm light-path. Binding of H_2O_2 to MetHb was monitored at different concentrations. Different increasing concentrations of H_2O_2 were added successively till saturation was reached. Soret spectrum was recorded immediately after each addition of H_2O_2. Hemin or β-hematin was then added in the above solution and spectrum was recorded. Equal concentration of hemin or β-hematin was added in the

reference cuvette to correct absorbance. For spin trapper study Phenyl N-tert-butylnitrone (PBN) was added before the addition of hemin or β-hematin.

2.15 Purification of pro-inflammatory factor from malaria culture supernatant-The *Plasmodium falciparum* 3D7 strain was grown in RPMI medium containing of O^{+ve} RBC and 0.5% albumax-II as described (Srivastava, Singh et al. 2007). The culture supernatant of 5% parasitemia was clarified with 0.22µm filter. The clarified supernatant was then subjected to 65% ammonium sulfate precipitation at 4°C. The supernatant obtained by centrifuging at 15,000xrpm for 10min was then saturated with 80% ammonium sulfate. Supernatant and pellet were collected by centrifugation. The pellet obtained after fractionation was resuspended in ice cold PBS. All the fractions were then dialyzed against 3 changes of PBS for 12h at 4°C. (Balaji and Trivedi 2013). The fractions contains variable level of MetHb which was determined as described. (Rodkey, Hill et al. 1979)

2.17 Appendix-I

Materials

Acridine Orange (AO), Agarose, Annexin-V FITC/PI Apoptosis detection kit, Bradford reagent, Catalase, Clotrimazole, Deferoxamine, 2′, 7′- dichlorofluorescein diacetate, Dulbecco's modified eagle's medium, Ethidium Bromide (Et-Br), Glutaraldehyde, Guanidine hydrochloride, Hemin chloride, Hematin (βH), Mannitol, Methemoglobin, N-acetylcysteine (NAC), Paraformaldehyde, Phenyl N-tert-butylnitrone (PBN), Quercetin, 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO), Tetramethylethylenediamine (TEMED), Thiobarbituric acid, Thiourea, 2',7'-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,1',3,3' tetraethoxy propane and 5,5' dithiobis (2- nitrobenzoic acid), were purchased from Sigma, St. Louis, MO, USA. DMSO, Fetal Bovine Serum (FBS), Guaiacol, Penicillin-Streptomycin (100X) antibiotic solution, Phosphate Buffer Saline (PBS), Proteinase K, RNase A, Sodium Azide, Trypan blue and Trypsin were purchased from Hi-media, Mumbai, India. Ethylenediaminetetraacetic acid (EDTA), Ethanol 100%, Ethyl acetate, Hydrogen peroxide (H_2O_2), Sodium chloride, Trichloroacetic acid (TCA) and Triton X-100 was purchased from Merk, Germany. All other reagents and chemicals were of analytical grade purity. All the cell culture plates and dishes were purchased from Corning, Lowell, MA, USA. Murine macrophage J774A.1 cell lines were procured from Tissue & Cell Culture Unit, Central Drug Research Institute (CDRI), Lucknow, India.

2.18 Appendix-II

Buffer, reagents and culture growth media

A. Luria Bertani broth (LB) - This media was used for growing bacterial culture. LB broth contains peptone (1%), yeast extract (0.5%), and sodium chloride (1%). Components were dissolve in 100ml of distilled water and autoclaved for 20min at 121°C and 15lb pressure. For LB agar solid media, agar was added in media to a final concentration of 1.5%.

B. Dulbecco's modified eagle's medium (DMEM) - This medium was used for macrophage J774A.1 cell culture. The composition and method of preparation is as follows:

Components	Composition
DMEM	13.4 gm/l
Sodium bicarbonate	3.7gm/l
Fetal bovine serum (FBS)	10%
100X Antibiotic (Penicillin –Streptomycin)	1%

Preparation - 80 - 90% of the final volume of cell culture grade (milliQ) water. Add the 13.4gm dry DMEM powder to the water slowly. Mix for 20min to dissolved it completely. For each liter of DMEM being prepared, 3.7gm of sodium bicarbonate was added and adjust the pH to 6.9 - 7.1 using 1N NaOH or 1N HCl (as during filtration the pH of bicarbonate buffered solutions usually rises by 0.1 - 0.2 units). Finally volume was adjusted with cell culture grade water. This incomplete medium was then passed through sterile filtration unit fitted with 0.22µm membrane filter. Supplements, such as 10% fetal bovine serum and 1% penicillin–streptomycin antibiotic solution (100units/ml penicillin and 100µg/ml streptomycin sulfate) was added to the sterilized solution using aseptic technique.

C. Buffer and their compositions

a. Lysis buffer- 100mM Tris-Cl pH 8 containing 2mM EDTA and 0.8% w/v SDS

b. Hypotonic buffer-10mM Tris buffer pH 7.4

D. SDS-PAGE

The electrophoretic analysis of protein was carried out using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Stock solution for SDS-PAGE were prepared as described in Table 2.1. The components for preparing running buffer of

electrophoresis and sample loading buffer is given in Table 2.2. The resolving gel and stacking gel were prepared using the components described in Table 2.1, 2.2 and 2.3. All the solutions were prepared in deionized water.

Table 2.1 Recipe for the preparation of stock reagents for SDS-PAGE.

Stock reagent	Preparation
Acrylamide solution (30%)	1gm of N'N'-methylene-bis acrylamide was dissolved in 50ml ultra-pure deionized water collected at 18 MΩcm (Millipore, Milli-Q water purification system) in amber colored bottle. On complete dissolving, 29gm acrylamide was added to it and stirred on a magnetic stirrer till the clear solution was formed. The final volume was adjusted to 100ml. The solution was filtered (Whatman No. 1) and stored at 4°C in dark.
Tris HCl (1.5 M, pH 8.8)	54.45gm Tris base was dissolved in 150ml deionized water. The pH of solution was adjusted to 8.8 using HCl and volume made to 300 ml. It was stored at 4°C.
Tris HCl (1M, pH 6.8)	Tris base 6gm was dissolved in 60ml deionized water. The pH of solution was adjusted to 6.8 using HCl and volume made to 100 ml. It was stored at 4°C.
SDS (10%, w/v)	10gm sodium dodecyl sulfate (SDS) was dissolved in 60 ml deionized water. The volume made to 100 ml.
APS (10%, w/v)	100mg ammonium per sulfate (APS) was dissolved in 1ml water.

Table 2.2 Recipe for the preparation of resolving gel of SDS-PAGE (10ml)

Components (ml)	8%	10%	12%	15%
Deionized water	2.3	1.9	1.6	1.1
30% acrylamide solution	1.3	1.7	2.0	2.5
1.5 M Tris (pH 8.8)	1.3	1.3	1.3	1.3
10% (w/v) SDS solution	0.05	0.05	0.05	0.05
10% (w/v) APS solution	0.05	0.05	0.05	0.05
TEMED	0.003	0.002	0.002	0.002

Table 2.3 Recipe for the preparation of 5% stacking gel

Components (ml)	5ml	10ml
Deionized water	3.4	6.8
30% acrylamide solution	0.83	2.0
1 M Tris (pH 6.8)	0.63	2.5
10% (w/v) SDS solution	0.05	0.1
10% (w/v) APS solution	0.05	0.1
TEMED	0.005	0.010

Table 2.4 Recipe for the preparation of 5x running buffer and loading buffer

Solution	Preparation
5x Running buffer	15gm Tris base, 5gm SDS and 72gm glycine were dissolved in 800ml of deionized water. The pH was adjusted to 8.3 and volume was adjusted to 1000ml. The solution was filtered (Whatman, Filter No. 1) and stored at 4°C. The buffer (5x) was diluted to 1x and pre-warmed at 37°C before use.
5x Loading buffer	10ml 0.5M Tris (pH 6.8), 1.6ml SDS 10%, 10 ml glycerol, 0.4ml β-mercaptoethanol and 0.4ml 0.5%(w/v) bromophenol blue were dissolved in 3ml deionized water and pH was adjusted to 6.8. The final concentration of buffer was 1x by mixing 1 volume of 5x sample loading buffer to 4 volumes of sample (protein) before loading in gel.

Table 2.5 Recipe for the preparation of staining and destaining solution (100ml)

Components	De-staining solution	Staining solution
Methanol	30ml	45ml
Acetic acid	10ml	10ml
Water	60ml	45ml
Coomassie Brilliant Blue R 250	-	0.25gm

Sample preparation and running of SDS-PAGE- The 40µl crude lysate was mixed with 10µl 5x loading buffer and heat for 5min in dry bath at 95°C and centrifuged at 12000xrpm for 5min to remove debris. Clarified sample is loaded on the 10% SDS-PAGE. Electrophoresis was carried out in 1x running buffer with a constant current of 50V for first

30min (to stack the protein) and then 100V after samples moves out of the stacking gel. Electrophoresis continued until dye front reaches to the other end of the gel.

E. Protein estimation by Bradford's method- To 50µl of protein solution 150µl of Bradford reagent is added and allow to incubate in dark for 37°C for 15min. The absorbance at 595nm (A_{595}) was then measured against a blank and the protein concentration was calculated from bovine serum albumin (BSA) standard curve of (0-1mg/ml).

F. Protein estimation by Lowry's method

Table 2.6 Recipe for preparation of reagents used in Lowry's method

Reagents	Components	Amount	Procedure
Reagent A	Sodium carbonate Sodium hydroxide	2.0gm. 0.4gm.	Both the components were dissolved in deionized water and volume was made to 100 ml.
Reagent B1	Sodium potassium tartrate	2%(w/v)	1gm was dissolved in 50ml deionized water.
Reagent B2	Cupric sulphate	1%(w/v)	0.5gm was dissolved in 50ml in deionized water.
Reagent C	Freshly prepared by mixing Reagent B1, Reagent A and Reagent B2 in the ratio 1:100:1.		
Folin-Ciocalteu reagent(2N)	2N Folin-Ciocalteu reagent (1ml) was dissolved in 1 ml deionized water.		

Procedure -To 200µl of sample containing protein, 1ml of reagent C was added. After 15 min, 100µl of phenol reagent was added and the solution was mixed. The absorbance at 660 nm (A_{660}) was measured after 30min against a blank and the protein concentration was calculated from BSA standard curve of (0-1mg/ml).

Methemoglobin Exhibits Toxicological Effects in Macrophages due to Multiple ROS Spike Induced Apoptosis

3.1 Introduction

Macrophages are the important cells of the mononuclear phagocyte system; distributed in various organs such as the liver, lung, spleen, kidney, and brain where they exhibit great structural and functional heterogeneity (Gordon 2007). Macrophages engulf and destroy foreign particles, invading microbes and cellular debris by the process of phagocytosis. Macrophages present antigenic peptide fragments of the pathogens along with class II MHC molecules to lymphocyte to produce robust immune response (Singhal, Sharma et al. 1998; Chang 2009). Malaria causes lysis of red blood cells, releasing hemoglobin (Hb) into the circulation (Boretti, Buehler et al. 2009). Free Hb is highly unstable and is readily converted into methemoglobin (Fe^{3+}) (Uko, Udoh et al. 2003; Lavergne, Darmon et al. 2006). At inflammatory sites, this process is more pronounced as neutrophils and activated macrophages are accumulated and release considerable amounts of reactive oxygen or nitrogen species. Normally, level of methemoglobin (MetHb) remains below 2% in healthy individual but during various patho-physiological conditions it reaches to toxic levels (Anstey, Marks et al. 1996). Extracellular MetHb accelerates the RBC destruction to further elevate the level of Hb, MetHb and Hb degradation products (Balaji and Trivedi 2012). MetHb is potentially toxic to various organ system such as renal, cardiac, pulmonary and CNS (Buehler and D'Agnillo ; Sadrzadeh, Anderson et al. 1987; Nath, Croatt et al. 2000; Burhop, Gordon et al. 2004). Contamination of cerebrospinal fluid (CSF) of malaria patients with MetHb derived from ruptured malaria infected RBC correlates with neurological symptoms or death (Sakamoto, Takaki et al. 2002). MetHb can activate endothelial cells to release inflammatory mediators IL-6, IL-8 and adhesion molecule E-selectin to cause endothelial cytotoxicity as in case of cerebral malaria (Liu and Spolarics 2003). MetHb also has potential to produce large amount of peroxide to accelerate oxidative-mediated tissue damage (Winterbourn 1990; Kelder, Fischer et al. 1991). In an *in-vitro* toxicity system, MetHb exhibits toxicity towards cortical neurons, astrocytes and spinal cord neurons in an oxidative stress dependent mechanism (Regan and Panter 1996; Regan and Guo 1998). But no reports are available about the effect of MetHb exposure on macrophage viability, cellular integrity and underlying molecular mechanism of its toxic response. Moreover, during

malaria, immune functions of the body get compromised. Therefore, in the proposed model given in Figure 3.1, it is hypothesized that the release of MetHb from ruptured RBC may increase the MetHb load in the local vicinity and it may affect macrophages viability resulting into the compromised immune functions. These consequences may ultimately leads to the disturb host body homeostasis. So in this chapter efforts have been put to understand **"How MetHb exposure affects macrophage viability and underlying molecular mechanism"?**

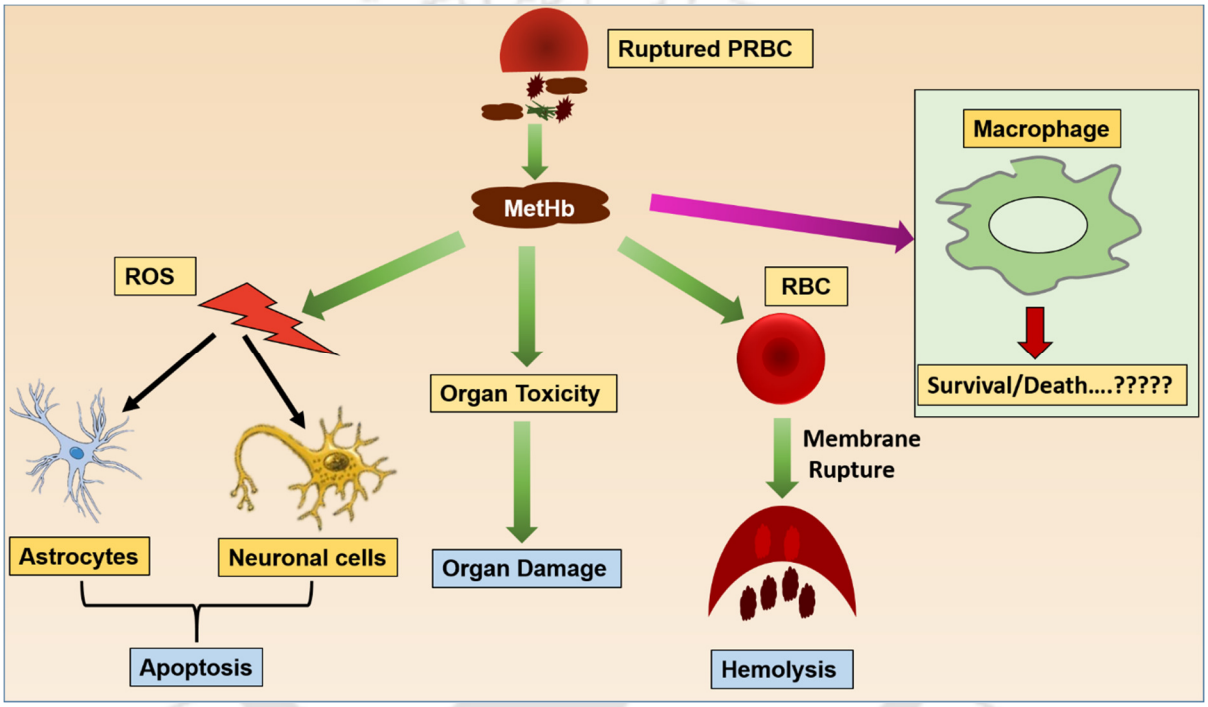

Figure 3.1: Effect of methemoglobin on various cells and organ of body and working hypothesis for its effects on macrophage biology. Rupture of RBC causes the increase in the MetHb concentration in the serum. The increased level of MetHb is known to cause toxicity and apoptosis of astrocytes and neuronal cells in an oxidative stress dependent pathway. MetHb is also known to cause toxic effects towards various organs such as kidney, heart and lungs. MetHb is also known to cause rupture of RBC by causing disturbance in membrane integrity. But till present date reports are not available about the effect of MetHb on macrophage physiology.

3.2 Materials and methods

3.2.1 Materials- Methemoglobin (MetHb), mannitol, N-acetyl cysteine (NAC), Phenyl N-tert-butylnitrone (PBN), 2',7'- dichlorofluorescein diacetate (DCFH-DA), Ethidium bromide (EtBr), Acridine orange (AO)**,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Annexin-V FITC Apoptosis detection kit, Agarose was purchased from Sigma, St. Louis, MO, USA. Other reagents and chemicals were of analytical grade purity.

3.2.2 Methods

3.2.2.1 Cell Culture- The detail method for cell culture is described in chapter II (Section 2.2; Macrophage cell culture and treatments; Page Number 23).

3.2.2.2 Cell viability assay- Ten thousand cells were seeded overnight in a 96-well in 0.2ml complete medium. On the day of experiment, cells were treated with different concentrations of MetHb (4µM-60µM) for 40h at 37°C with 5% CO_2 in serum free medium. To probe the role of intracellular ROS with cell death, cells were pre-incubated for 1h with NAC (0-10mM), mannitol (0-10mM) or PBN (0-200µM) and treated with MetHb (20µM) for 40h at 37°C with 5% CO_2 in serum free medium. MTT reduction test was used for cell viability assay as described in chapter II (Section 2.3; Cell viability assay; Page number 23).

3.2.2.3 Light microscopy- The morphological changes in untreated or treated macrophage cells as described above were observed under Nikon eclipse TS 100F inverted microscope using 20x and 40x objectives and images were taken with high resolution Nikon L22 camera.

3.2.2.4 Scanning electron microscopy (SEM) - Macrophages were untreated or treated with 20µM of MetHb for 40h and subjected to SEM analysis. The detail experimental procedure for SEM analysis was described in chapter II (Section 2.5; Scanning electron microscopy; Page number 23). The instrumental conditions like EHT, magnification, Width and signal were 12 kV, 3.0K X, 12 mm and SE1 respectively.

3.2.2.5 Intracellular ROS measurement- One lakh cells were untreated or treated with MetHb (20µM) for various time points (30min- 24h) at 37°C with 5% CO_2 in serum free medium and intracellular ROS was measured as described in of chapter II (Section 2.9; Intracellular ROS measurement; Page number 25).

3.2.2.6 Annexin V-FITC and Propidium Iodide (PI) staining - One lakh cells were untreated or treated with MetHb (20µM) for 40h at 37°C with 5% CO_2 in serum free medium in presence or absence of NAC (5mM), mannitol (5mM) and rest was same as described in chapter II (Section 2.6; Annexin V-FITC and Propidium Iodide (PI) staining of apoptotic and dead cells; Page number 24).

3.2.2.7 Acridine orange and Ethidium Bromide (AO-EtBr) staining- One lakh cells were treated with MetHb (20µM) for 40h at 37°C with 5% CO_2 in serum free medium in presence

or absence of NAC (5mM), mannitol (5mM) and analyzed in FACS as described in chapter II (Section 2.7; Acridine orange and Ethidium Bromide (AO-EtBr) staining; Page number 24).

3.2.2.8 Detection of DNA fragmentation on agarose gel- Five lakh cells were treated with varying concentrations of MetHb (0µM-20µM) for 40h at 37°C with 5% CO_2 in serum free medium. Cells were lysed, treated with RNAse, Proteinase K, run in agarose gel and visualized under UV-light as described in chapter II (Section 2.8; Detection of DNA fragmentation on agarose gel; Page number 25).

3.3 Results

3.3.1 Methemoglobin shows cyto-toxicity towards macrophages.

In normal physiological state, ~1% of total hemoglobin (15gm/dl) is in MetHb state. But during malaria it rises maximum up to 4% (0.6gm/dl) or 90µM (Anstey, Marks et al. 1996; Uko, Udoh et al. 2003). Therefore in the present study, patho-physiological concentrations of MetHb (0- 60µM) were used to study its effect on the viability of mouse macrophage cell line J774A.1. Macrophage treated with different concentrations of MetHb (0-60µM) in serum free medium shows decrease in cellular viability from 100% to 9 % in a dose dependent manner as compared to macrophage exposed to serum free medium (Figure 3.2A). Macrophage exposed to 20µM of MetHb was found to be 38% viable as compared to untreated cells. Hence 20µM of MetHb was taken as optimum concentration for further studies.

The cellular morphology of macrophages exposed to MetHb (20µM) was observed under light microscope using 20x objective lens to further confirm the results of MTT toxicity in which the number of macrophages reduced significantly in the field as compared to control (Figure 3.2B). Control cells were healthy, dividing and show normal cell morphology. In contrast MetHb treated cells were found to be unhealthy and showed abnormal morphology. More critical observation of macrophages at 40x objective lens showed cell shrinkage and accumulation of small vesicular bodies in the cytoplasm (Figure 3.2B inset). Strikingly, a dark brown colored particulate matter was also been observed within the cytoplasm of MetHb treated macrophages which was completely absent in control cells.

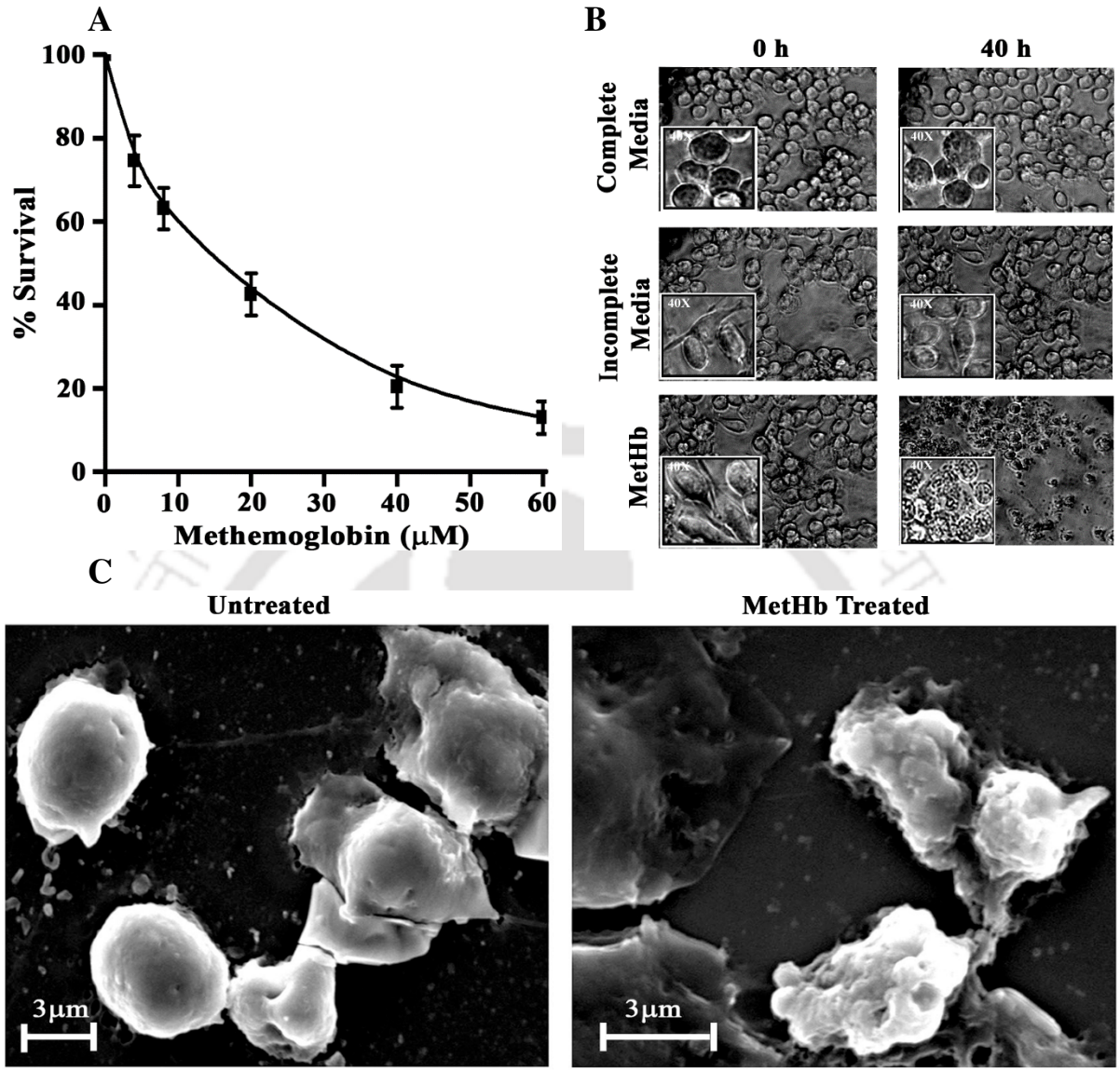

Figure 3.2: MetHb reduces viability and produces toxicological cellular abnormalities in macrophages. (**A)** Macrophages exposed to different concentration of MetHb (0–60μM) for 40h shows dose dependent reduction in cellular viability. Data are the mean ± SD of three independent experiments with triplicate measurement. Statistical analysis, ANOVA ($P < 0.001$). The viability at different MetHb treatment in each independent experiment ($n = 3$) is used to calculate average macrophage viability and standard deviation (SD). **(B)** Macrophage were incubated in complete medium, incomplete medium or in MetHb (20μM) for 40h. Cells were observed before treatment (0h) or after incubation (40h) with a 20x objective (Nikon TS100F). Cells in all treatments were healthy at 0h but at 40h MetHb treatment shows the less number of cells with visible defects in cellular integrity. Inset in each panel shows observation of cells with 40x objective. An enlarged view of cell exhibits accumulation of particulate matter and vesicular structure in cytosol. **(C)** Scanning Electron Microcopy (SEM) images of macrophages untreated (incomplete medium) or treated with MetHb (20μM) for 40h. Images from a total of 10 different fields in both samples were captured with LEO 1430VP Scanning Electron Microscope. The instrumental setting EHT, width and signal were 12 kV, 12 mm and SE1 respectively. A representative image of untreated cells (magnification; 3000, scale bar = 3μm) or MetHb treated macrophage cell (magnification; 5000, scale bar = 3μm) is given.

Structural integrity of untreated and MetHb treated macrophages by scanning electron microscopy (SEM) indicated that macrophages treated with 20µM of MetHb exhibit abnormal morphology such as membrane blebbing with severely distorted shape and cell shrinkage, a classical characteristic of apoptosis. In contrast, untreated macrophages were found to be normal healthy morphology with extended filopodia (Figure 3.2C). Together, the data in Figure 3.2 indicated cytotoxicity of MetHb toward macrophages.

3.3.2 Methemoglobin toxicity towards macrophage is due to induction of apoptosis

Cellular death is controlled by two molecular events: necrosis and apoptosis. Necrosis is characterized by cell swelling, injured cytoplasm organelles leading to membrane lysis and content release (Nanji and Hiller-Sturmhofel 1997). Whereas apoptosis is a programmed cell death characterized by phosphatidylserine (PS) externalization to outer leaflet, membrane blebbing, cell shrinkage, nuclear pyknosis, chromatin condensation, DNA fragmentation and formation of apoptotic bodies (Bonfoco, Krainc et al. 1995). Membrane blebbing with severely distorted shape and cell shrinkage of MetHb treated cells pointed out a potential role of apoptosis in death of macrophages (Figure 3.2C).

The differential population of apoptotic cells (early or late), necrotic/ dead cells were identified by annexin V-FITC/PI double staining method. Annexin V-FITC stained cells with phosphatidylserine (PS) on the external surface were used to detect early or late stages of apoptosis (Trotter, Orchard et al. 1995). Propidium iodide (PI) binds to the DNA of necrotic/ dead cells to give orange fluorescence (Crompton, Peitsch et al. 1992). Macrophage treated with 20µM MetHb for different time periods 0–48h at 37°C with 5% CO_2 in serum free medium and annexin V-FITC/PI double stained cells were analyzed by flow cytometry. Cells incubated in serum free (incomplete) or serum containing (complete) medium for 48h showed 100% healthy cells (Figure 3.3A, untreated). Macrophage cells treated with MetHb (20µM) for 0–48h showed a time dependent appearance of early, late apoptotic and necrotic-dead cells (Figure 3.3A, MetHb). Apparently, MetHb treated macrophages were healthy until 8h but after 16h, ~37% early and 42% late apoptotic stage cells were observed with no necrotic/dead cells therein (Figure 3.3A). In the longer exposure of 24, 32, 40 and 48h, MetHb treated macrophage cells gradually shifted from early apoptotic to predominately late apoptotic stage (Figure 3.3A). Annexin V-FITC/PI staining revealed that MetHb (20µM) for 40h gives about 70% cells with PS externalization on the outer membrane of macrophage in

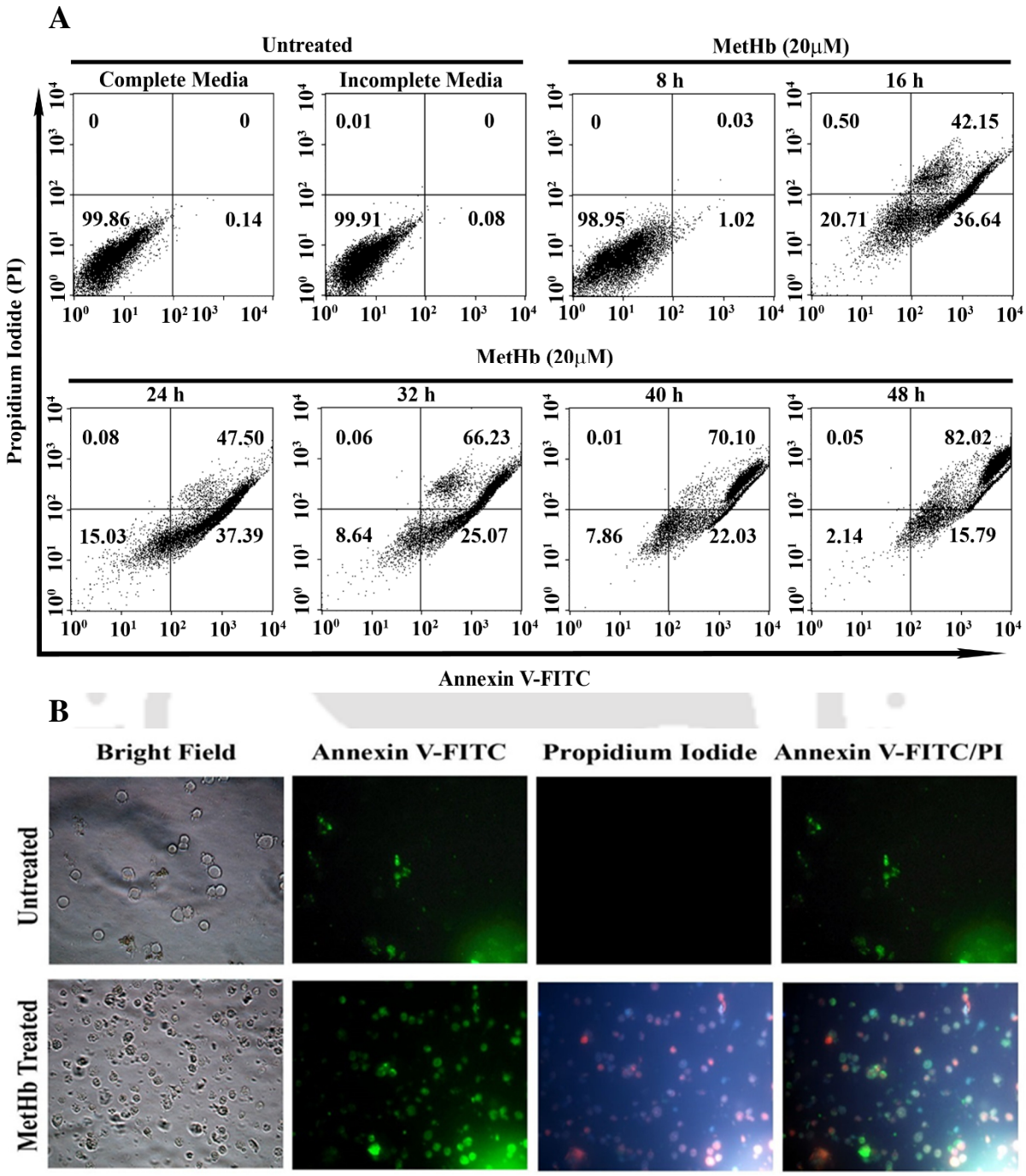

Figure 3.3: MetHb mediated cytotoxicity is due to induction of apoptosis in macrophages. (**A)** Macrophage exposed to MetHb (20μM) for 0–48h at 37℃ with 5% CO_2 in incomplete medium. Apoptosis/necrosis phenotypes in cellular population were detected by flow cytometric analysis of annexin V-FITC/PI stained cells. MetHb time dependently causes induction of apoptosis in macrophage with late apoptosis and necrosis at late time points. Macrophage exposed to either incomplete medium or complete medium (untreated) for 48h at 37℃ with 5% CO_2 as control. **(B)** Macrophage treated with MetHb (20μM) for 40h as described in **A** was used to perform fluorescence imaging of annexin V-FITC/PI stained cells with fluorescence microscope Nikon 80Ti. It exhibited early or late apoptotic cells (annexin V-FITC) and double stained (annexin V-FITC/PI) necrotic/dead cells. Untreated cells are healthy and show no sign of apoptosis/necrosis.

comparison to no cells in incomplete or complete medium treated cells. Fluorescence microscopic observation of annexin V-FITC/PI stained untreated or treated macrophage indicates a bright fluorescence of annexin V-FITC (early or late apoptosis) and a minor population as PI stained cells (dead) or annexin V-FITC/PI doubly stained cells (necrotic or dead cells) as compared to untreated cells (Figure 3.3B).

The differential percentage of apoptotic cells (early or late), necrotic/dead cells were also identified and calculated by AO-EtBr double staining. AO stained cells in early or late stage of apoptosis to give green fluorescence whereas Et-Br binds to DNA of necrotic/dead cells to give orange fluorescence. Macrophage treated with different concentrations of MetHb (0-20μM) for 40h and AO-EtBr double stained cells were analyzed in flow cytometry. Quadrant analysis of dot plots discriminate healthy, apoptotic and dead cells (Figure 3.4A). Untreated cells show ~99% healthy cells whereas 20μM MetHb treated cells show ~13 % healthy, ~53% apoptotic and ~33% necrotic/dead cells.

DNA fragmentation is known to be hallmark for apoptosis therefore apoptosis of macrophage cell treated with MetHb was further confirmed by a DNA fragmentation experiment (Saraste and Pulkki 2000). Macrophage treated with different concentrations MetHb (0-20μM) for 40h and DNA laddering assay was performed. A characteristic DNA laddering profiles with multiple DNA fragments was observed in MetHb treated macrophages (Figure 3.4B, lane 2-4). Untreated macrophages were processed under identical condition didn't show any DNA fragmentations (Figure 3.4B, lane 1). Mitomycin C (Mito-C) is an anticancer drug known to cause apoptosis (Wu, Wang et al. 2008) and used as positive control (Figure 3.4B, lane 5). Taken together, data strongly suggested that MetHb toxicity toward macrophage was due to death by following an apoptotic pathway.

3.3.3 Macrophage undergo apoptosis due to generation of multiple ROS spikes

MetHb is a pro-oxidant molecule which induces oxidative stress in exposed cells through generation of reactive oxygen species (ROS). To probe such a phenomenon behind the MetHb toxicity towards macrophages, intracellular ROS was measured using fluorescent ROS probe 2', 7'dichlorofluorescein diacetate (DCFH-DA). Macrophages treated with MetHb (20μM) or with incomplete medium (control) for 30min. The cellular machinery is capable of detoxifying ROS through their antioxidant enzymes and small molecule antioxidants (Ates, Abraham et al. 2008; Wu, Wang et al. 2008).

MetHb treatment show a significant ROS generation within their cytoplasm as compare to control cells (Figure 3.5A). Hence, the level and duration of ROS (up to 24h) was further explored from macrophage treated with MetHb. Incomplete medium treated cells were used as control to calculate fold change in ROS level. It was observed that MetHb treated cells accumulates large amount of ROS in 30min which reduces back to control level in next 30min (Figure 3.5B).Again at 2h, a high ROS was accumulated and which reduced back to normal level in next 2h. Similar multiple ROS accumulation was found inside the macrophage at 6h, 10h and it continues up to 22h and is probably responsible for MetHb mediated toxicity towards macrophages (Figure 3.5B). It would therefore be logical to assume that multiple ROS spike events might exhaust the antioxidant enzymes and reduce cellular ability of macrophage to overcome any more ROS (metabolically or other event mediated). Thus the inability to maintain a constant internal environment (ROS level) could have driven the cells towards death via apoptosis.

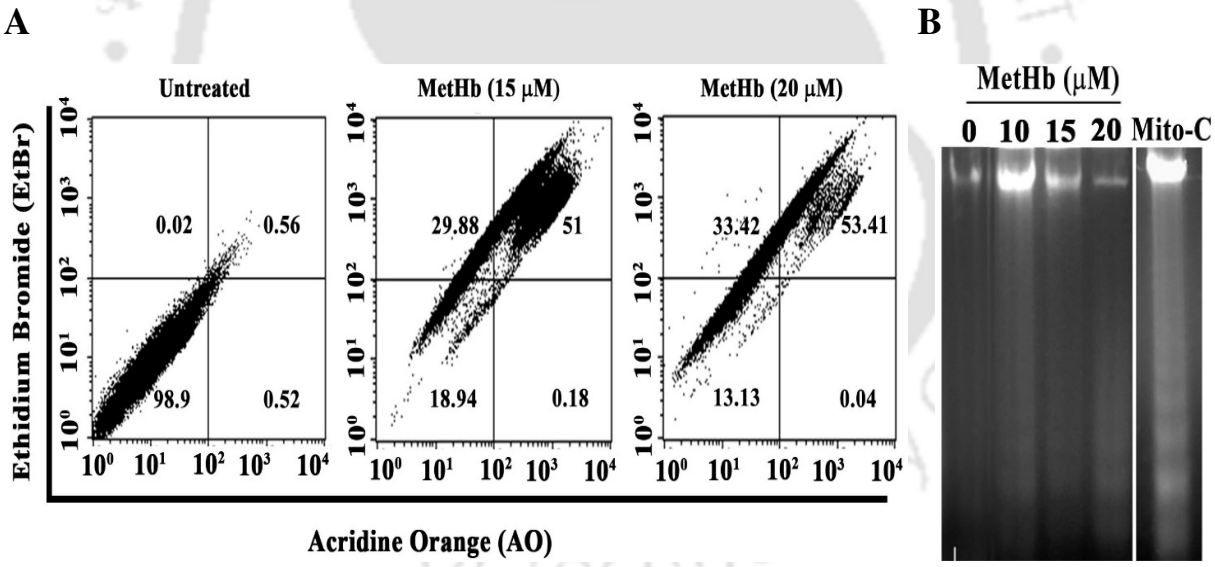

Figure 3.4: Death of macrophage was because of apoptosis. Cells were left untreated or treated with 0-20μM of MetHb and analyzed after 40h and apoptosis was determined by **(A)** AO-EtBr staining. Dot plot of AO-EtBr staining, untreated or cells treated with MetHb (15 and 20μM) were analyzed after 40h with flow cytometry. Fluorescence dot plot represent events within green FL-1(x axis) versus red FL-3 (y axis).Quadrant represent AO^{low}-Et-Br^{low} (LL), AO^{high} (LR), AO^{high}-Et-Br^{high} (UR), Et-Br^{high} (UL) respectively. Fluorescence events were collected on a logarithmic scale. **(B)** DNA fragments of macrophage untreated (lane 1), MetHb 10μM (lane 2), MetHb15μM (lane 3), MetHb 20μM (lane 4) and positive control mitomycin-C (lane 5) for 40h. DNA fragmentation was performed as described in the experimental procedure section of chapter II. A representative gel from the four independent experiment (n = 4).

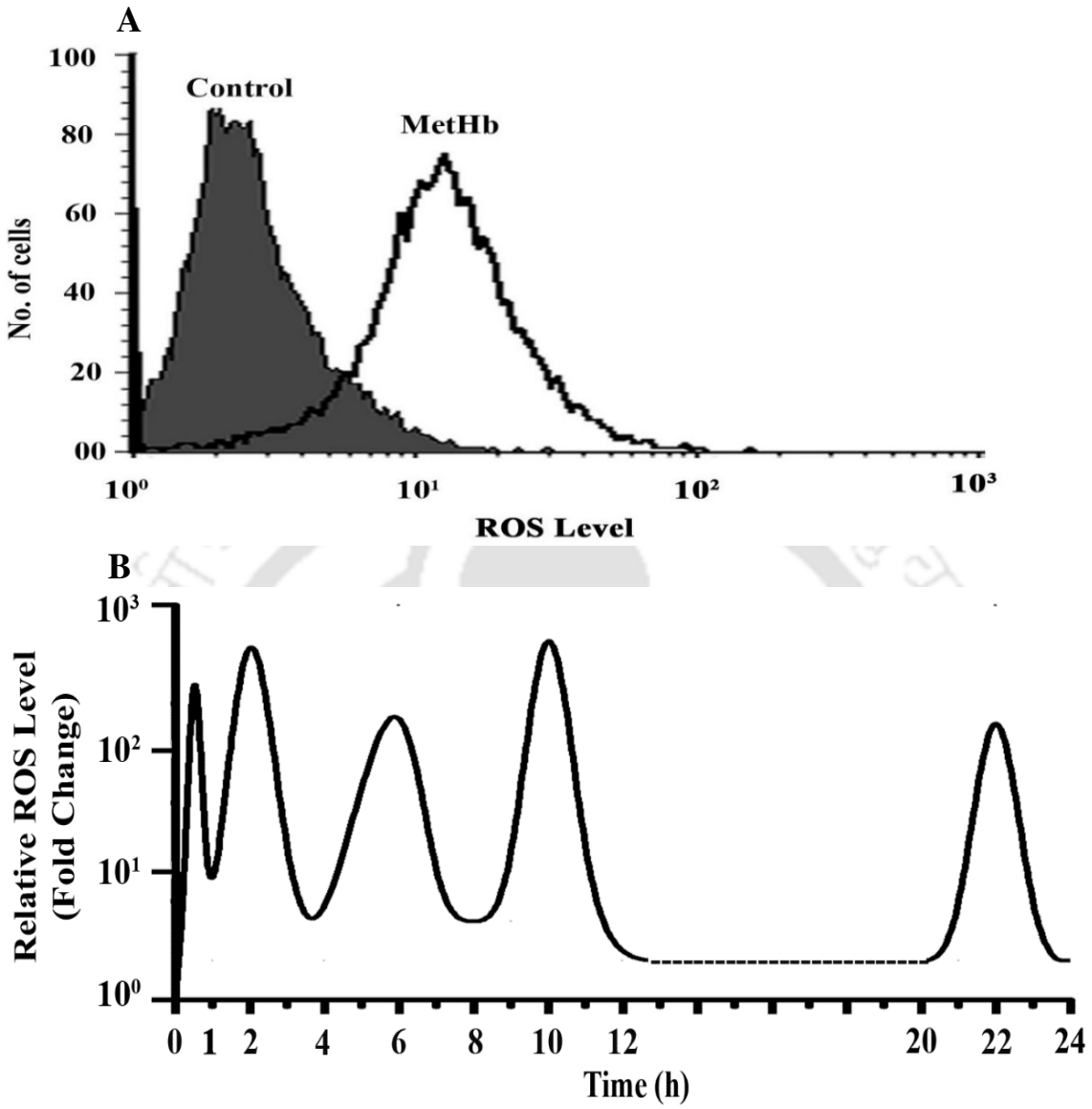

Figure 3.5: Death of Macrophage was due to multiple ROS spike accumulation. Treatment of macrophage J774A.1 cell with MetHb causes generation of ROS intracellularly which leads to the death. **(A)** Cells were left untreated or treated with 20µM of MetHb and analyzed after 30min by flow cytometry. ROS produced inside the cell was measured by the fluorescence exhibited by fluorescence probe (DCF). Filled histogram represents untreated cells and black line histogram represents MetHb treated cells. **(B)** Measurement of the level and duration of ROS intracellularly in 20µM MetHb treated macrophage. ROS produced inside the cell was measured at different time points (15min-24h) by the fluorescence exhibited by ROS probe (DCF). Multiple high ROS spike spreads over long duration during MetHb toxicity to macrophages.

3.3.4 Antioxidants prevent macrophage from MetHb mediated apoptosis and death

To explore the role of ROS in MetHb mediated macrophage toxicity, ROS was removed by two natural antioxidants N-acetyl cysteine (NAC), or mannitol and macrophage apoptosis was analyzed by annexin V-FITC/PI staining (Pigault, Follenius-Wund et al.

1994).Flow cytometric analysis of the samples after annexin V-FITC/PI staining, pre-incubation of MetHb treated cells with NAC (5mM) or mannitol (5mM) showed a significant decrease in apoptosis as compared to MetHb treated cells. Macrophages treated with MetHb (20µM) for 40h give 73% apoptotic cells but NAC and mannitol pre-treatment showed reversal of MetHb toxicity with appearance of 100% healthy cells (Figure 3.6).

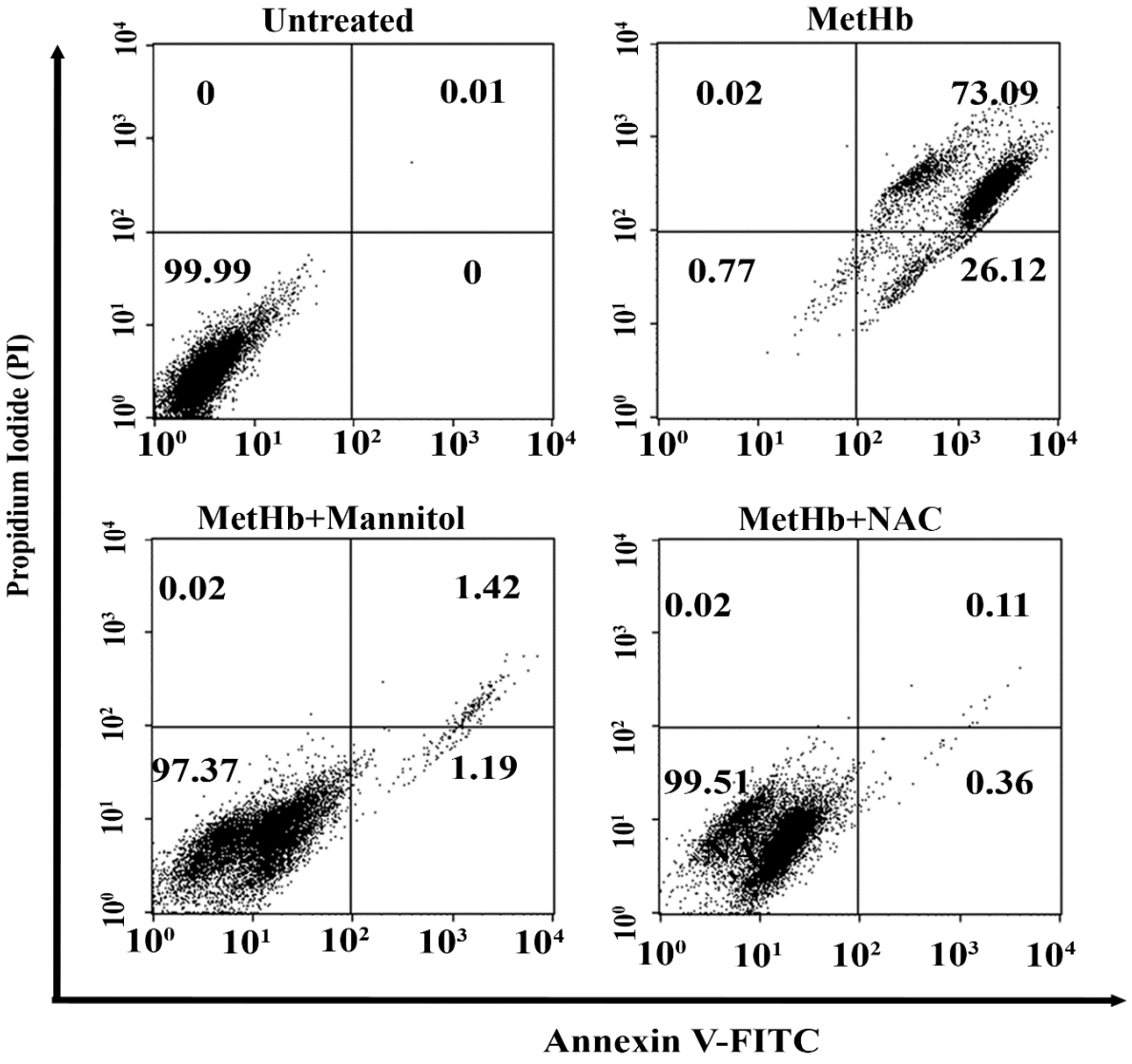

Figure 3.6: Antioxidants prevent the macrophage cells from apoptosis. Macrophage treated with 20µM MetHb for 40h in the absence or presence of NAC (5mM) or mannitol (5mM) and apoptosis/necrosis phenotypes cells were detected by flow cytometeric analysis of annexin V-FITC/PI stained cells. Fluorescence events were collected on a logarithmic scale and dot plot analysis of events within FL-1 (annexin V-FITC) versus FL-3(PI, y axis) channel gives cellular population with A^{-ve} PI^{-ve} (healthy cells, LL), A^{high} PI^{-ve} (early apoptotic, LR), A^{high} PI^{high} (late apoptotic, UR), A^{-ve} PI^{high} (necrotic/dead, UL).The % cell present in each quadrant is given.

Similarly in AO-EtBr staining, pre-incubation of MetHb treated cells with NAC (5mM) or mannitol (5mM) shows a significant decrease in apoptosis as compared to MetHb treated cells. As described earlier macrophages treated with MetHb shows ~13 % viability. In contrast, NAC and mannitol pre-treatment shows complete reversal of MetHb toxicity and gives 100% viable cells (Figure 3.7A).

If apoptosis is reversed by antioxidant treatment, then it is expected that pre-incubation of MetHb treated cells with antioxidants should recover the macrophage from death in MTT assay. As expected, pre-incubation of cells into different concentration of NAC (0-10mM), mannitol (0-10mM) reversed the cytotoxic effects of MetHb in a dose dependent manner. NAC at 5mM is potent enough to reverse the MetHb toxic effects completely whereas mannitol at 5mM gives only ~80% reversal (Figure 3.7B). A probable reason of being mannitol less efficient than NAC could be due to its faster metabolism and lesser stability. Both antioxidants have restored the viability of macrophage by reducing intracellular ROS level.

MetHb and by labile hemin released from it, is known to be associated with oxidative damage and inflammation (Wagener, Volk et al. 2003).To further explore if a specific free radical species is involved in the cyto-toxicity of MetHb, cells were pre-incubated with different concentration of PBN and cellular viability was measured by MTT. Macrophage pre-incubated with PBN gives a dose dependent survival from MetHb mediated cyto-toxicity and a complete reversal at 200µM (Figure 3.7B). It indicates role of specific free radical species in MetHb mediated toxicity towards macrophages.

Antioxidant and spin trap pre-incubated cells exhibit reversal of MetHb mediated cyto-toxicity and they were analyzed by light microscopic to rule out the possibility of artifacts associated with MTT (as MTT asses mitochondrial oxido-reductase activity rather than death). As observed earlier, after 40h of incubation, MetHb treatment shows macrophages with apoptotic phenotype as compare to healthy cells at 0h. But cells pre-incubated with NAC, mannitol or PBN were viable, healthy and depicting normal cellular architecture (Figure 3.7C).Taken together study demonstrates that MetHb released during malaria exhibits toxicity towards macrophage through development of oxidative stress to causes death by apoptosis.

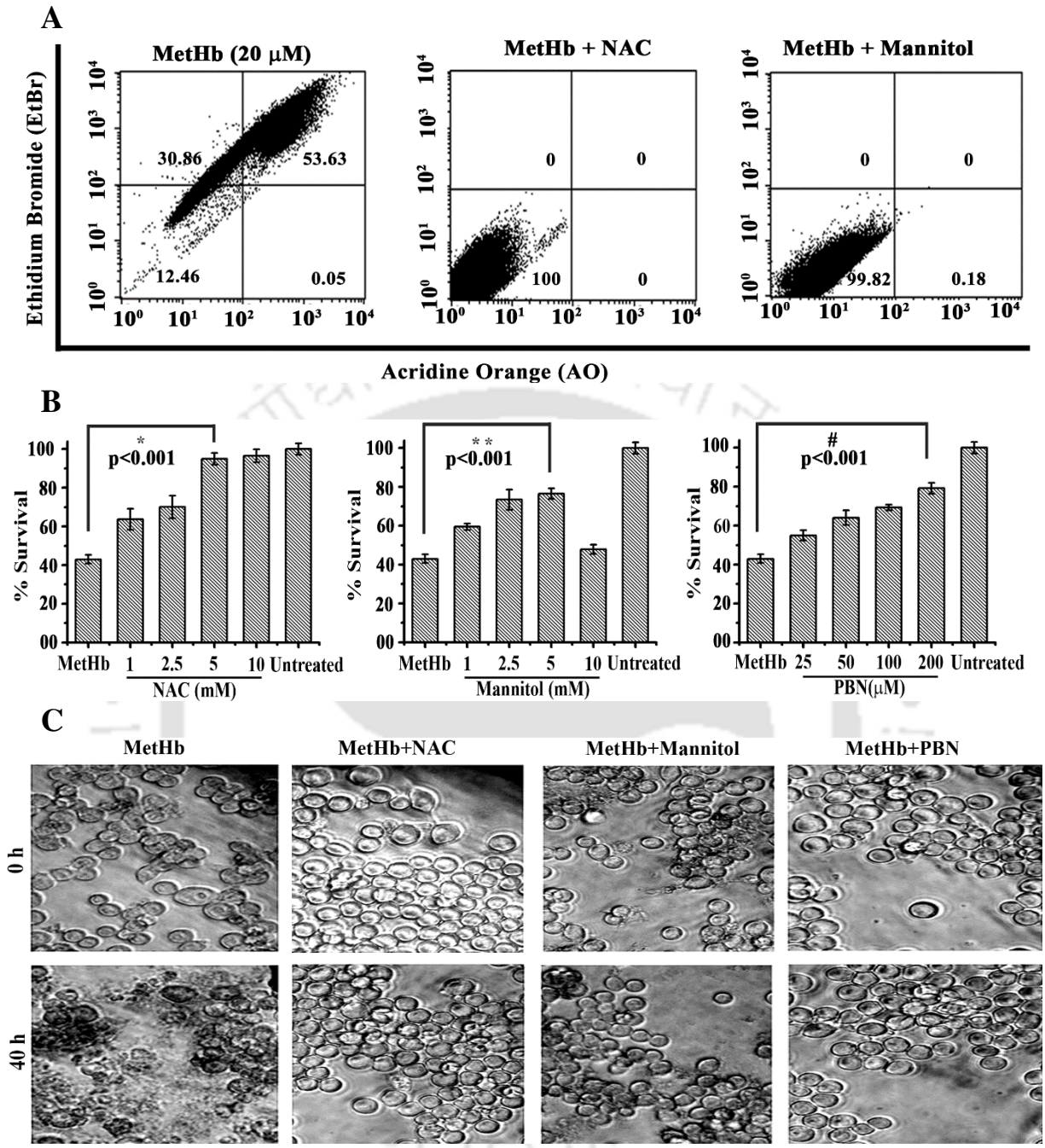

Figure 3.7:Pre-incubation of MetHb treated cells with antioxidants or spin trapper reversed the cytotoxic effects of MetHb and prevents the cells from going to apoptosis. (A) Dot plot of AO-EtBr staining, cells treated with 20μM MetHb or pre-incubation of MetHb treated cells with NAC (5mM) or mannitol (5mM) and analyzed after 40h with flow cytometry. **(B)** Cell viability (MTT assay) of MetHb treated macrophage cells or pre-incubated MetHb treated cells with NAC (0-10mM), mannitol (0-10mM) or PBN (0-200μM) for 40h. Antioxidant pre-incubation dose dependently reverses the MetHb mediated cytotoxicity in macrophages. Data are the mean ± SD of three independent experiments (n = 3) with triplicate measurement. The pairwise results were analyzed with anova & student t-test and it was statistically significant with *P < 0.001, **P < 0.001, #P < 0.001. **(C)** Light microscopic observation with a 20x objective of MetHb treated macrophage or pre-incubation of MetHb treated cells with NAC (5mM), Mannitol (5mM) or PBN (200μM) to detect cellular morphology and toxicological effects in macrophages at 0h and 40h.

3.4 Discussion

The current study presented a novel mechanism of MetHb mediated multiple ROS spike generation in macrophage to exhibit apoptosis and reduced viability. Hemolysis and accumulation of cell free hemoglobin/methemoglobin in plasma are common with many patho-physiological conditions including malaria (Anstey, Hassanali et al. 1996). Results of the study showed that MetHb exposure reduced macrophage viability and caused structural abnormalities (Figure 3.2). The MetHb concentration used in the current study was within the range reported during patho-physiological conditions (4.5–5.8% in malaria patients) (Uko, Udoh et al. 2003; Sobolewski, Gramaglia et al. 2005). MetHb has ability to scavenge peroxide to protect the cells and its ability to produce free radicals (cytotoxic) is also known, and this dual behavior of MetHb has been linked with nature of substrate molecules present within the tissue microenvironment (Winterbourn 1990). Although the mechanism of MetHb mediated ROS production was not explored in the present chapter, MetHb peroxidase activity has been reported to be associated with disturbance of oxidative/antioxidant balance of the cell to cause ROS production (Balaji and Trivedi 2012). A number of studies have suggested that the release of the enzyme bound hemin or iron is associated with the production of free radicals in the external microenvironment (Wagener, Volk et al. 2003). It would therefore not be incoherent to speculate that the MetHb might be utilizing hemin as a substrate to produce large amount of ROS. PBN mediated reversal of MetHb cytotoxic effects toward macrophage suggested the role of single electron species in this process (Figure 3.7B). But the generation of multiple ROS for prolonged period appeared to be crucial events for MetHb mediated cyto-toxic events in macrophage (Figure 3.5B). Generation of ROS spikes and incremental appearance of apoptosis in macrophage exposed to MetHb (20µM) for a period of 0–48h (Figure 3.3A) indicated the role of oxidative stress with MetHb associated cyto-toxicity. Macrophages have been reported to produce a ROS spike in response to bacteria and release it to the external environment to produce cytolytic effects against invading foreign organism. Once the protective response is over, they become competent enough to subside ROS spikes through the action of antioxidant enzymes and small molecule antioxidants (Harrison 2009). Production of antioxidant enzymes and small molecule antioxidant is an energy driven process and that's why multiple ROS spikes might exhaust energy equivalent (NADH) of the cell to induce the apoptosis (Buttke and Sandstrom

1995). Most of the molecular events downstream to multiple ROS generation are still at the level of speculation and a detailed study is required to explore the exact mechanism.

3.5 Conclusion

The present chapter focused on the consequences of macrophage exposure to the MetHb being released from ruptured RBCs. As per the proposed hypothesis in the beginning of the chapter (Figure 3.1), it was postulated that MetHb can affect the macrophage viability to contribute into the disturbance of the host homeostasis. Experimental result shows that the multiple ROS spikes were observed in macrophage cells. It is probably exhausting the antioxidant machinery of the cell and inducing apoptosis to exhibit cell death. Apoptosis caused by MetHb may disturb body homeostasis (Figure 3.8).the following ways.

1. Decrease in macrophage population leads to decrease in phagocytosis of pathogen, results in various microbial infections leading to disease conditions.
2. Macrophages are unable act as an APC as a result T-Lymphocytes will not be able to produce a robust immune response.
3. Decrease in macrophage population severely affects the clearance of apoptotic and senescent RBC which may results in several toxicological consequences.The above mention effects are the logical assumptions as per the known literature and need to further validate experimentally.

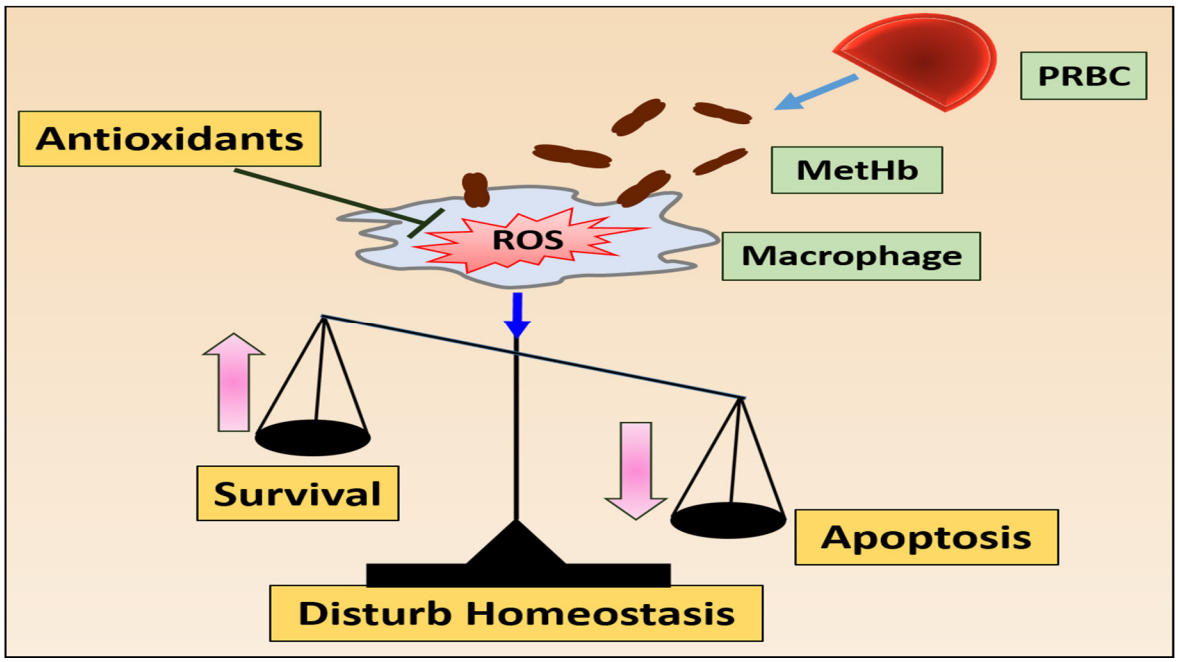

Figure 3.8: A Schematic model to summarize of MetHb mediated cytotoxicity towards macrophages during malaria like conditions.

Mutual Cross-talk Modulates Immuno-toxic Potentials of Pro-oxidant Molecules towards Macrophages

4.1 Introduction

Malaria caused by *plasmodium falciparum* completes its life cycle in the vertebrate host human and invertebrate host mosquito (Hay, Okiro et al. 2010). In the vertebrate host, parasite spends a major part of its life-cycle inside red blood cell (RBC) and depends on Hb for nutrition. Rupture of parasite containing RBCs lead to release of a large amount of Hb/MetHb, free hemin, haemozoin, malaria toxins and other uncharacterized metabolic by-products (Kamchonwongpaisan, Samoff et al. 1997). Hemin, haemozoin, MetHb and other iron containing hemoglobin degradation products are pro-oxidant in nature and has potential to cause oxidative damage to the cells and tissues (Nuchsongsin, Chotivanich et al. 2007; Reeder 2010). Exposure of pro-oxidant molecules to the immune cells exhibit change in their cytokine secretion profile and contributes to the inflammation during malaria (Jaramillo, Godbout et al. 2005; Dostert, Guarda et al. 2009). Circulatory phagocytes (monocytes), tissue associated macrophages provide defense from invading pathogens. Macrophage exposed to the haemozoin or synthetic haemozoin (β-hematin) exhibits depression of phagocytosis, inhibition of phagosome/lysosome maturation, disturbance of pro-inflammatory/anti-inflammatory cytokine balance and is responsible for immune-depression during malaria (Pichyangkul, Saengkrai et al. 1994; Scorza, Magez et al. 1999). Immune-depression during malaria is also due to the depression of proliferative behavior of PBMC to antigen, decreased number of T-lymphocytes, circulating phagocytes, neutrophils and macrophages (Schwarzer, Turrini et al. 1992; Schwarzer and Arese 1996; Barrera, Skorokhod et al. 2011). Pro-oxidant molecules released during malaria also contributes to the pathological complications of cerebral malaria, vascular complications, immune-depression following multiple mechanism, but the exact mechanism behind this is still not conclusive (Hunt and Grau 2003; Balaji and Trivedi 2013).

A comparative analysis of *P.falciparum* infected patient with control healthy volunteer indicates the high degree of spontaneous apoptosis in mononuclear phagocytes (Toure-Balde, Sarthou et al. 1996; Balde, Aribot et al. 2000). RBCs infected with *P.falciparum* express phosphatidylserine on their outer surface (Eda and Sherman 2002) and phagocytosis of infected RBCs induces macrophage apoptosis following a redox imbalance (Cambos and

Scorza 2011). The macrophage apoptosis was also known to be independent to the phagocytosis of RBCs, where cellular factor(s) from RBC are responsible for development of oxidative stress mediated induction of apoptosis. Hb and hemin derived from Hb is the causative agent responsible for macrophage apoptosis (Cambos and Scorza 2011). *In-vitro* exposure of macrophages with MetHb causes dose-dependent induction of apoptosis with generation of multiple intracellular ROS spikes for a prolonged period (Deshmukh and Trivedi 2013). MetHb has an intrinsic peroxidase activity and can accept free hemin as substrate and form heme polymer. MetHb produced heme polymer stimulates macrophage to secrete large amount of ROS in the external microenvironment (Deshmukh and Trivedi 2013). A model for hypothesis is given in Figure 4.1. According to this model, rupture of RBC causes release of haemozoin and MetHb in the circulation. Haemozoin stimulates various cells of immune system to modulate cytokines. But cyto-toxicity and apoptosis of macrophage caused by β-hematin (synthetic analog of haemozoin which is structurally and spectroscopically identical to haemozoin) in presence of MetHb have so far received no attention. Therefore in present chapter mechanistic details of mutual synergistic interaction of different pro-oxidants (MetHb and β-hematin) molecules to exhibit severe cyto-toxic effect toward macrophage has been explored out.

4.2 Material and Methods

4.2.1 Materials- Methemoglobin (MetHb), β-hematin (βH), Mannitol, N-Acetylcysteine (NAC), phenyl N-tert-butylnitrone (PBN), 2',7'- dichlorofluorescein diacetate (DCFH-DA), Catalase, Deferoxamine, Ethidium bromide, Acridine orange, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Thiobarbituric acid, 1,1',3,3' tetraethoxy propane , Guanidine hydrochloride, 5,5' dithiobis (2-nitrobenzoic acid), and Agarose was purchased from Sigma, St. Louis, MO, USA. Dinitrophenyl hydrazine, Ethyl acetate, Dimethylsulfoxide (DMSO), Triton X-100, Hydrogen peroxide, Trichloroacetic acid (TCA) was procured from Merck, Germany. Other reagents and chemicals were of analytical grade purity.

4.2.2 Methods

4.2.2.1 Cell culture and treatments- Mouse macrophages J774A.1 cells were cultured as described in chapter II (Section 2.2; Macrophage cell culture and treatments; Page number 23). On the day of experiment, cells were washed twice with cell culture grade PBS and treated with MetHb (7.75μM) or different concentrations of HP or β-hematin (βH) (0-

300µg/ml) or combination of MetHb (7.75µM) with different concentrations of βH (0-120µg/ml) for 6h at 37°C with 5% CO_2 in serum free medium. To test the effects of various antioxidants, spin trap or iron chelator, cells were pre-incubated with NAC (5mM), Mannitol (5mM), PBN (0-300µM), catalase (0-500units) or deferoxamine (0-500µM) for 1h and treated with different agonists as described above.

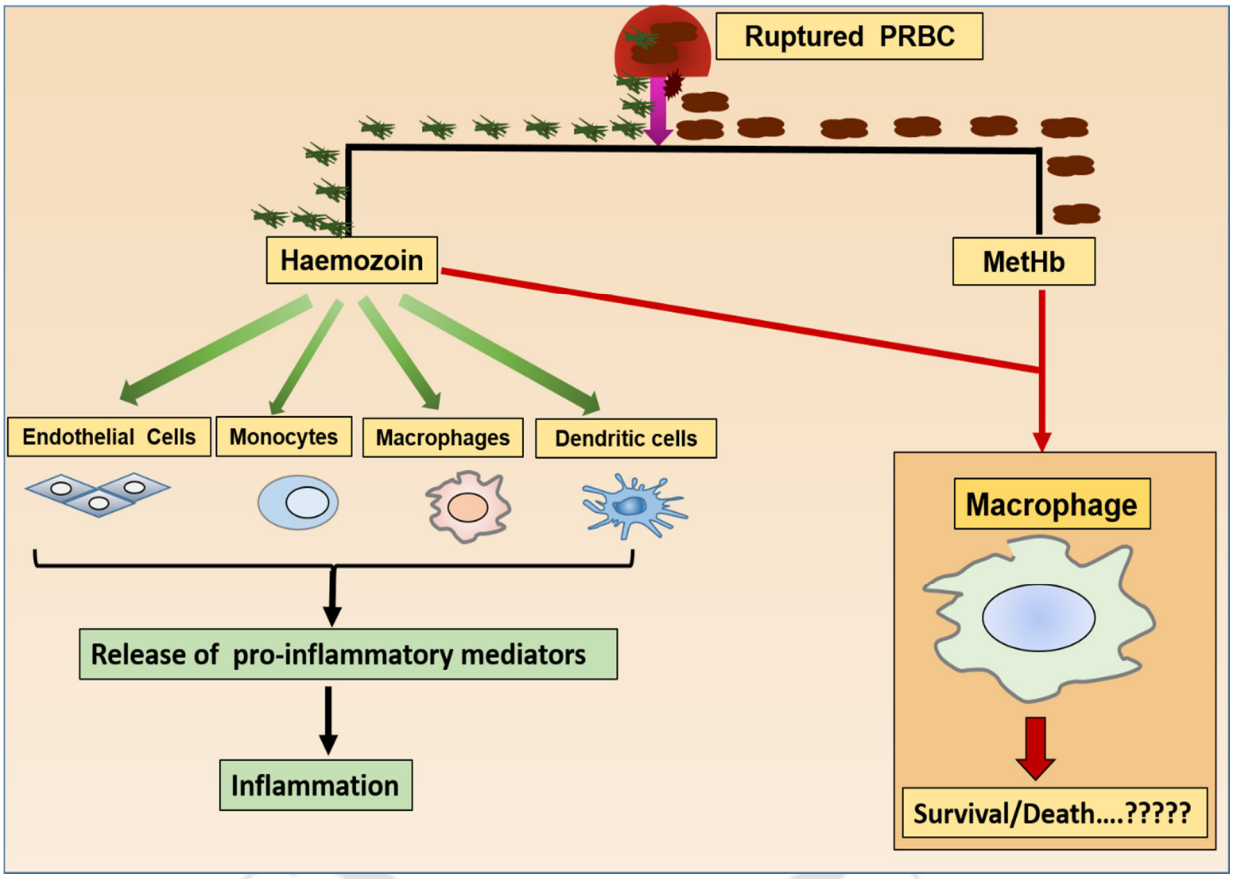

Figure 4.1: Working hypothesis for the mutual interaction between different pro-oxidant molecules on the macrophage biology.

4.2.2.2 Measurement of cellular viability- Ten thousand cells were seeded overnight in a 96-well in 0.2ml complete medium. On the day of experiment, cells were treated as described above. MTT reduction test was used for cell viability assay as described in chapter II (Section 2.3; Cell viability assay; Page number 23). Macrophage treated with incomplete medium was considered as 100% viable and used to express viability. To probe the role of oxidative stress or single electron containing species, cells were pre-incubated for 1h with antioxidants or spin trap and then treated with different agonists as described above.

4.2.2.3 Microscopic observation of macrophages- Cells were treated with different agonists and observed with Nikon eclipse TS 100F inverted microscope using 20x objective pre and post treatment. Images were captured with a high resolution Nikon L22 camera.

4.2.2.4 Preparation and fractionation of malaria culture supernatant- Purification of pro-inflammatory factor from malaria culture supernatant was done as described in chapter II (Section 2.15; Purification of pro-inflammatory factor from malaria culture supernatant; Page number 27).

4.2.2.5 Estimation of β-hematin inside the macrophages- Macrophages were treated with βH (60µg/ml), MetHb (7.75µM) or combination of βH (60µg/ml)/MetHb (7.75µM) for 6h at 37°C with 5% CO_2. Post treatment, the cells were washed twice with PBS to remove uningested βH. Cells were lysed by adding 0.1% triton X-100 in PBS and lysate was clarified by centrifugation at 1000xrpm for 5min at 4ºC. The supernatant was again centrifuged at 15,000xrpm for 10min at 4°C to pellet out the βH present in the cytoplasm. Pellet was washed twice to remove contaminating species and free hemin. The resulting pellet was dissolved in 2N NaOH and absorbance was measured at 400nm. Absorbance of β-hematin (60µg/ml) was taken as 100% to calculate % βH uptake in macrophage.

To study the phagocytosis mediated βH uptake, macrophage were equilibrated at particular temperature (37°C/10°C) for 30min and treated with different pro-oxidant molecules as described before. Post treatment, βH was extracted and quantitated as described.

4.2.2.6 Scanning electron microscopy (SEM)- Macrophages were untreated or treated with βH (60µg/ml), or combination of βH (60µg/ml)/MetHb (7.75µM) for 6h at 37°C and subjected to SEM analysis as described in chapter II (Section 2.5; Scanning electron microscopy; Page number 23). The instrumental conditions like EHT, magnification, width and signal were 10 kV, 2.5KX, 15 mm and SE1 respectively.

4.2.2.7 Measurement of intracellular ROS- Macrophages were treated with different pro-oxidant molecules in presence or absence of NAC (5mM), mannitol (5mM) or PBN (250µM) as described above and intracellular ROS was measured with the help of a ROS sensitive fluorescent probe 2′, 7′- dichlorofluorescein diacetate (DCFH-DA) as described in chapter II (Section 2.9; Intracellular ROS measurement; Page number 25).

4.2.2.8 Lipid peroxidation assay- Peroxidation of biological membranes results in formation of a major reactive aldehyde called malondialdehyde (MDA) which was used for the estimation of damage caused by reactive oxygen species. The content of lipid peroxides in macrophages was estimated by measuring thiobarbituric acid reactive substances (TBARS) (Trivedi, Chand et al. 2005). Cells were treated with β-hematin, MetHb or combination of β-hematin with MetHb as described. Post treatment, macrophages were washed twice with PBS and lysed with lysis buffer containing 0.1% triton X-100. Protein present in supernatant was precipitated by the addition of ice cold 10% trichloro acetic acid (TCA) and incubation for 15min at 4°C. The protein precipitate was centrifugation at 3000xrpm for 15min at 4°C. Resulting supernatant (150µl) was supplied with the equal volume of thiobarbituric acid (0.67%) and boiled in dry bath for 15min. The formation of malonaldehyde (thiobarbituric acid-reactive substances) was measured at 535nm in microtitre plate reader Spectromax M2^{e}. A small aliquot from macrophage lysate was used to measure the protein by Lowry's method as described (Lowry, Rosebrough et al. 1951). The lipid peroxidation was measured as thiobarbituric acid-reactive substances formed/mg of protein.

4.2.2.9 Protein carbonyl estimation- Protein carbonyl was estimated as described previously (Dalle-Donne, Rossi et al. 2003). Macrophages were treated with β-hematin, MetHb or combination of β-hematin with MetHb as described. Post treatment, macrophage were washed twice with PBS and lysed by lysis buffer containing 0.1% triton X-100. Protein present in supernatant was precipitated by the addition of ice cold 10% TCA and incubation for 15min at 4°C. The precipitated protein was pelleted down by centrifugation at 8000xrpm for 10min at 4°C. To the pellet 50µl of 2% 2, 4-dinitrophenyl hydrazine (DNPH) was added and incubated for 1h at 37°C to form a stable dinitrophenyl (DNP) hydrazone product. After incubation, protein pellet was recovered from reaction mixture by TCA (100%) and washed 3 times with a cocktail of ethanol, ethyl acetate mixture (1:1, v/v) and centrifuged at 8000xrpm for 10min. The final protein pellet was dissolved in 600µl of 6M guanidine hydrochloride, and measured at 372nm. Protein carbonyl was measured as µM of DNP formed/mg of protein.

4.2.2.10 Measurement of Reduced Glutathione (GSH) - GSH was estimated as described previously with slight modification (Trivedi, Chand et al. 2005). Macrophages were treated with β-hematin, MetHb or combination of β-hematin with MetHb as described. Post treatment, macrophage were washed twice with PBS and lysed by lysis buffer containing

0.1% triton X-100. Protein present in supernatant was precipitated by the addition of ice cold 10% TCA and incubation for 15min at 4°C. The precipitated protein was pelleted down by centrifugation at 8000xrpm for 10min at 4°C. To the supernatant an equal volume of 0.8M Tris-HCl, pH 9, containing 20mM 5–5'-dithionitrobenzoic acid was added to yield the yellow chromophore of thionitrobenzoic acid which was measured at 412nm. GSH content was measured as µM of thionitrobenzoic acid formed/mg of protein.

4.2.2.11 Acridine orange and Ethidium Bromide (AO-EtBr) staining- Macrophages were treated with different pro-oxidant molecules in presence or absence of NAC (5mM), mannitol (5mM) or PBN (250µM) as described above and apoptotic and dead cells were differentiated by staining the cells with acridine orange (AO) and ethidium bromide (Et-Br) and analyzed in FACS as described in chapter II (Section 2.7; Acridine orange and Ethidium Bromide staining; Page number 24).

4.2.2.12 Detection of DNA fragmentation on agarose gel- Five lakh cells were treated with different pro-oxidant molecules in presence or absence of NAC (5mM), mannitol (5mM), PBN (300µM) catalase (200units) or deferoxamine (10µM). Post treatment, cells were lysed, treated with RNAse, Proteinase K, run in agarose gel and visualized under UV-light as described in chapter II (Section 2.8; Detection of DNA fragmentation on agarose gel; Page number 25).

4.3 Results

4.3.1 Pro-oxidant molecules mutually interact to potentiate toxicity towards macrophage

Malaria culture supernatant is a mixture of Hb, MetHb, heme, haemozoin (polymeric heme) and malaria toxin. Initially, the hypothesis that mutual co-operative relationship of MetHb with heme polymer is responsible for enhanced toxicity of malaria culture supernatant towards macrophages was tested out. Malaria culture supernatant was fractionated by ammonium sulfate gives fractions with varying amount of MetHb, where fraction P2 was found to contain maximum amount of MetHb. Macrophage treated with combination of P2/hemin (P2/hemin mixture) or heme polymer (P2/heme polymer) for 6h has reduced cellular viability, indicating interaction of MetHb (pro-oxidant) with other pro-oxidant molecules (hemin or heme polymer) to enhance cellular toxicity (Table 4.1). The toxicological amplification is more pronounced for heme polymer rather than hemin.

Table 4.1: Effect of mixing P2 with other pro-oxidant molecules on macrophage viability.

Treatments	Survival (%) ±SD	Δ Cellular viability
Hemin	46.96 ± 7.64	NA
Heme polymer (HP)	71.06 ± 6.82	NA
Fraction P2	96.88 ± 4.21	100
Fraction P2+ Hemin	34.77 ± 3.85	70
Fraction P2+ Heme polymer (HP)	43.09 ± 4.66	60

Macrophage are exposed to P2 (90µg) alone or in combination with other pro-oxidant molecules present in malaria culture supernatant for 6h and viability was determined by MTT assay. Macrophage exposed to hemin (60µg/ml), HP (40µg/ml) was used as control. Change in cellular viability after reconstitution was calculated considering viability of P2 exposed macrophage as 100%. NA= "Not applicable".

MetHb utilizes its pseudoperoxidase activity to oxidize and polymerize aromatic/halide substrate into the polymeric products. MetHb oxidizes hemin to form heme polymer (HP) and it has similar crystal packing, bonding pattern and structural features with synthetic βH. Heme polymer (HP) or βH exposure to macrophages for 6h dose dependently reduces cellular viability with a lethal concentration (LC_{50}) of 132µg/ml and 182µg/ml respectively (Figure 4.2A). Treating the macrophages with different concentrations of βH in the presence of a non-toxic concentration of MetHb (7.75µM) gives an enhanced level of cellular toxicity with LC_{50} of 58µg/ml. At the highest concentration of βH (120µg/ml), MetHb enhances its cyto-toxicity by 2.75 folds. (Figure 4.2B). Similarly, heme polymer in the presence of MetHb (7.75µM) gives a cellular toxicity with LC_{50} of 32µg/ml. In an earlier study, MetHb exhibits toxicity towards macrophages in 40h, but it is non-toxic at earlier time points (<20h) (Deshmukh and Trivedi 2013). In the presence of sub-lethal concentration of βH (60µg/ml), MetHb is dose-dependently exhibiting toxicity towards macrophages with LC_{50} of 7.75µM (Figure 4.2C). At the highest concentration of MetHb (40µM), βH enhances its cyto-toxicity by 10 folds. Hence, pro-oxidant molecules co-operate synergistically with each other, results in enhanced level of cellular toxicity in an *in-vitro* toxicity model towards macrophages.

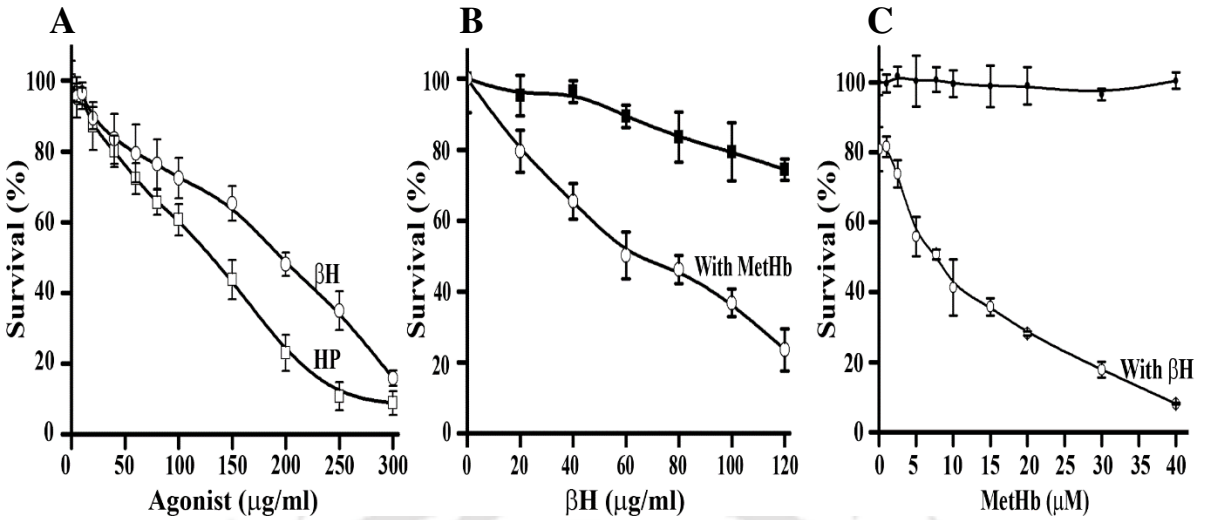

Figure 4.2: Pro-oxidant molecules co-operate with each other to exhibits enhanced toxicity towards macrophages. (A).Macrophage treated with different concentrations of agonists HP or βH for 6h at 37℃ and viability was determined by MTT. **(B)** MetHb potentiates the toxicity of βH towards macrophage. Macrophage treated with different concentrations of βH for 6h in absence or presence of MetHb (7.75μM) at 37℃ and viability was determined by MTT assay. **(C)** βH potentiates the toxicity of MetHb. Macrophage treated with different concentrations of MetHb (0-40μM) in absence or presence of βH (60μg/ml) at 37℃ and macrophage viability was determined by MTT. Macrophage treated with incomplete medium was considered as 100% viable. Data are the mean ± SD of three independent experiments with triplicate measurement.

4.3.2 MetHb exposure causes βH accumulation and morphological deformities

Macrophage exhibits high level of phagocytic activity and macrophage populations with high phagocytic activity are more susceptible to functional defects and damage from pro-oxidant molecules. Macrophage treated with a combination of βH (60μg/ml)/MetHb (7.75μM) gives ~40% more cells associated with βH than macrophage treated with βH alone (Table 4.2). The change in level of cell associated βH is statistically significant (p-value 0.001, βH vs. βH/MetHb). A light microscopic observation of macrophage further confirms the higher level of βH associated with macrophages treated with combination of βH (60μg/ml)/MetHb (7.75μM) in comparison to macrophage treated with βH only (Figure 4.3A, βH crystal indicated by arrow heads). In addition, careful observation of macrophages treated with βH, or combination of βH (60μg/ml)/MetHb (7.75μM) indicates a pronounced cyto-toxic effect in βH/MetHb treated cells (Figure 4.3A). Scanning electron microscopic images of macrophages treated with βH (60μg/ml)/MetHb (7.75μM) for 6h show an enhanced level of damage to cellular structure, cell shrinkage with severely distorted shape and membrane blebbing (Figure 4.3B).

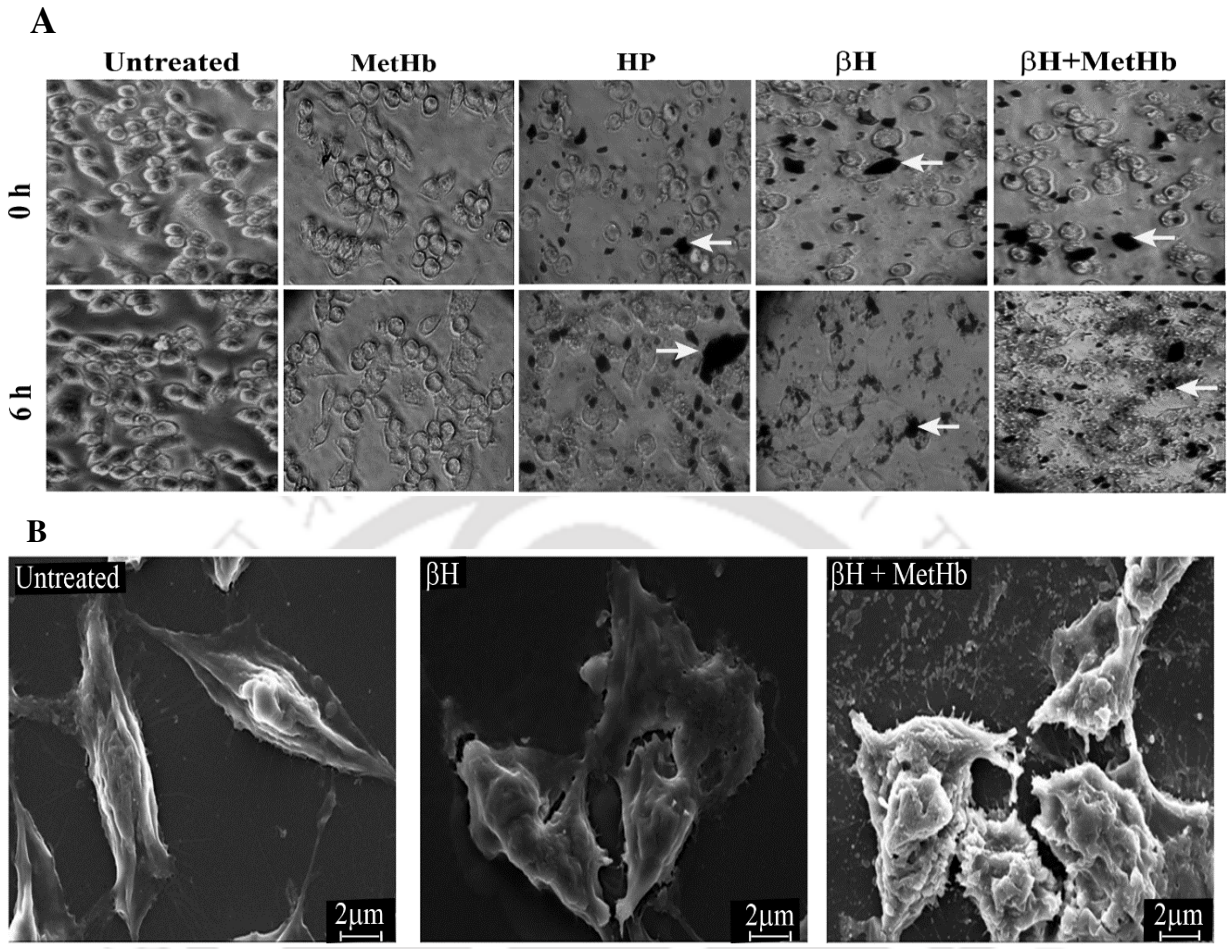

Figure 4.3: Microscopic observation of βH particles and cellular damage in macrophages. (A) Macrophages either untreated or treated with MetHb, HP,βH and βH (60μg/ml) / MetHb (7.75μM) mixture respectively for 6h at 37℃ and images of random 10 fields were captured with a 20x objectives using an inverted microscope TS100F (Nikon, Japan). βH particles are denoted by arrows in each panel. **(B)** SEM analysis of macrophages. Macrophages either untreated or treated with βH (60μg/ml) in the absence or presence of non-toxic concentration of MetHb (7.75μM) for 6h at 37℃ and a total of 10 different fields were captured using LEO 1430VP Scanning Electron Microscope with the instrument setting as EHT, width and signal were 10 kV, 15 mm and SE1 respectively. A representative image of untreated, treated with βH (60μg/ml) and βH (60μg/ml)/MetHb (7.75μM) mixture respectively (magnification; x2500, scale = 2μm) is given.

β-hematin is a crystalline material, and its uptake inside the macrophage follows phagocytosis mediated internalization and subsequent release into the cytoplasm through lysis of phagosome membrane (Schwarzer, Turrini et al. 1992). Phagocytosis is an energy dependent process, and it is severely been comprised at subpermisive temperature (Salman, Bergman et al. 2000). At 10°C, number of cells associated with βH is low (~1% of total) as compared to uptake studies performed at 37°C. This indicates a direct role of phagocytosis in the process (Table 4.2).

Table 4.2: Amount of β-hematin associated with macrophages.

Treatments	Total β-hematin (% ± SD)
Uptake at 37°C	
Methemoglobin	0.33 ± 0.1
β-hematin	10.43± 0.57
β-hematin + Methemoglobin	14.37± 1.09
Uptake at 10°C	
Methemoglobin	0.73± 0.18
β-hematin	1.04 ± 0.39
β-hematin + Methemoglobin	2.14 ± 0.31

Macrophages were treated with β-hematin (60µg/ml), MetHb (7.75µM) or combination of β-hematin (60µg/ml)/MetHb (7.75µM) for 6h at 10℃ or 37℃. Post treatment, the cells were washed, lysed and clarified and centrifuged to pellet out the β-hematin present in the cytoplasm. Pellet was washed twice to remove contaminating species and free hemin. The resulting pellet was dissolved in 2N NaOH and absorbance was measured at 400nm. Absorbance of β-hematin (60µg/ml) was taken as 100% to calculate % β-hematin uptake in macrophage.

To probe the phagocytosis mediated βH uptake with the toxicity, macrophages were treated with βH (60µg/ml)/MetHb (7.75µM) at 10°C for 6h and cellular viability was determined by MTT assay. Untreated cells incubated at 10°C served as control and used to calculate the change in survival for treated cells. At 37°C in the presence of MetHb (7.75µM), βH gives ~50% more toxicity towards macrophage whereas at 10°C, the increase in toxicity is only 10% (Table 4.3). Hence, data in Figure 4.2 clearly highlights the mutual interactions of pro-oxidant molecules which results into the severe damage to cellular structure through higher uptake of βH.

Table 4.3: Pro-oxidants toxicity toward macrophages at different temperature.

Treatments & Temperature	Survival of macrophage (%) ± SD
At 37°C	
Untreated	100.0 ± 4.88
Methemoglobin	95.00 ± 3.78
β-hematin	68.62 ± 2.54
β-hematin + Methemoglobin	47.47 ± 0.54
At 10°C	
Untreated	100.0 ± 15.43
Methemoglobin	94.00 ± 11.94
β-hematin	74.00 ± 1.39
β-hematin + Methemoglobin	67.00 ± 3.30

Macrophages were untreated or treated with β-hematin (60µg/ml), MetHb (7.75µM) or combination of β-hematin (60µg/ml)/MetHb (7.75µM) for 6h at 10℃ or 37℃ and viability was determined by MTT assay as described in chapter II.

4.3.3 Pro-oxidants molecules interaction give enhanced oxidative insult to macrophages

Pro-oxidant molecules disturb cellular physiology and causes development of oxidative stress through inhibition of cellular antioxidant machinery. Macrophage exposed to the MetHb (7.75µM) or βH (60µg/ml) for 6h causes up regulation of ROS level within macrophage cytoplasm as compared to the untreated control cells. Whereas, macrophage treated with a combination of βH (60µg/ml)/MetHb (7.75µM) for 6h exhibits significant high level of intracellular ROS as compared to the untreated cells (Figure 4.4A). MetHb exposure produces multiple ROS spikes within the macrophage cytoplasm to exhibit toxicological effects (Deshmukh and Trivedi 2013). In contrast, macrophage treated with a combination of βH (60µg/ml)/MetHb (7.75µM) did not exhibits multiple ROS spike pattern.

Development of oxidative stress leads to the protein damage, oxidation of membrane lipids and depletion of antioxidants (GSH) inside the macrophages. As compared to untreated cells, MetHb, βH or combination of βH/MetHb treated cells shows 5.5%, 20.5% and 61% increase in lipid peroxidation level. The increase in increase in lipid peroxidation correlates well with macrophage viability with a correlation coefficient (r^2) of -0.82 (Negative sign indicates the increase in lipid peroxidation with decrease in macrophage survival) (Figure 4.4B). Similarly, treated cells show a 32% increase in protein carbonyl level, whereas MetHb (7.75µM) or βH (60µg/ml) treated cells show only 3.4% and 20% respectively (Figure 4.4C). The correlation coefficient between protein carbonyl level and macrophage survival was found to be -0.86. An increase in lipid peroxidation or protein carbonyl level is due to reduction of GSH level of cytoplasm inside the cell. Cells stimulated with MetHb (7.75µM), βH (60µg/ml) or combination of βH (60µg/ml)/MetHb (7.75µM) show decrease in GSH level inside the cell by 29%, 34% and 94% respectively (Figure 4.4D). The correlation coefficient between decrease in GSH level and macrophage survival was found to be -0.97 which means the death is because of exhaust of antioxidant machinery inside the macrophages.

The data presented clearly indicates a synergistic relationship between MetHb and βH to develop oxidative stress inside the macrophage, and probably be responsible for cellular damage and death.

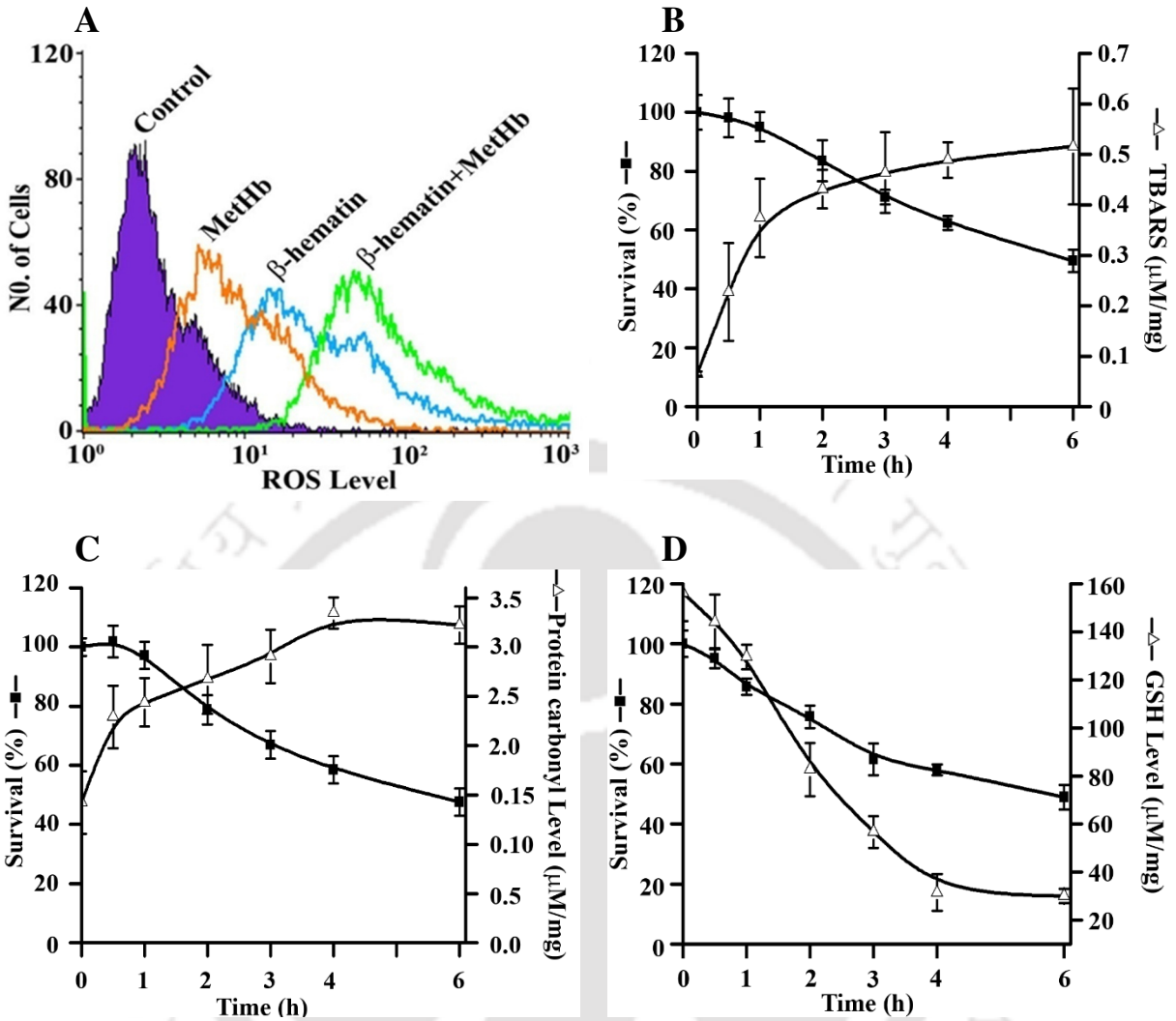

Figure 4.4: Proxidant molecules give oxidative insult to cells leading to damage of cellular machinery. Macrophages either untreated or treated with MetHb (7.75μM), βH (60μg/ml) or combination of βH (60μg/ml)/MetHb (7.75μM) for 6h at 37˚C. **(A)** Intracellular ROS was measured by fluorescent ROS probe DCFH-DA. **(B)** Correlation of macrophage survival with Lipid Peroxidation after treatment with combination of βH (60μg/ml)/MetHb (7.75μM) **(C)** Correlation of macrophage survival with Protein carbonyl after treatment with combination of βH (60μg/ml)/MetHb (7.75μM) **(D)** Correlation of macrophage survival with reduced GSH measurement after treatment with combination of βH (60μg/ml)/MetHb (7.75μM) as described in "material and method" section.

4.3.4 Mutual interaction of proxidant molecules show an enhanced level of apoptosis

DNA Fragmentation is used to assess the degree of apoptosis in macrophage cells. Macrophage treated with different concentrations of βH (0-60μg/ml), MetHb (7.75μM) or combination of βH (60μg/ml)/MetHb (7.75μM) for 6h, genomic DNA was extracted from treated cells and analyzed on 1.8 % agarose gel. Untreated or MetHb treated cell shows an intact genomic DNA with very little or no visible appearance of DNA fragments (Figure 4.5A, lane 1 and 3). In contrast, cells treated with βH give the appearance of DNA fragments

(Figure 4.5A, lane 2) whereas the level was significantly high in cells treated with combination of βH (60µg/ml)/MetHb (7.75µM) (Figure 4.5A, lane 4). Mitomycin-C was used as a positive control (Figure 4.5A, lane 5).

The differential population of apoptotic cells (early or late), necrotic and dead cells were identified by acridine orange-ethidium bromide (AO-EtBr) double staining method (Liegler, Hyun et al. 1995). Acridine Orange (AO) stains cells with early or late stage of apoptosis to give green fluorescence whereas ethidium bromide (Et-Br) binds to the DNA of necrotic/dead cells to give orange fluorescence. Macrophage treated with incomplete medium (control), MetHb (7.75µM), βH (60µg/ml) or combination of βH/MetHb for 6h, and AO-EtBr double stained cells were analyzed by flow cytometry. Untreated or MetHb treated cells shows 100% healthy cells (Figure 4.5B) whereas β-hematin treated cells showed 71 % healthy, 28.2% apoptotic cells (Figure 4.5B, β-hematin). In the presence of MetHb, β-hematin treated cells exhibit 28.17 % healthy, 22% early apoptotic and 49.83% late apoptotic/necrotic cells (Figure 4.5B, β-hematin/MetHb).

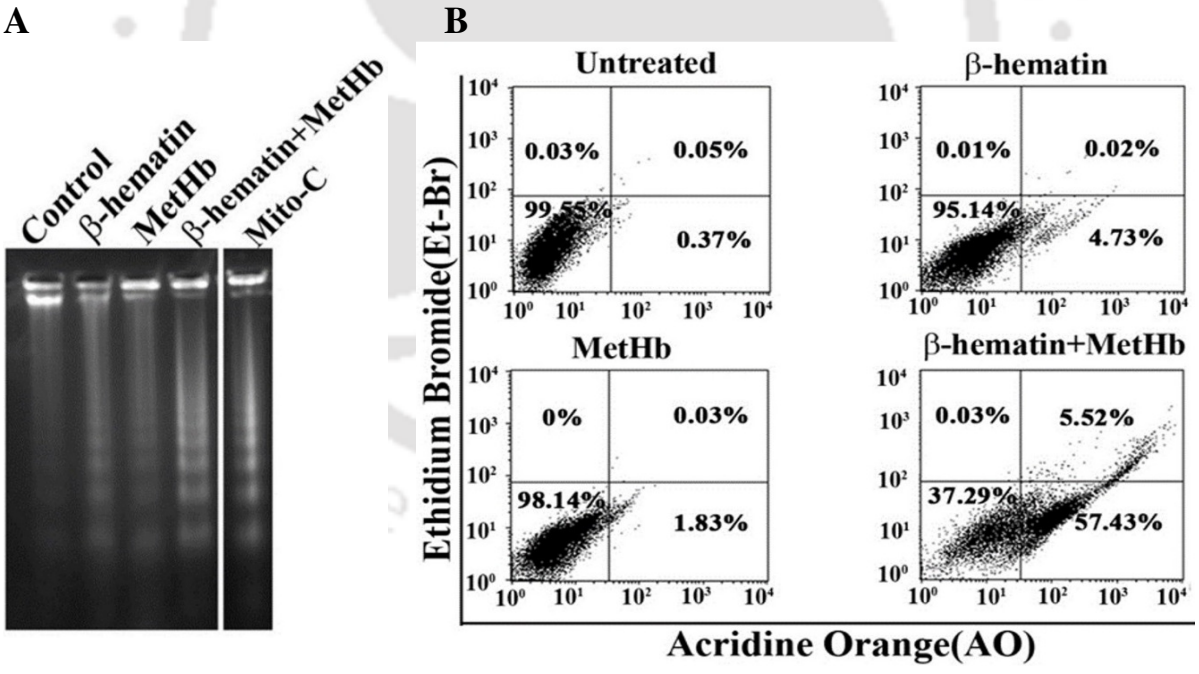

Figure 4.5: Generation of intracellular ROS leads to apoptosis. (A) DNA fragmentation pattern of untreated (lane 1), β-hematin 60µg/ml (lane 2), MetHb 7.75µM (lane 3), combination of 60µg/ml β-hematin with MetHb 7.75µM (lane 4), and positive control mitomycin-C (lane 5). **(B)** Dot plot of AO-EtBr staining, untreated or cells treated with β-hematin 60µg/ml, MetHb 7.75µM, combination of 60µg/ml β-hematin with 7.75µM MetHb were analyzed after 6h with flow cytometry. Fluorescence dot plot represent events within FL-1(Et-Br; x axis) versus FL-3 (AO; y axis).

4.3.5 Antioxidants scavenge the intracellular ROS and prevent macrophage apoptosis

To verify the ROS mediated toxicity in treated macrophage, ROS was removed by antioxidants. One lakh cells were treated with combination of βH (60µg/ml)/ MetHb (7.75µM for 6h in presence or absence of NAC (5mM), mannitol (5mM) or PBN (200µM). Post treatment, cells were incubated with DCFH-DA for 30min and 50,000 cells were stained with AO-EtBr and acquired and analyzed in FACS Calibur. Macrophage treated with serum free medium was considered as control. Pre-incubation of βH (60µg/ml)/MetHb (7.75µM) treated cells with NAC (5mM), mannitol (5mM) or PBN (200µM) show a significant decrease in ROS as compared to treated cells (Figure 4.6A).

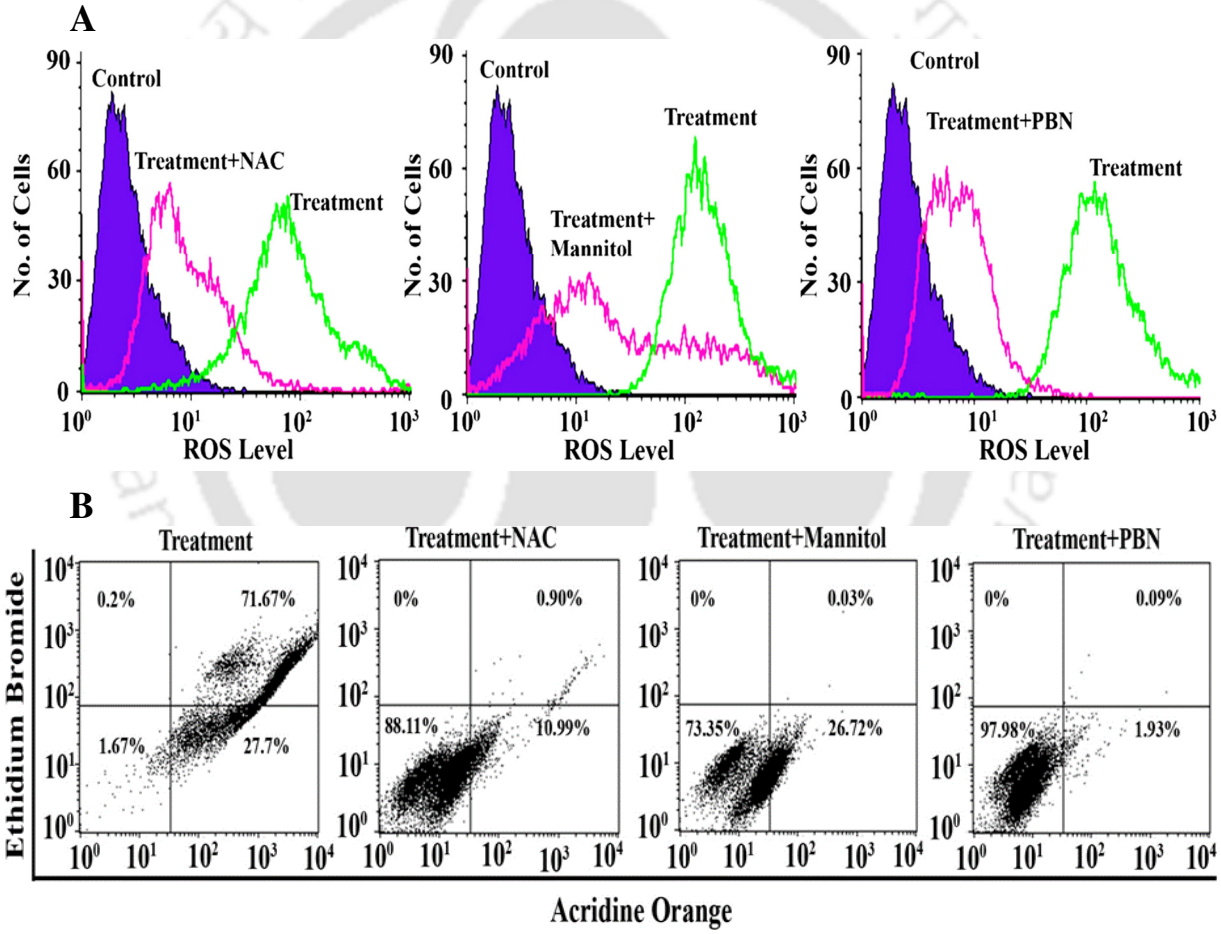

Figure 4.6: Pre-incubation with antioxidants or spin trapper reversed the ROS and prevents from apoptosis in macrophage. **(A)** Cells were left untreated or treated with MetHb 7.75µM or 60µg/ml βH or combination 60µg/ml βH with 7.75µM MetHb or pre-incubation of treated cells with NAC (5mM), mannitol (5mM) or PBN (200µM) and analyzed after 6h by flow cytometry. ROS produced inside the cell was measured by the fluorescence exhibited by fluorescence probe DCF. **(B)** Dot plot of AO-EtBr staining, cells treated with combination of 60µg/ml βH and 7.75µM MetHb or pre-incubation of treated cells with NAC (5mM), mannitol (5mM) or PBN (200µM) and analyzed after 6h with flow cytometry.

The differential percentage of healthy, apoptotic and death cells were studied by AO-EtBr staining. Pre-incubation of βH (60µg/ml)/MetHb (7.75µM) treated cells with NAC (5mM), mannitol (5mM) or PBN (200µM) shows a significant decrease in apoptosis as compared to treated cells (Figure 4.6B). Treated cells shows ~2% healthy cells whereas NAC shows 88%, mannitol 73% and PBN 98% healthy cells.

4.3.6 Antioxidant pre-incubation prevents macrophage from apoptosis

Recovery of death from βH (60µg/ml)/MetHb (7.75µM) treated cells was measured by MTT assay. As expected, pre-incubation of treated cells with NAC (5mM) or mannitol (5mM) for 6h shows a significant recovery of death from treated cells in MTT assay. NAC and mannitol reversed the toxic effects of βH/MetHb by 73% and 38% respectively (Figure 4.7A). Light microscopic observation of macrophage treated with βH/MetHb in the presence of antioxidants (NAC or mannitol) further confirms the reversal of observed cellular damages (Figure 4.7B). After 6h of treatment macrophages shows cellular damage phenotype whereas NAC or mannitol pre-incubated cells were healthy with normal morphology (Figure 4.7B). It highlights the role of oxidative stress with the cyto-toxic potentials of βH/MetHb but probably oxidative stress does not regulate βH uptake inside the macrophages. Moreover, DNA fragmentation pattern appeared in βH/MetHb treated cells was reversed completely in the presence of antioxidants (Figure 4.7C).

4.3.7 Extracellular H_2O_2 generation is crucial for toxicological effects of βH/MetHb

β-hematin has an iron coordinately associated with the porphyrin ring system (Pagola, Stephens et al. 2000). MetHb is a metallo-protein and contains iron (Fe^{3+}) in the bound hemin (Reeder 2010). Release of free iron from the βH or MetHb leads to the production of hydrogen peroxide (H_2O_2) by a fenton reaction, and known to be associated with the toxicity of both pro-oxidant molecules (Regan and Panter 1996; Burhop, Gordon et al. 2004). To probe the role of extracellular hydrogen peroxide generation with toxicological phenotype, H_2O_2 was scavenged by catalase and macrophage viability was studied by MTT assay. Pre-incubation of treated cells with catalase (0-500units) shows a dose dependent reversal of cyto-toxic effects of βH/MetHb (Figure 4.8A). In comparison to treated cells with ~57% viability, catalase pre- incubated cells shows reversal of toxicity in a dose dependent manner and gives ~100% reversal to give viable cells (Figure 4.8A).

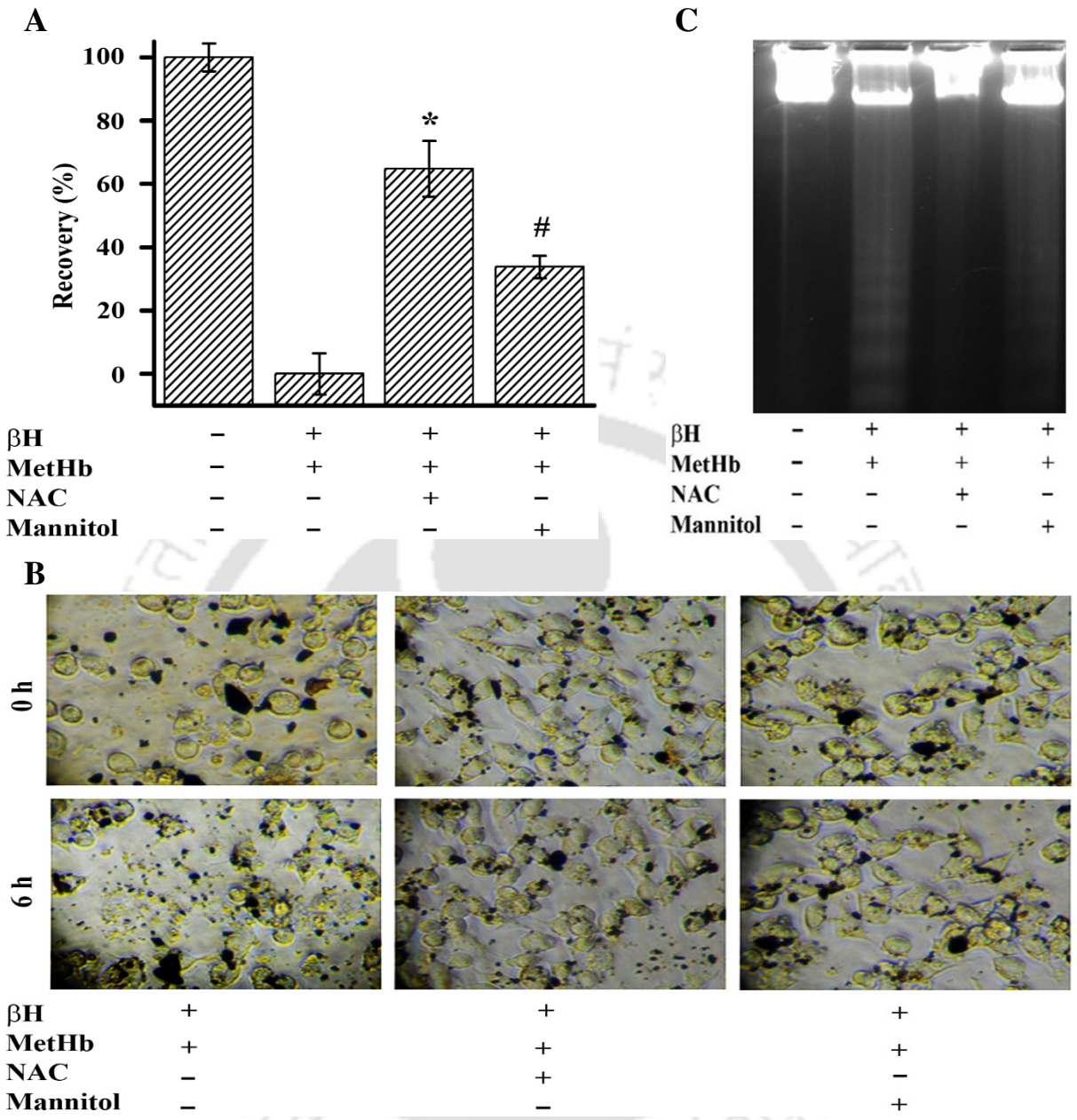

Figure 4.7: Pre-incubation of MetHb/βH treated cells with antioxidants prevents the cells from death. (**A)** Macrophages either untreated or treated with βH (60µg/ml)/MetHb (7.75 µM) for 6h at 37℃ in the absence or presence of NAC (5mM) and mannitol (5mM) respectively. Cell viability was measured by MTT assay. Macrophage treated with incomplete medium considered as 100% viable. Recovery of cellular viability in the presence of NAC (5mM) or mannitol (5mM) was calculated in terms of cellular viability of macrophage treated with combination of βH (60µg/ml)/MetHb (7.75 µM) and expressed as % recovery ±SD. Data are the mean ± SD of three independent experiments (n=3) with triplicate measurement. The pairwise results were analyzed with anova & student t-test and it was statistically significant with *P <0.001, #P <0.001. **(B)** Light microscopic observation of macrophage treated in A with a 20x objective to detect cellular morphology at 0h and 6h.**(C)** DNA fragmentation analysis of macrophages either remains untreated or treated with combination of βH (60µg/ml)/MetHb (7.75 µM) for 6h at 37℃ in the absence or presence of different antioxidant molecules (NAC/mannitol).

Macrophage treated with catalase (0-500units) alone has no stimulatory or pro-supportive growth effect ruled out any such possibility with the observed reversal of cyto-toxicity (Figure 4.8A). To test the possibility of released free iron as a source of H_2O_2, cells were pre-incubated with deferoxamine (0-500µM) to chelates free iron. Interestingly, chelating iron has no effect on the reversal of βH/MetHb mediated cyto-toxicity towards macrophages (Figure 4.8B). Macrophage treated with deferoxamine (0-500µM) alone has no cyto-toxic effects up to 10µM but exerts cyto-toxicity beyond this concentration in a dose-dependent manner. The non-toxic concentration of deferoxamine (0-10µM) is used to provide recovery in the cellular system through chelation of free iron released from hemo-proteins (Lee, Lee et al. 2007).

DNA fragmentation study showed that removal of external hydrogen peroxide by catalase restored the integrity of the genomic DNA (Figure 4.8C, lane 3). But macrophages pre-incubated with deferoxamine gives a DNA fragmentation pattern which was almost similar to the βH/MetHb treated cells (Figure 4.8C, lane 4). The above results clearly indicate a pivotal role of extracellular H_2O_2 generation but not the release of free iron from βH/MetHb mixture in eliciting the cyto-toxicity towards the macrophages.

4.3.8 MetHb to oxidize βH to βH radical (βH*) via a single electron transfer mechanism

MetHb in the presence of H_2O_2 oxidizes a number of aromatic and halide substrate through single electron oxidation mechanism. When H_2O_2 added to native MetHb (FeIII), there was a shift of soret peak from 406nm to 417nm (Figure 4.9) indicating the formation of an intermediate higher oxidation ferryl state (Fe-IV=O) complex, compound II. When βH (10µM) added to this complex (417nm), it gets reduced to the native FeIII state (406nm) by one electron transfer process. An equal amount of βH was added to the reference cuvette to avoid artifact of shift in soret peak. The UV-Visible spectral studies strongly support the finding that the βH probably been oxidized to single electron containing species βH* following a single electron oxidation mechanism.

4.3.9 Single electron containing species (βH*) is responsible MetHb mediated toxicity

Different concentrations of βH in the presence of MetHb (7.75µM) give a dose dependent killing of macrophages if remains untreated. Whereas, cells incubated with the different concentrations (0-300µM) of PBN to remove single electron species generated in external milieu, showed a dose dependent recovery from the cyto-toxic effect of β-hematin/MetHb with a full recovery at 250µM (Figure 4.10A). A light microscopic

observation of PBN pre-incubated macrophage showed a full recovery from cellular damage. At the initial time point (0h), all cells exhibit normal phenotype but at 6h, PBN pre-incubated macrophages give recovery from the toxicological phenotype of βH/MetHb (Figure 4.10B). In addition, scavenging of a single electron containing species (βH^*) by PBN reversed the DNA fragmentation pattern to give an intact genomic DNA which confirms the role of βH^* in potentiating cyto-toxic effect of β-hematin (Figure 4.10C, lane 3). Thus, the above result confirms that generation of a single electron species via one electron transfer from MetHb is responsible for the enhanced cyto-toxicity of βH to the macrophage. In order to identify the single electron species (βH*), EPR experiment was tried out, but high molecular weight and less solubility of βH in aqueous buffer system are the limitation to perform EPR spectroscopy.

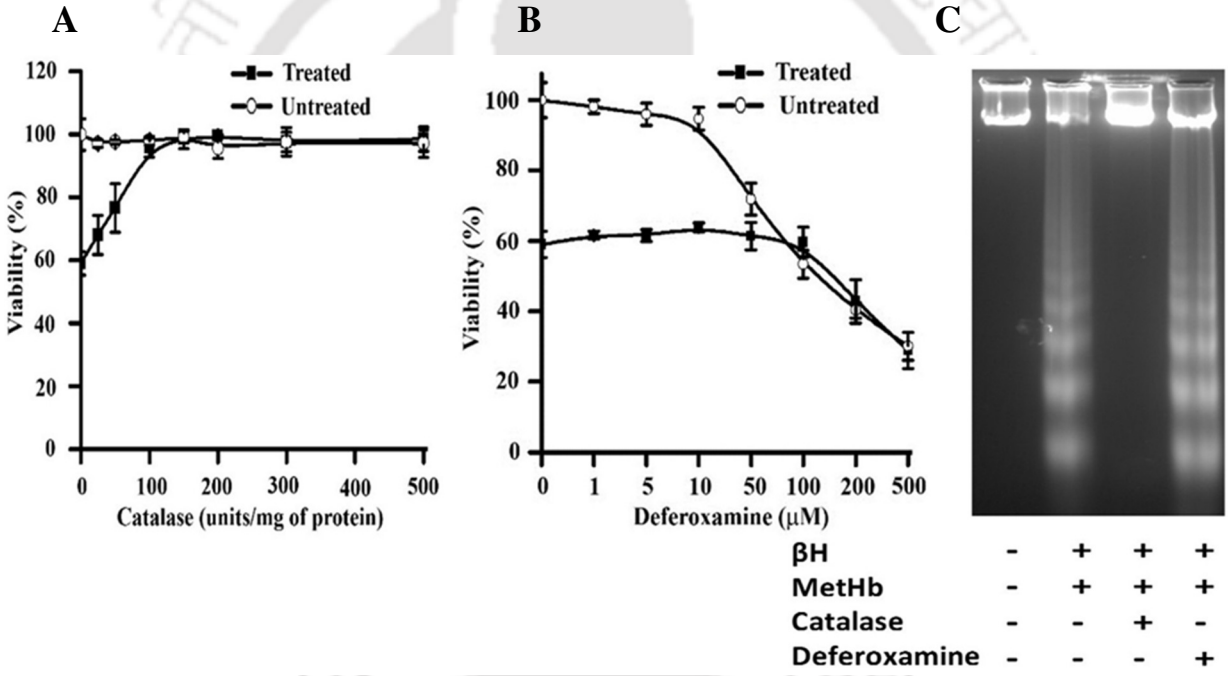

Figure 4.8: Extracellular H_2O_2 generation is responsible for MetHb mediated βH toxicological potentiation towards macrophages. (A) Removal of extracellular H_2O_2 gives recovery from cyto-toxic effects of βH towards macrophages. Macrophage was pre-incubated with different amount of catalase (0-500units) and either remains untreated or treated with combination of βH (60μg/ml)/MetHb (7.75μM) for 6h at 37℃. Catalase (0-500units) alone has no growth stimulatory effects and scavenging H_2O_2 has reversed the toxicity of βH (60μg/ml)/MetHb (7.75μM). **(B)** Scavenging free iron has no effects on reversal of viability from cyto-toxic effects of βH towards macrophages. Macrophage was pre-incubated with different amount of deferoxamine (0-500μM) and either remains untreated or treated with combination of βH (60μg/ml)/MetHb (7.75μM) for 6h at 37℃. Macrophage viability was determined by MTT assay and expressed as % viability. Data is the mean ± SD of three independent experiments (n=3) with triplicate measurement. **(C)** DNA fragmentation analysis of macrophage remains untreated or treated with combination of βH (60μg/ml)/MetHb (7.75μM) for 6h at 37℃ in absence or presence of catalase (200units) or deferoxamine (10μM).

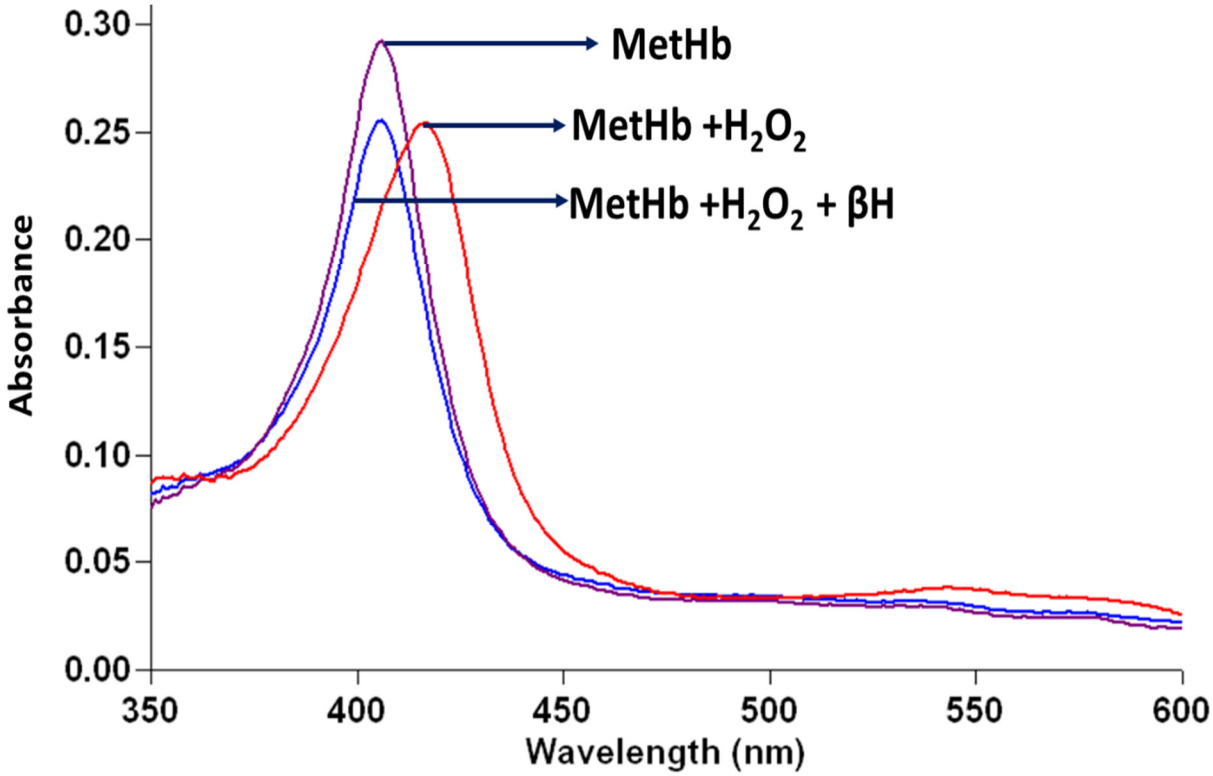

Figure 4.9: MetHb and βH interaction generates single electron containing species (βH*) to exhibits cyto-toxicity towards macrophages. Optical spectra of βH oxidation by MetHb. Soret spectra were recorded in 100 mM Tris-HCl buffer, pH 7.4, in a total volume of 0.8ml. Soret spectrum of MetHb (1µM) (violet color spectrum); MetHb + H_2O_2 (100µM) (red color spectrum); MetHb + H_2O_2 + βH (10µM) (blue color spectrum). Equal concentration of βH (10µM) was added in the reference cuvette to correct absorbance.

4.4 Discussion

Propagation of malaria parasite within host blood releases a mixture of different pro-oxidant molecules such as MetHb, heme, haemozoin (heme polymer) and malaria toxins (Kamchonwongpaisan, Samoff et al. 1997; Krishnegowda, Hajjar et al. 2005; Pamplona, Hanscheid et al. 2009). Haemozoin is an inert non-biodegradable polymer and macrophage treated with polymer exhibits significant change in cytokine profile (pro-inflammatory/anti-inflammatory), phagosome maturation and other functional defects (Pichyangkul, Saengkrai et al. 1994; Sherry, Alava et al. 1995; Scorza, Magez et al. 1999; Parroche, Lauw et al. 2007; Lucchi, Sarr et al. 2011). Macrophage J774A.1 exposed to hemin and heme polymer exhibits a significant change in cellular viability (Table 4.1). In the current study, heme polymer and βH exposure to macrophage causes dose-dependent reduction in cellular viability with LC_{50} of 132 µg/ml and 182 µg/ml respectively (Figure 4.2).

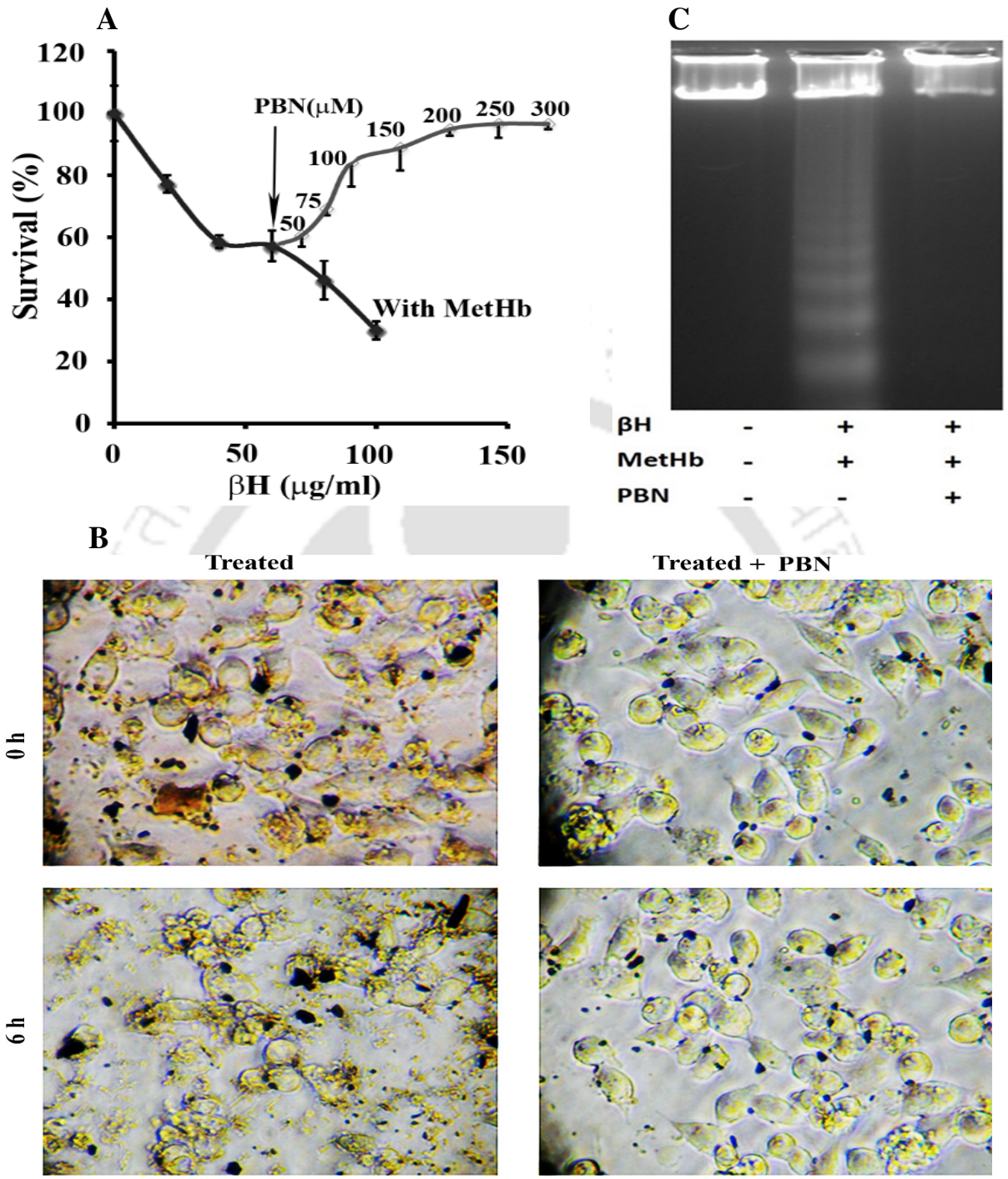

Figure 4.10: Scavenging single electron containing species (βH*) restores cellular viability of macrophages. (A) Cells were treated with different concentration of βH (0-100μg/ml)/MetHb (7.75μM) mixture in the presence of different concentrations of PBN (0-300μM) to scavenge single electron containing species generated in reaction mixture or remained untreated. Cellular viability was determined by MTT assay. Cells treated with incomplete medium was considered as 100% viable. Data is the mean ± SD of three independent experiments (n = 3) with triplicate measurement. **(B)** Light microscopic observation of macrophage treated in "A" with a 20x objective to detect cellular morphology at 0h and 6h.**(C)** DNA fragmentation analysis of macrophages untreated or treated with combination of βH (60μg/ml)/MetHb(7.75 μM) for 6h at 37℃ in absence or presence of PBN (300μM).

In an *in-vitro* incubation system, MetHb accepts hemin as substrate and polymerizes to the heme polymer, and further contribute to the level of toxic heme polymer (Deshmukh and Trivedi 2013). In the presence of extracellular MetHb, βH toxicity towards macrophage is increased several folds with a LC_{50} of 58 µg/ml (Figure 4.2). Pro-oxidant molecule released during malaria has ability to potentiate macrophage phagocytosis (Leitner and Krzych 1997; Chua, Brown et al. 2013) and may explain higher uptake of βH in macrophage treated with βH/MetHb (Figure 4.3). MetHb exposure to the macrophage causes production of multiple ROS spikes within cytoplasm to develop oxidative stress (Deshmukh and Trivedi 2013). Development of oxidative stress activates stress linked signaling components to secrete pro-inflammatory cytokines (Jaramillo, Godbout et al. 2005). Macrophage exhibits higher level of phagocytosis in the presence of pro-inflammatory cytokines (Leitner and Krzych 1997; Chua, Brown et al. 2013). Hence, a series of molecular events down-stream to MetHb mediated ROS production may in-turn activates macrophage machinery to stimulate higher uptake of βH through phagocytosis. Macrophage isolated from malaria patients exhibits different degree of phagocytotic activity and accumulation of intracellular ROS, and functional defects within macrophage correlates well with degree of phagocytotic activity. A detail study may be needed to understand molecular events responsible for higher phagocytotic activity which result in accumulation of βH within macrophages.

Peroxidases play a pivotal role in protecting cells from generated peroxide and other free radicals. MetHb has pseudoperoxidase activity and it has potentials to oxidize aromatic and halide substrates (Reeder, Svistunenko et al. 2004; Balaji and Trivedi 2013; Deshmukh and Trivedi 2013). A dual role of MetHb in cyto-protective or cyto-toxic effects is documented and it is linked to the availability and ability of MetHb to oxidize particular substrate (Buehler and D'Agnillo 2010). Substrate oxidation through peroxidases utilizes large amount of peroxide to form stable product, and protect the cells from high level of peroxides. In cases where peroxidase is incompetent to oxidize the substrate or substrate oxidation product is fast acting (unstable), enhances cyto-toxicity (Reeder 2010). MetHb accepts primaquine as substrate and oxidizes it to the very fast acting 5-hydroxy-primaquine and enhances the hemo-toxicity of parent molecule towards RBCs (Balaji and Trivedi 2013). Following similar mechanism, MetHb utilizes extracellular H_2O_2 to oxidize βH to generate $βH^*$ (Figure 4.9). It is interesting that extracellular H_2O_2 is important for enhanced toxicity of βH in the presence of MetHb but source of H_2O_2 is not free metal released from it (Figure

4.8). MetHb to hemoglobin turn over through oxidation/reduction reactions (by several pro-oxidant/antioxidant molecules) are releasing free electron into the aqueous environment which might be responsible for generation of H_2O_2 (Reeder 2010). PBN mediated reversal of cyto-toxic effects of βH partially supports such a mechanism but it does not ruled out the obvious role of $βH^*$ radical as well (Figure 4.10).

4.5 Conclusions

The present chapter explored the cyto-toxicity and underlying mechanism towards macrophage when exposed to β-hematin in presence of MetHb during malaria. MetHb synergistically potentiate the toxicity of β-hematin towards macrophage and causes death. A schematic model to summarize the current finding is presented in Figure 4.11. MetHb mediated toxicological potentiation of pro-oxidant β-hematin towards macrophage involves multiple steps: Step 1; MetHb oxidizes βH to form $βH^*$ in the presence of excess of H_2O_2 in extracellular milieu. Simultaneously, it is potentiating macrophage phagocytotic activity to engulf large amount of βH crystals. Step 2; Irrespective of β-hematin uptake mechanism, βH accumulation inside the macrophage causes development of oxidative stress. Oxidative stress causes induction of apoptosis mediated death of macrophages. Antioxidants and spin trap treatment revert ROS and protect macrophages from apoptosis.

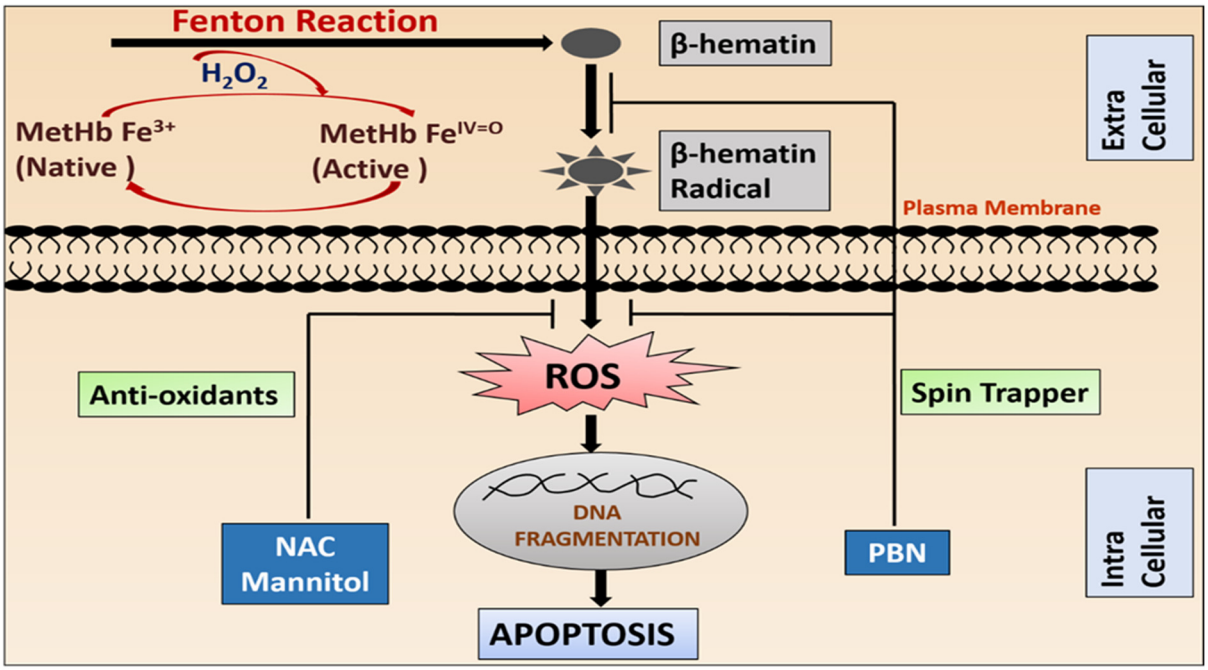

Figure 4.11: A schematic model to summarize the methemoglobin mediated immuno-toxicity modulation of pro-oxidant molecules towards macrophages during malaria.

Methemoglobin Mediated Homeostatic Disturbance through Hemin Oxidation and Polymerization.

5.1 Introduction

Malaria is one of the most devastating disease responsible for around 1–3 million deaths per year, mainly in children. Cerebral Malaria (CM) is associated with pathological complications such as coma, seizures, respiratory distress, severe anemia, spontaneous bleeding, severe metabolic acidosis, renal failure and pulmonary edema (Weatherall and Abdalla 1982). The pathogenesis of CM is due to mechanical blockage by parasitized red blood cells (PRBC) or inflammation at the site of brain injury (Schofield and Grau 2005). The presence of erythrocyte membrane protein 1 (PfEMP1) on surface of parasitized RBC (PRBC) causes binding PRBC to brain endothelium cells and contribute to vesicular blockage and leakage of blood brain barrier (BBB) (Hisaeda, Yasutomo et al. 2005). This leaky behavior BBB leads to inflammation and requirement of leukocyte at the cerebrospinal fluid (Kim 2003). Moreover $CD4^+$ T_H mediated response produces a high level of pro-inflammatory cytokines, TNF-α and LT-α (Sarra, Cupi et al. ; Hunt and Grau 2003). Histological examination of brain tissues of CM patients indicates accumulation of stress proteins, urokinase type plasminogen activator from activated macrophages (Fauser, Deininger et al. 2000). Inflammatory and resident macrophages are the two subset of macrophage found in the brain site during CM (Sun, Rao et al. 2007). Both these macrophage have a role in CM pathology. Inflammatory macrophage release pro-inflammatory cytokines whereas activation of resident macrophage is responsible for requirement of $CD8^+$ T lymphocytes which initiate perforin mediated cytotoxic events (Nitcheu, Bonduelle et al. 2003; Pais and Chatterjee 2005).

Malaria culture supernatant or infected serum is pro-inflammatory in nature. During malaria rupture of PRBC releases various factors (proteinous or non-proteinous) into the blood stream which is a complex mixture consisting of host derived proteins, parasite secreted proteins and other metabolically derived degraded peptides (Shio, Kassa et al. ; Kamchonwongpaisan, Samoff et al. 1997). The non-proteinous part contains hemin, haemozoin and other toxic metabolic byproducts. These molecules activate the immune cells to release pro-inflammatory cytokines, stimulate endothelial cells and affects blood brain barrier (BBB) (Jaramillo, Gowda et al. 2003; Dostert, Guarda et al. 2009). Cerebro-spinal

fluid (CSF) of malaria patients contaminated with proteinous factors derived from PRBC rupture and their level correlates with neurological symptoms or death (Sakamoto, Takaki et al. 2002). Malaria toxin (glycophosphoinositol) can cause production of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) and reactive oxygen species (ROS) (Krishnegowda, Hajjar et al. 2005). Macrophage activation to induce ROS production and release into the external microenvironment contributes to the inflammation within brain tissue (Ekdahl, Claasen et al. 2003).

RBC destruction is accelerated in most of the pathological conditions due to poor antioxidant protection. During malaria, every infected RBC causes lysis of 10 or more uninfected RBCs (Balaji and Trivedi 2012). As a result, a large amount of released hemoglobin is converted to methemoglobin (MetHb). MetHb, a Fe^{3+} containing metallo-protein is a pro-oxidant molecule and is responsible for production of the large amount of peroxide to accelerate tissue damage (Boretti, Buehler et al. 2009).

Hemin to heme polymer formation is considered to be catalyzed by a peroxidation reaction involving production of heme free radicals. Using a model enzyme, horse raddish peroxidase (HRP), mechanistic details of the polymerization process has already been established (Trivedi, Chand et al. 2005). But whether peroxidase present at brain site exploits such a mechanism to contribute patho-physiological effects are not known. MetHb released from infected RBC has a pseudo peroxidase activity but it is not clear if it has the potential to polymerize hemin to heme polymer following a peroxidation mechanism. In addition, it is not clear how presence of MetHb at brain site amplifies the ROS production from the macrophages. These possibilities are depicted in the proposed hypothesis given in Figure 5.1 and give us the opportunity to explore the complex molecular details of observed inflammation at the brain site.

5.2 Material and methods

5.2.1 Materials- Methemoglobin (MetHb), Hemin (He), clotrimazole (CLT), Mannitol, N-Acetyl cysteine (NAC), Phenyl N-tert-butylnitrone(PBN) and 2,2,6,6-Tetramethylpiperidine 1-oxyl (TEMPO) were purchased from Sigma, St. Louis, MO, USA. Sodium azide and Guaiacol were purchased from Hi-media, India. Hydrogen peroxide (H_2O_2) was purchased from Merck, Germany. All other reagents and chemicals were of analytical grade purity.

5.2.2 Methods

5.2.2.1 Cell line and cell culture- The detail method of cell culture is given in chapter II (Section 2.2; Macrophage cell culture and treatments; Page number 23).

5.2.2.2 Heme polymerization assay- Heme polymerization assay was done as described in chapter II (Section 2.10; Heme polymerization assay; Page number 25). To test the effect of peroxidase inhibitors (clotrimazole or sodium azide) or substrate competition studies (guaiacol, potassium chloride or potassium iodide); indicated inhibitors or small molecules electron donors were added to incubation mixture containing MetHb, prior to hemin and hydrogen peroxide additions.

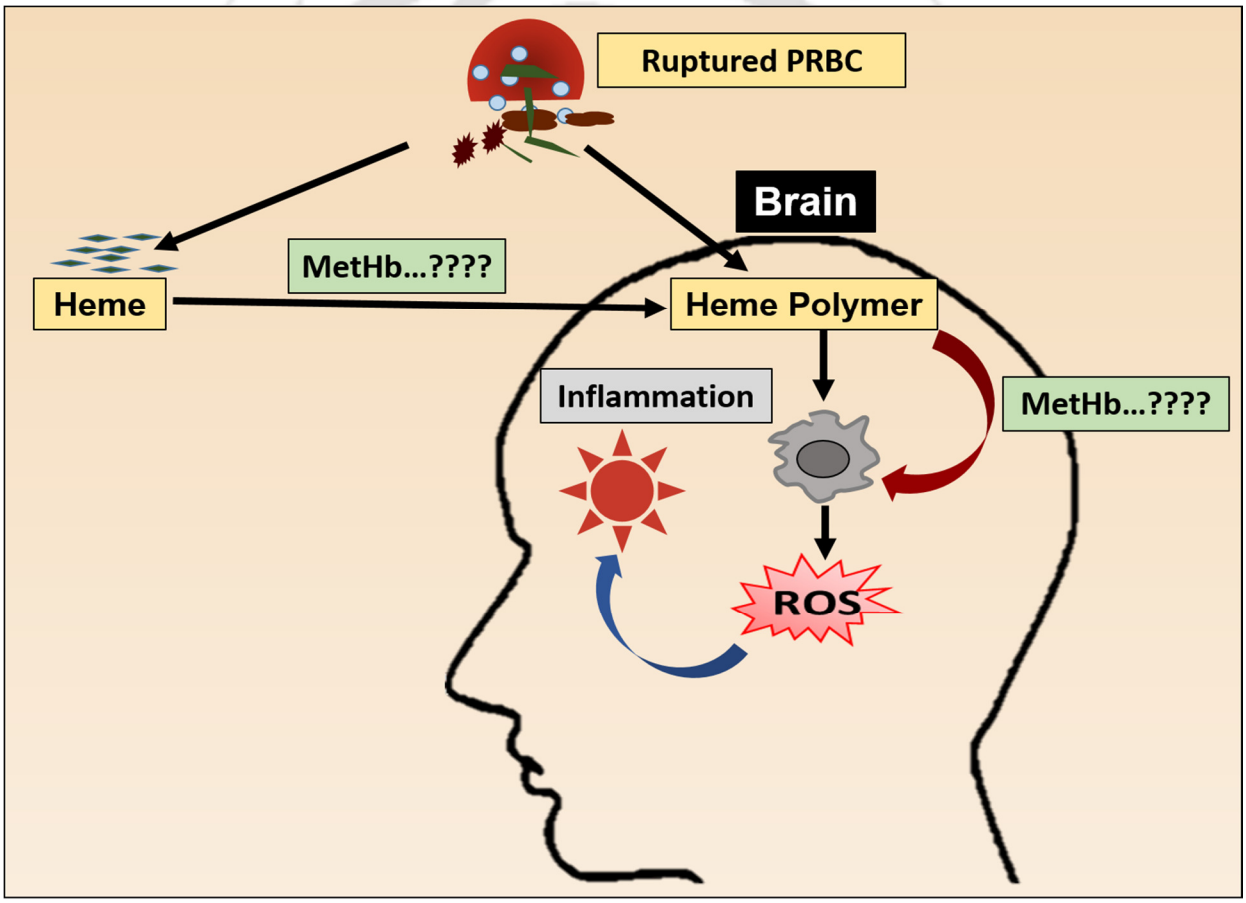

Figure 5.1: Working hypothesis for the pro-stimulatory role of methemoglobin in inflammation through hemin oxidation and polymerization.

5.2.2.3 Macrophage stimulation with heme polymer and ROS measurement- Ten thousand cells were stimulated with heme polymer or β-hematin (20µM) in a total volume of 200µl of PBS and incubated for different time period. 25µl of sample supernatant was taken out at specific time period (0-1h) and 10µM DCFH-DA was added to it. Fluorescent intensity

was measured by taking excitation wavelength at 488nm and emission wavelength at 520nm. To assess the pro-stimulatory effects of MetHb, purified heme polymer was added alone or in combination with 100µg MetHb in a total volume of 0.2ml and extracellular ROS was measured as described. Macrophage treated with PBS served as control and was used to calculate changes in ROS level (fold).

5.3.2.4 Purification and characterization of activity from malaria culture supernatant- Purification of pro-inflammatory factor from malaria culture supernatant was done as described in chapter II (Section 2.15; Purification of pro-inflammatory factor from malaria culture supernatant; Page number 27). The fractions were dialyzed and checked for their heme polymer activity, extracellular ROS generation from macrophage and protein content. Bradford method was used for determination of protein content in the pellet and supernatant taking BSA as standard. Optical spectrum of P2 fraction was recorded in a total volume of 1ml in PBS pH 7.4, in a Cary 100 UV/VIS spectrophotometer at 25°C with quartz cells of 1 cm light-path. All fractions were analyzed on 10% SDS-PAGE to follow the purification.

5.2.2.5 Peroxidase assay- Peroxidase activity of MetHb was measured by oxidation of guaiacol (Trivedi, Srivastava et al. 2005). Peroxidase activity was assayed in 300µl guaiacol assay solution containing 50mM Tris-Hcl buffer pH 7.2, guaiacol (20mM), MetHb (0.6µM) and H_2O_2 (0.27mM). Reaction was initiated by the addition of H_2O_2 and enzyme activity was measured by the formation of tetraguaiacol at 460nm. Enzyme activity was expressed as total activity (µmol/min) by measuring tetraguaiacol formed using millimolar absorption coefficient of tetraguaiacol as described (Bandyopadhyay, Bhattacharyya et al. 1995).

5.2.2.6 Clotrimazole modified methemoglobin preparation- The detail of preparation of CLT-modified MetHb was described in chapter II (Section 2.11; Clotrimazole modified methemoglobin preparation; Page number 26).

5.2.2.7 Optical spectral studies- Optical spectra were recorded in a total volume of 1ml containing 10µM of MetHb as described in chapter II (Section 2.14; Optical spectra studies; Page number 26). Different concentration of H_2O_2 (5µM to 1mM) and hemin (10-100µM) was used for the study. For spin trapper study, PBN (30nM) was added before the addition of hemin.

5.2.2.8 Difference spectra studies- For measurement of difference spectra of MetHb – H_2O_2 versus MetHb, both the reference and sample cuvettes were filled with 800µl of MetHb solution (10µM) to provide the baseline trace. This was followed by addition of a small volume of H_2O_2 to the sample cuvette with concomitant addition of the same volume of buffer to the reference cuvette. The equilibrium dissociation constant (K_D) for complex formation was calculated from the following expression. $1/\Delta A = (K_D/\Delta A_\alpha)\ 1/S + 1/\Delta A_\alpha$, where K_D is the dissociation constant of the hemin-H_2O_2 complex, S is the concentration of H_2O_2, ΔA is the observed absorption change at a particular wavelength, and ΔA_α is the absorption change at a saturating concentration of the ligand (H_2O_2).

5.2.2.9 FR-IR spectroscopy- The pellet obtained from heme polymerization assay was washed thoroughly with deionized water and lyophilized. The dried lyophilized heme polymer was mixed with dried potassium bromide (KBr) to make a KBr disc by press pellet technique. This KBr disc was used to take FT-IR spectra with Perkin Elmer IR spectrophotometer. β-hematin was used as standard reference.

5.2.2.10 Atomic Force Microscopy (AFM) studies- The morphological characteristics of the samples were monitored using AFM. Samples for AFM were prepared by adding heme polymer and β-hematin to freshly cleaved mica and kept for few minutes for adsorption of the samples on to the mica sheet. 2µl of 10mM $MgCl_2$was added to facilitate efficient adsorption of the sample onto the mica sheets. Samples were then rinsed with deionized water to remove the unabsorbed sample. The samples were then dried overnight at 37°C and AFM measurements were taken using Picoplus microscope (Molecular Imaging, USA) operating under non-contact mode.

5.2.2.11 Spectral characterization of heme polymer- A complete scan (300-700nm) was recorded for heme polymer and β- hematin, dissolved in Tris-HCl buffer pH 7.2, in a Cary 100 UV/VIS spectrophotometer at 25°C with quartz cells of 1 cm light-path. The fraction P2 of malaria culture supernatant was also subjected to UV/Vis scan (300-700nm).

5.2.2.12 Intracellular ROS measurement and fluorescence microscopy- Two Lakh cells were grown in 96 well plate cells and loaded with 10µM 2', 7'- dichlorofluorescein diacetate for 30min at 37°C and 5%CO_2. Cells were stimulated with 20µM of heme polymer for 15min at 37°C and 5%CO_2. ROS produced inside the cell was measured as described in the chapter II (Section 2.9; Intracellular ROS measurement; Page number 25). βH (20µM) was used as a

positive control. A small aliquot of cells from intracellular ROS measurement experiments were observed under 40x objectives with a fluorescence microscope (Nikon eclipses 80Ti microscope).

5.3 Results

5.3.1 Malaria culture supernatant contains pro-inflammatory factors

The *P. falciparum* culture supernatant has factor(s) to produce oxidative stress. To probe this, macrophage cells line J774A.1 were stimulated with malaria culture supernatant and intracellular ROS was measured using fluorescent ROS probe, 2, 7- dichlorofluorescein diacetate (DCFH-DA). Macrophages treated with malaria culture supernatant for 30min shows significant intracellular ROS as compared to PBS (control) treated cells (Figure 5.2A). The malaria culture supernatant was clarified with 0.22µm filter and subjected to ammonium sulfate fractionation. The purification strategy was given in Figure 5.2B. Various fractions were dialyzed against PBS for 12h and analyzed on 10% SDS-PAGE. The fraction P2 showed a single band with approximate molecular weight 64KDa as calculated from drawing a calibration curve from the standard molecular weight marker proteins (Figure 5.2C). All the fractions (pellets and supernatants) were checked for their extracellular ROS secretion from macrophages. Fraction P2 (which shows single band of protein in SDS-PAGE) exhibit 16 fold increase in ROS level as compared to the PBS stimulated cells (Figure 5.2D). The specific activity and fold purification of all fractions are given in Table 5.1. Among the various fractions, the fraction P2 stimulate macrophage to and give highest extracellular ROS. In addition, fraction P2 gives 6.5 folds purification with 28%yield.

Table 5.1: Ammonium sulfate fractionation of malaria culture supernatant.

Fractions	Total ROS (AFU)	Total protein (mg)	Specific activity (AFU/mg)	Purification (fold)	Yield (%)
S1	14486213	0.67	21700501.23	1	NA
P1	4793469	0.35	13798713.93	0.63 ±0.05	52 ±4.3
P2	25915648	0.18	141034162.99	6.5 ±0.7	28 ±3.8
S3	251809.7	0.13	1856811.98	0.086 ±0.001	20 ± 2.2

Malaria culture supernatant was clarified and fractionated by ammonium sulfate precipitation. Different fractions were dialyzed and protein content was measured by Bradford method. Macrophages were stimulated with these fractions and extracellular ROS was measured as described in “materials and methods” section. AFU= arbitrary fluorescence unit

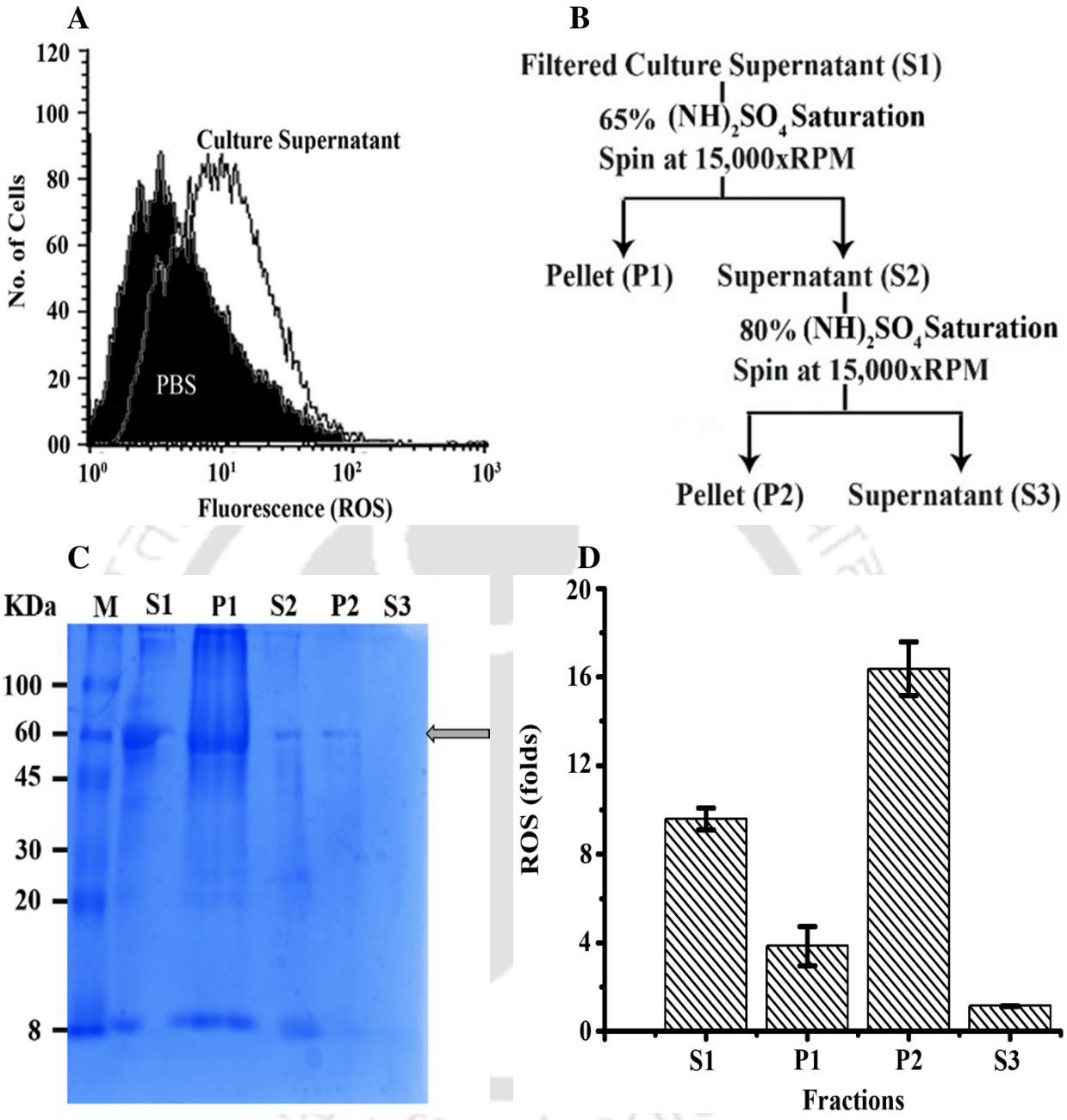

Figure 5.2: Malaria culture supernatant has a factor to produce intracellular ROS in macrophages. **(A)** Cells were left untreated or treated with malaria culture supernatant for 30min and analyzed by flow cytometry. Intracellular ROS was measured by the fluorescence exhibited by ROS probe DCFH-DA. **(B)** Fractionation of malaria culture supernatant for identification and purification of pro-inflammatory. Scheme for the purification from culture supernatant by ammonium sulfate fractionation method **(C)** Analysis of molecular weight marker (8-100kDa) malaria culture supernatant S1(Lane 2), 65% cut supernatant S2 (Lane 3), 65% cut pellet P1 (Lane 4), 80% cut pellet P2 (Lane 5), 80% cut supernatant S3 (Lane 6). **(D)** Measurement of potentials of various fractions to cause ROS secretion from macrophages. Macrophages were stimulated with different fractions obtained by fractionation of malaria culture supernatant and external ROS was measured after 1h by measuring DCF fluorescence. All error bars indicated standard deviation (SD) and calculated from triplicate measurements.

5.3.2 Partially purified protein is an iron (III) containing metallo-protein

An optical scan (300-700nm) of P2 fraction shows a soret peak at 408nm (Figure 5.3A) indicates presence of hemin as a cofactor present in the protein. Moreover, a characteristic visible peak at 536nm and 572nm denotes it globular protein (Figure 5.3A inset). Malaria culture supernatant is rich in hemoglobin, methemoglobin and hemoglobin degradation products (Kamchonwongpaisan, Samoff et al. 1997). Therefore, the factor present in P2 fraction can be hemoglobin (Hb) with iron in Fe-(II) form or MetHb with iron in Fe-(III) form. Moreover, a red shift in the soret peak at 408nm (Figure. 5.3B spectrum a) after addition of azide (Figure 5.3B, spectrum b) indicates the presence of Fe-(III) in hemin moiety. This confirms that the pro-inflammatory factor present in malaria culture supernatant is a globular hemo-protein with the molecular weight 64Kda. Considering the results obtained in the current study and on the basis of previously known literature, it is possible that the pro-inflammatory factor might be methemoglobin

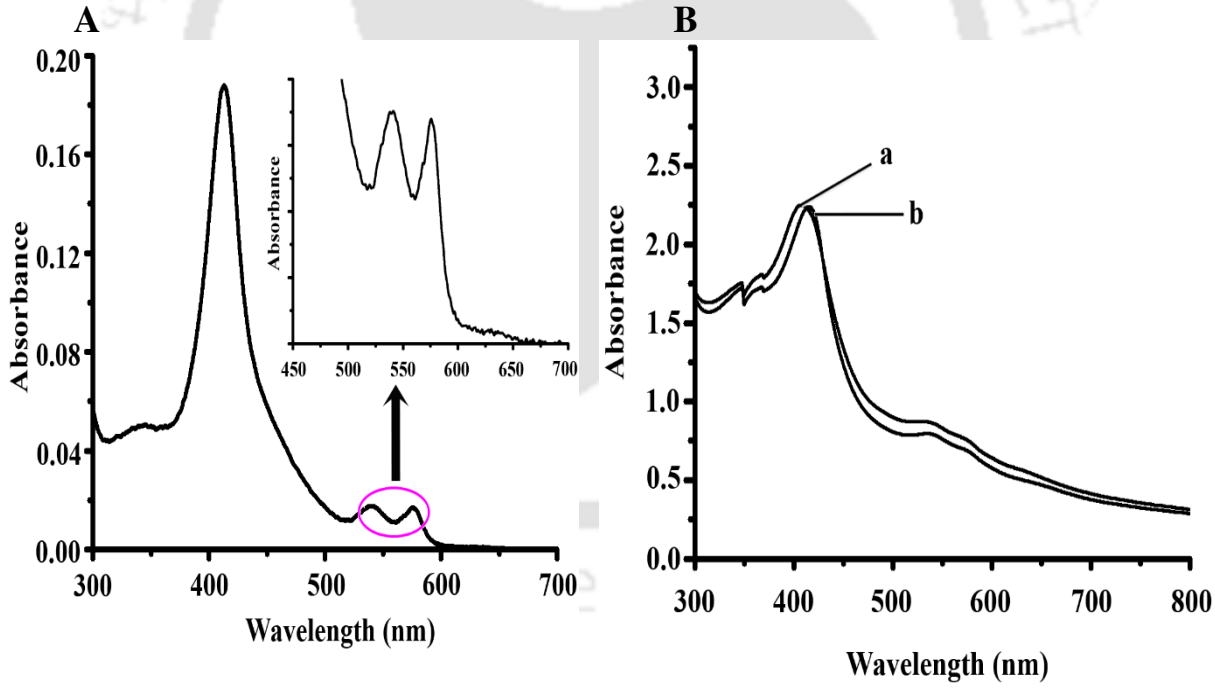

Figure 5.3: Methemoglobin is the active factor present in P2 Fraction as evidence by UV–Vis spectroscopy. (A) Fraction P2 was dissolved in PBS pH 7.4 and UV-Visible spectrum was recorded from 300-700nm, Inset showed the visible spectrum peak at 536 and 572 nm which represent peak for globular proteins. **(B)** Azide binding spectra, (a) P2 (b) a + azide (10µM).Binding of azide to P2 fraction causes red shift from 408nm (spectrum a) to 414nm (spectrum b).

5.3.3 Pro-inflammatory factors have potentials to catalyze hemin to heme polymer.

Malaria culture supernatant was also subjected to heme polymerization assay. Surprisingly, malaria culture supernatant can catalyze conversion of hemin to heme polymer (HP). It was observed that as the volume of supernatant increases from 0-100µl, HP formation also increases from 0-23µM in a dose dependent manner. (Figure 5.4A). Further, the various fractions obtained after ammonium sulfate precipitation were subject to heme polymerization. Among the various fractions, P2 fraction (which shows single band of protein in SDS-PAGE) gives highest heme polymerization activity at 18µM, followed by fraction S1, P1 and S3 (Figure 5.4B).

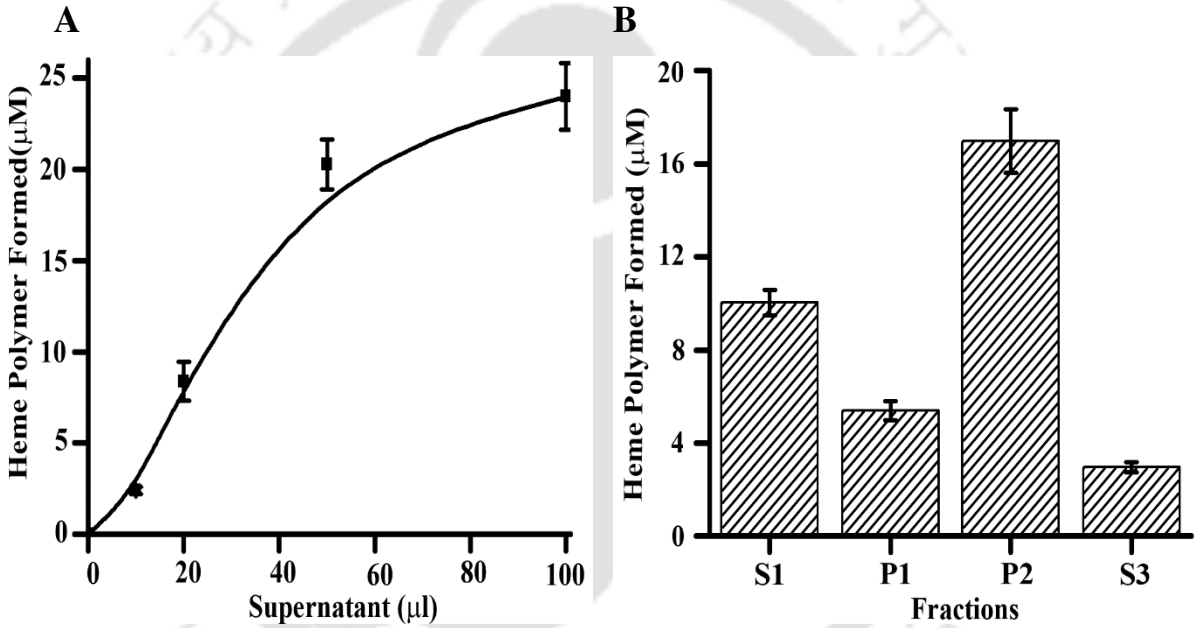

Figure 5.4: Malaria culture supernatant catalyzes heme polymer formation (A) Heme polymer formation by different volume of malaria culture supernatant (0-100µl). Different volume of malaria culture supernatant was incubated in a total volume of 1 ml containing sodium acetate buffer 100mM pH 5.2, hemin (100µM) and H_2O_2 (5mM) for 12h at 37℃. All error bars indicate standard deviation (SD) and calculated from triplicate measurements **(B)** Heme polymer formation by different fractions obtained by fractionation of malaria culture supernatant. 100µl of different fractions were incubated in a total volume of 1ml containing sodium acetate buffer 100mM pH 5.2, hemin (100µM) and H_2O_2 (5mM) for 12h at 37℃. All error bars indicated standard deviation (SD) and calculated from triplicate measurements.

5.3.4 MetHb catalyzes heme polymer formation in a dose and time dependent manner

Heme polymerization assay was performed with different concentrations of MetHb (0-10µM) and at various time points (0-12h) as described. It was observed that MetHb dose dependently catalyzes heme polymerization and give 26.6µM of HP at highest concentration of 10µM (Figure 5.5A). At 5µM MetHb ~17µM heme polymer formation takes place and

this concentration of MetHb was used for the further study. Similarly, time response for heme polymerization was done and it was observed that 12h is the optimal time for heme polymerization (Figure 5.5B).

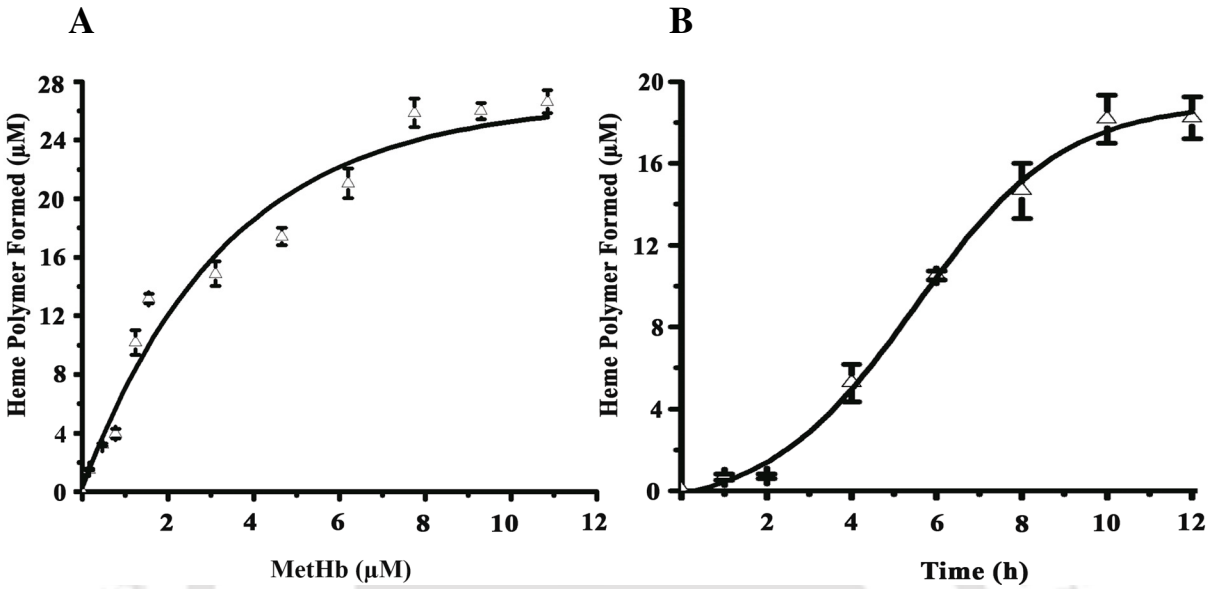

Figure 5.5: Time and dose dependent heme polymerization by MetHb. (A) HP formation by different amount of MetHb (0-10µM). Different concentration of MetHb was incubated in a total volume of 1ml containing sodium acetate buffer 100mM pH 5.2, hemin (100µM) and H_2O_2 (5mM) for 12h at 37℃. **(B)** HP formation at different time intervals (0-12h). 5µM of MetHb was incubated in a total volume of 1ml containing sodium acetate buffer 100 mM pH 5.2, hemin (100 µM) and H_2O_2 (5 mM) for different time starting from 0-12h at 37℃. Heme-polymerization assay was described in chapter II. Data is from two independent experiments (n=3) performed in triplicate. All error bars indicated standard deviation (SD) and calculated from triplicate measurements.

5.3.5 Peroxidase activity of methemoglobin is responsible for heme polymer formation.

Horseradish peroxidase mediated hemin to heme polymer formation involves hemin free radical generation through a single electron transfer mechanism (Sakamoto, Takaki et al. 2002; Trivedi, Chand et al. 2005). MetHb has pseudoperoxidase activity, and it oxidizes aromatic and halide substrates (Reeder 2010). This provoked us to ask whether pseudoperoxidase activity of MetHb is responsible for heme polymer formation. To test such a possibility, peroxidase activity of MetHb and clotrimazole (CLT) - modified MetHb (inactive) was measured by oxidation of guaiacol as described in "materials and methods" section. MetHb shows peroxidase activity whereas CLT-MetHb shows no or very little peroxidase activity (Table 5.2). Similarly, CLT-MetHb shows very low heme polymerization activity as compared to MetHb (Table 5.2). Hence, it proves that MetHb is utilizing its peroxidase activity to form heme-polymer from monomeric hemin.

Table 5.2: Peroxidase and heme polymerization activity of MetHb and CLT-MetHb

Enzymes	Peroxidase activity ± SD	Heme polymerization activity ± SD
MetHb	47.6 ± 0.9	45.3 ± 1.1
CLT-MetHb	12.9 ± 0.7	14.4 ± 4.2

Peroxidase activity was done using 300µl of guaiacol assay solution containing 50mM Tris-Hcl buffer pH 7.2, guaiacol (20mM), MetHb or CLT-MetHb (0.6µM) and H_2O_2 (0.27mM) as described under "materials and methods" section. For heme polymerization activity of MetHb and CLT-MetHb (0.6µM) was incubated in a total volume of 1ml containing sodium acetate buffer 100 mM pH 5.2, hemin (100 µM) and H_2O_2 (5 mM) for 12h at 37℃ and heme polymerization assay was done as described under "materials and methods" section. All error bars indicated standard deviation (SD) and calculated from triplicate measurements.

To confirm further that pseudoperoxidase activity of MetHb is responsible for heme polymer formation, a series of peroxidase inhibitors and substrate was screened against MetHb for heme polymerase activity (Table 5.3).

Table 5.3: Sensitivity of MetHb mediated heme polymerization towards peroxidase inhibitors and substrates

Conditions	Heme-polymerized (µM) ± SD
Hemin	0.02± 0.009
Hemin +MetHb	0.03 ± 0.01
Hemin +H_2O_2+MetHb	18.54 ± 0.78
Hemin +H_2O_2+CLT+MetHb	0.68 ± 0.12
Hemin +H_2O_2+Azide+MetHb	0.58 ± 0.14
Hemin +H_2O_2+Guaiacol+MetHb	0.23 ± 0.04
Hemin +H_2O_2+KI+MetHb	2.55 ± 0.06
Hemin +H_2O_2+KCl+MetHb	1.27 ± 0.52

MetHb mediated heme polymerization assay was performed in the absence or presence of H_2O_2 , Clotrimazole (1mM), Sodium azide (100µg), Guaiacol (20mM), Potassium iodide (1mM), Potassium chloride (100µM).Inhibitors or substrate were added 30min prior to the addition of hemin and H_2O_2 was added last to initiate the reactions. Heme-polymerization was assayed as described under experimental procedures section of chapter II. All error bars indicated standard deviation (SD) and calculated from triplicate measurements.

In presence of peroxidase inhibitors such as CLT and azide a negligible amount of heme polymer was formed. Peroxidase substrate such as KI, KCl and guaiacol competes with the substrates in reaction mixture and did not allow efficient heme polymerization thus reducing the formation of heme polymer. The small molecule electron donor competes with hemin for

MetHb and might be responsible for observed inefficient heme polymerization. No polymer was formed when free hemin was incubated alone, or with MetHb in absence of H_2O_2. This confirms the need of H_2O_2 and MetHb for the heme polymerization assay. Hence, MetHb peroxidase activity plays a crucial role in hemin oxidation and subsequent polymerization.

5.3.6 Methemoglobin process hemin and hydrogen peroxide with specificity

In MetHb mediated heme polymerization assay, two substrates were involved therefore their kinetics was also studied. Concentration response of H_2O_2 was determined for heme polymerization assay starting from 0-8mM (Figure 5.6A). Saturation for the formation of heme polymer was reached at the concentration of 5mM. The K_m of MetHb for H_2O_2 was calculated from the Line weaver- Burk plot and it was found to be 2mM at pH 5.2 keeping the concentration of hemin constant of 100µM (Figure 5.6A, inset). The binding affinity (K_D) of H_2O_2 with MetHb was studied by optical difference spectroscopy (Figure 5.6C). The apparent K_D values of H_2O_2 with MetHb was 166 calculated from 1/ΔAbsorbance vs. 1/ [H_2O_2] plot (Figure 5.6C, inset). Hemin concentration response was also performed from 0-500µM (Figure 5.6B). Adequate amount of heme polymer (~18µM) was formed from 100µM of hemin. The substrate affinity (K_M) a value of MetHb for hemin was 40.33µM at pH 5.2.

5.3.7 MetHb oxidizes hemin to hemin radical by single electron transfer

Hemin is an aromatic molecule and it is oxidized by MetHb through one-electron oxidation. Optical spectroscopic studies reveal that MetHb compound II kinetically oxidizes free hemin by one-electron transfer. When H_2O_2 was added to native MetHb (Fe-III), there was a shift of soret peak from 406nm (Figure. 5.7A, spectrum a) to 417nm (Figure. 5.7A, spectrum b) which indicates the formation of an intermediate complex, compound II where iron remains as higher oxidation ferryl state (Fe-IV=O). In order to investigate the oxidation of free hemin by compound II, hemin was added to this intermediate to reduce it to the native Fe-III state by one electron transfer process. The result shows that the addition of hemin reduced the ferryl form to the ferric state as indicated by the time-dependent return of the spectrum at 417nm back to the native form at 406nm (Figure 5.7A, spectrum c). This indicates that the hemin was probably oxidized by one electron oxidation. To confirm again that oxidation of free hemin by active MetHb intermediates was responsible for heme polymer formation; heme polymerization assay was done in presence of Phenyl N-tert-

butylnitrone (PBN), a spin trap. MetHb mediated heme polymerization in the presence of different increasing concentrations of PBN (0.001µM-10µM) dose dependently inhibits the heme polymerization with an IC_{50} of 30nM (Figure 5.7B). All the above results support the notion that MetHb oxidizes hemin with one electron transfer mechanism involving high oxidation state enzymatic intermediates (compound II/compound I).

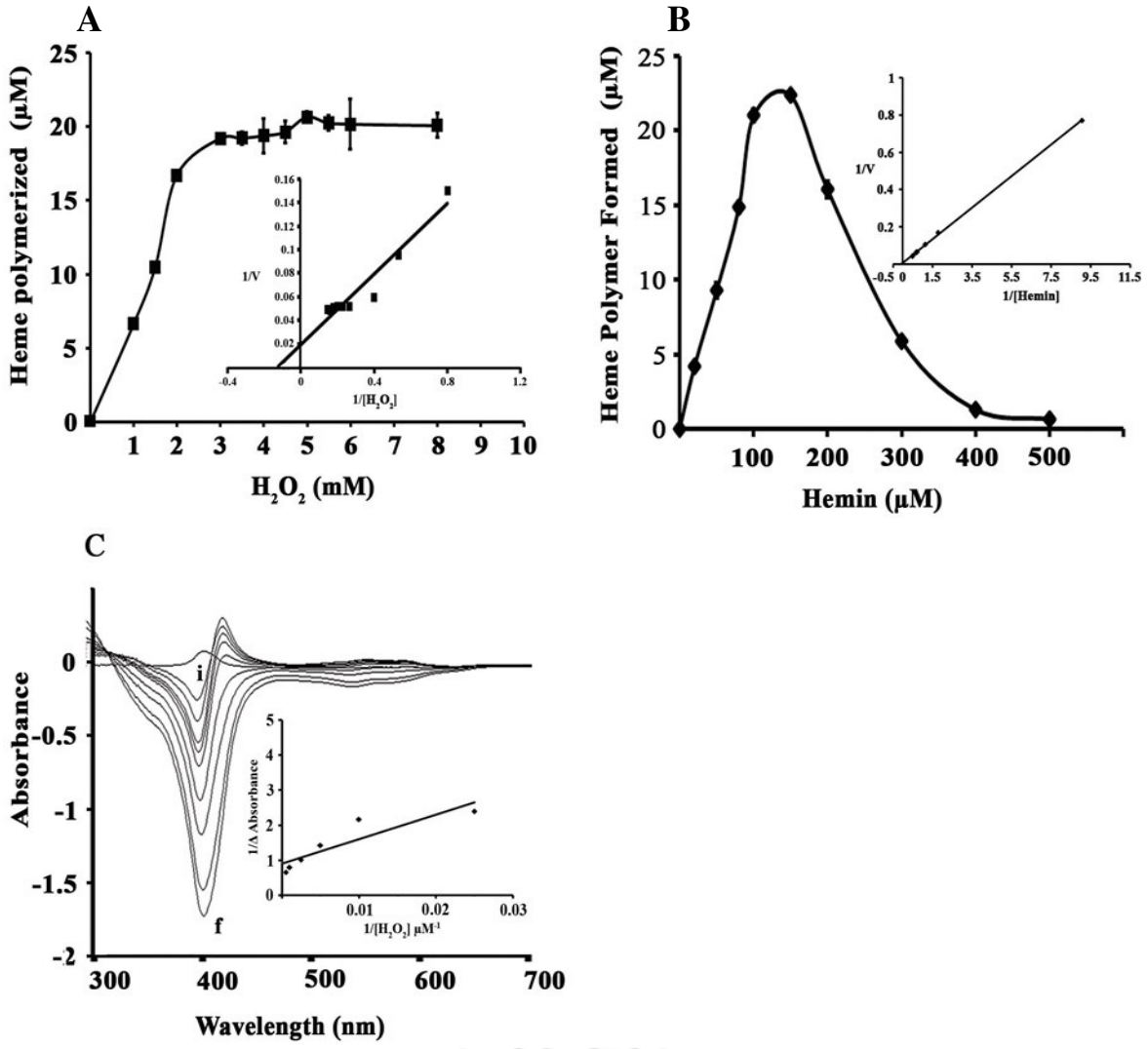

Figure 5.6: Kinetic parameters of heme polymerization by MetHb. (A) HP formation by different concentration of H_2O_2 (1-8mM). Different concentration of H_2O_2 (1-8mM) was incubated in a total volume of 1ml containing sodium acetate buffer 100mM pH 5.2, MetHb (5µM) and hemin (100µM) for 12h at 37℃.. The inset shows the Lineweaver- Burk plot of 1/*v* versus 1/ [H_2O_2.]. **(B)** HP formation by different concentration of hemin (0-500µM). Different concentration of hemin (0-500µM) was incubated in a total volume of 1ml containing sodium acetate buffer 100mM pH 5.2, MetHb (5µM) and H_2O_2 (5mM), for 12h at 37℃. The inset shows the Lineweaver- Burk plot of 1/*v* versus 1/[S]. Heme-polymerization was assayed as described in chapter II. (**C)** Optical difference spectroscopy for MetHb- H_2O_2 complex formation (i, 50µM; f, 10mM). The inset shows the plot of 1/Δ Absorbance (nm) versus 1/ [H_2O_2] to calculate the K_D. All error bars indicated standard deviation and calculated from triplicate measurements.

5.3.8 Hemin free radical is essential for heme polymer formation

PBN dose dependently inhibits the heme polymerization with an IC_{50} of 30nM. Optical spectroscopic studies suggest that PBN interacts with compound II (MetHb-H_2O_2 active complex) and binds to protein radicals to prevent efficient electron transfer from compound II for hemin oxidation (Figure 5.8). PBN mediated inhibition of heme polymerization can be of multi-factorial and there could be a different mechanism (chelation of hemin free radicals, binding of PBN to enzyme or bound hemin etc.), but regardless of the mechanism involved, PBN treatment reduces hemin oxidation and polymerization. Hence, the spin trap sensitivity indicates formation of hemin free radical as an essential intermediate for MetHb mediated heme polymerization

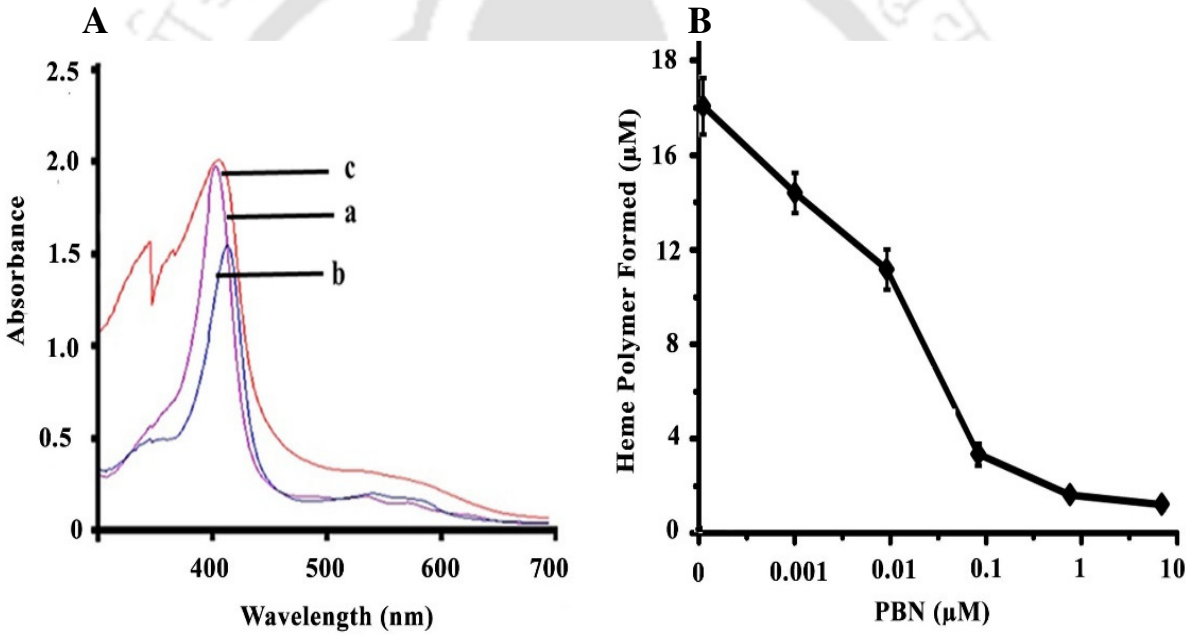

Figure 5.7: MetHb oxidized hemin to form heme polymer. (A) Optical spectra of hemin oxidation by MetHb (compound II). Soret spectra were recorded in 100mM Tris-HCl buffer, pH 7.2, in a total volume of 1ml. Soret spectrum (a) of MetHb (10μM); (b) a+ H_2O_2 (1mM); (c) b+hemin (100μM). Equal concentration of hemin (100μM) was added in the reference cuvette to correct Soret absorbance contributed by hemin. **(B)** Inhibitory effect of PBN (spin trap) on the heme polymer formation. PBN dose dependently inhibits MetHb mediated heme polymerization. PBN (0-10μM) was added into the reaction mixture containing MetHb (5μM), 100μM hemin and 5mM H_2O_2.The heme polymerization assay was performed as described in chapter II. Error bars indicated standard deviation (SD) and calculated from triplicate measurements.

5.3.9 Heme polymer formed by MetHb is identical to the synthetic β-Hematin

The chemical structure, crystal packing and surface topology was studied by optical spectroscopy, IR spectroscopy and AFM analysis. The synthetic heme polymer β-hematin

was used as a reference molecule for comparison purposes. A complete scan (300-800nm) was recorded for heme polymer and β-Hematin. Samples were dissolved in Tris-HCl buffer pH 7.2 and spectrum was recorded. The UV-visible spectrum of heme polymer has two peaks with absorption maxima at 426nm and 602nm (Figure 5.9A) and matches with the absorption spectrum of synthetic β-hematin (Figure 5.9B). It also matches well with reported absorption spectra of haemozoin (malaria pigment) extracted from a malaria parasite (Orjih and Fitch 1993). The pellet obtained from heme polymerization assay was lyophilized, dried and then mixed with KBr and made a KBr disc which was used to take FT-IR spectra. An IR spectral analysis of heme polymer has a prominent peak at 1207cm^{-1} and 1660cm^{-1} indicative of the typical iron carboxylate bond, also present in synthetic β-hematin (Figure 5.9C, D). Surface topology of heme polymer and β-hematin was identical as evidenced by AFM studies (Figure 5.9E, F). Hence, heme polymer and β-hematin exhibits similar bond pattern, structural properties and crystal packing.

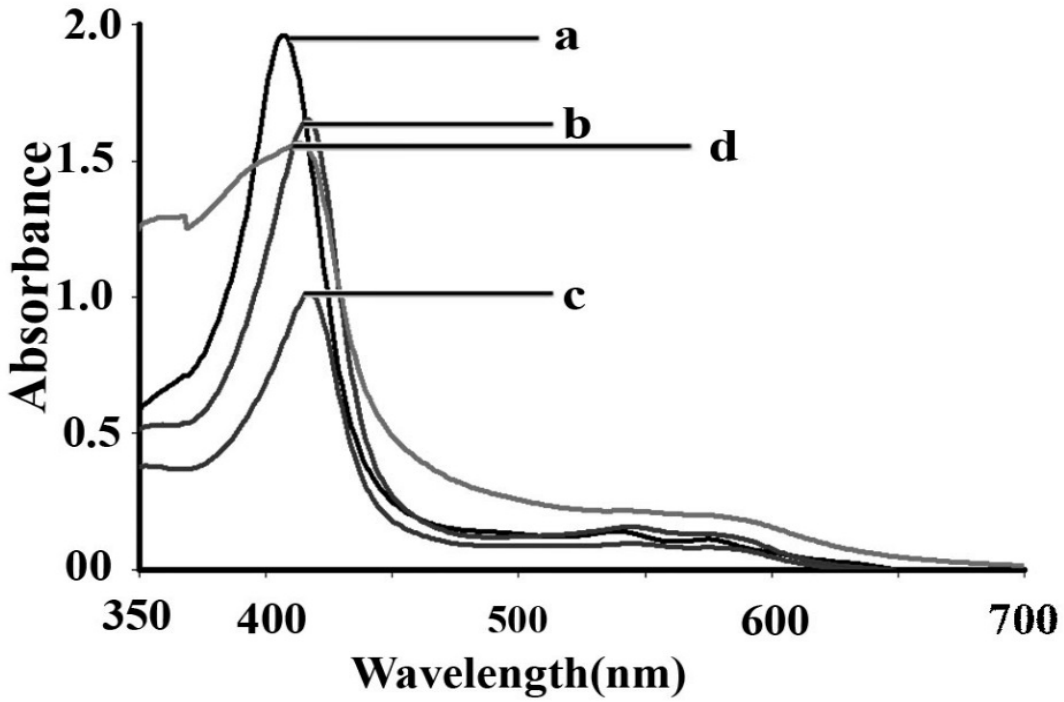

Figure 5.8: PBN (Spin trap) binds to protein radicals and blocks enzyme transition to inhibit MetHb mediated heme oxidation. Optical spectra of heme oxidation by MetHb in the presence of IC_{50} concentration (30nM) of PBN. In a total volume of 1ml containing100 mM Tris-HCl buffer, pH 7.2, soret spectrum of (a) native MetHb (10μM), (b) a+ H_2O_2 (1mM), (c) b+PBN (30nM); (d) c+hemin (20μM) were recorded. An equal concentration of hemin (20μM) was added in the reference cuvette to correct absorbance of hemin.

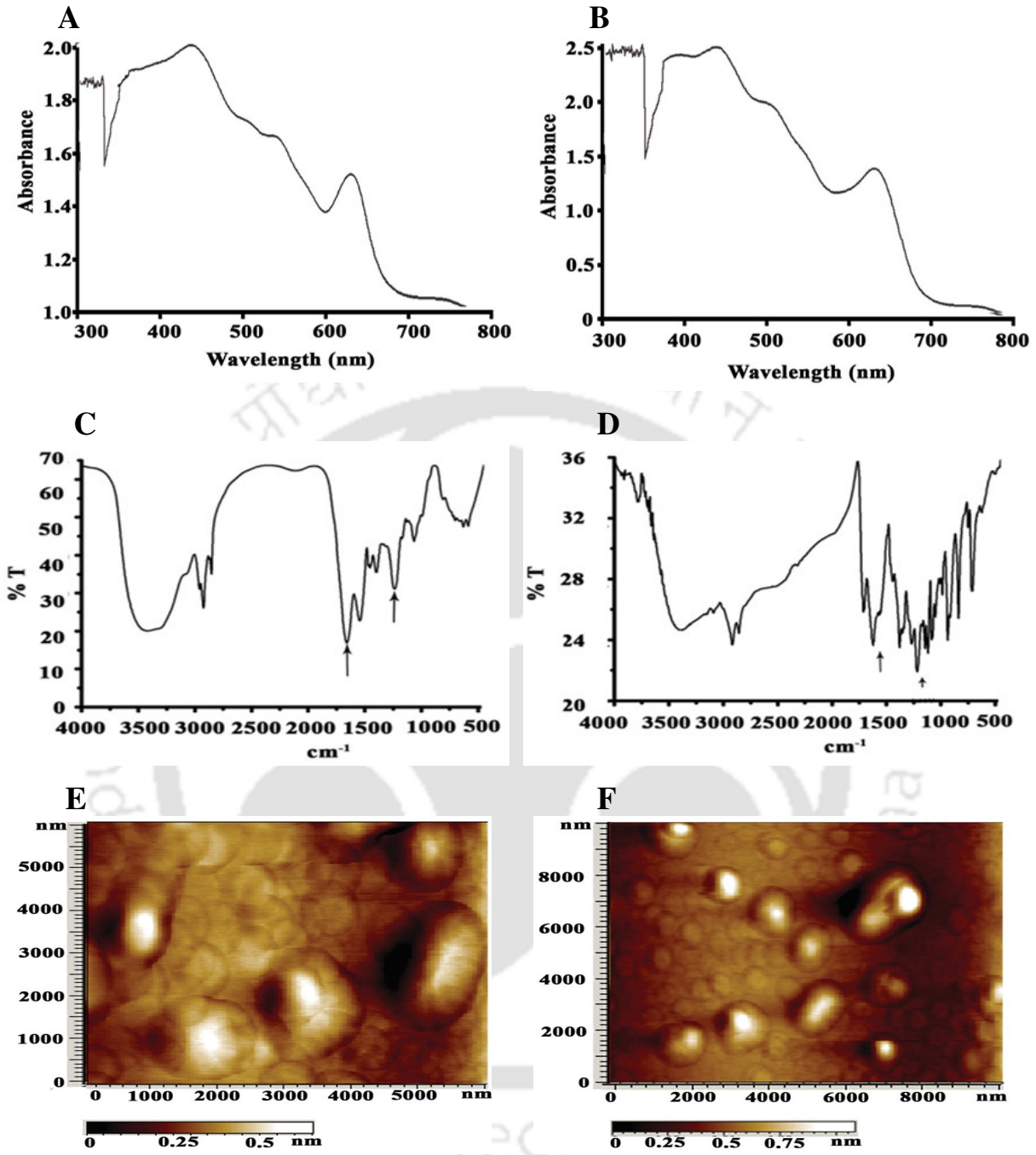

Figure 5.9: Comparison of heme polymer formed by MetHb with synthetic β-Hematin for its bonding pattern, structure and spectral properties. (A) UV-Visible spectra of heme polymer formed by MetHb. **(B)** Synthetic β-Hematin. The lyophilized powder of the heme polymer formed by MetHb was dissolved in Tris-HCl buffer and a spectrum was recorded from 300-800nm. **(C)** FT-IR spectra of heme polymer formed by MetHb. **(D)** Synthetic β-Hematin. The lyophilized powder of the heme-polymer formed MetHb was dehydrated and FT-IR spectrum was recorded. **(E)** AFM image of heme polymer formed by MetHb. **(F)** Synthetic β-Hematin. 10µl of sample (heme polymer and β-hematin) was added to freshly cleaved mica and kept for few minutes for adsorption then 2µl of 10mM $MgCl_2$ was added the sample and rinsed with deionized water and dried overnight at 37℃ and AFM measurements were taken under non-contact mode.

5.3.10 Heme polymer is pro-inflammatory in nature

The *P. falciparum* culture supernatant has factor(s) to stimulate macrophage to produce ROS. Hemin or haemozoin (heme polymer) stimulates macrophage to release ROS into microenvironment and contributes to inflammation (Manucha and Valles 2012). To probe pro-inflammatory nature of heme polymer ROS was measured using fluorescent ROS probe, 2, 7- dichlorofluorescein diacetate (DCFH-DA). Macrophages were treated with 20µM of heme polymer or β-hematin or with PBS (control) for 30 min. Heme polymer causes ROS production from macrophages, comparable to synthetic heme polymer, β-hematin. Macrophage stimulated with heme polymer causes more than 80% cell population with high ROS in the cytoplasm as compared to PBS treated cells (Figure 5.10A). The rate of ROS secretion was increases with time and was highest at 15min and decreases gradually and become zero at 1h (Figure 5.10B).

.5.3.11 MetHb potentiates the heme polymer inflammatory responses

Macrophage stimulated with HP causes more than 80% cell population with high ROS as compared to PBS treated cells (Figure 5.11A). Macrophages secrete ROS into the external microenvironment with varying degree when stimulated with MetHb, heme polymer or βH, in comparison to unstimulated (PBS) cells. βH mediated ROS secretion is most effective among all other agonists. Moreover, purified HP (20µM) was mixed with MetHb (100µg) in 0.2ml sterile PBS and added to the macrophages. Surprisingly, macrophage stimulated with a mixture of MetHb + HP causes rapid ROS secretion and accumulates a large amount (several folds higher) of ROS in the outer microenvironment in comparison to their individual responses (Figure 5.11A). Addition of H_2O_2 to MetHb, βH or HP alone does not give any significant stimulation to secrete ROS from macrophages. It clearly excludes any role of H_2O_2 generation (through fenton reactions) or release of iron during incubation. In the absence of macrophage, combination of MetHb, hemin, H_2O_2 did not provide an enhanced level of ROS in the supernatant which ruled out the mutual interaction of the reaction components responsible for ROS in supernatant. Hence, MetHb has potential to cause ROS production, and it can be able to amplify the pro-inflammatory effects of free hemin or pre-formed HP. Macrophage stimulated with HP exhibits distribution of bright ROS fluorescence throughout the cytoplasm (Figure 5.11B).

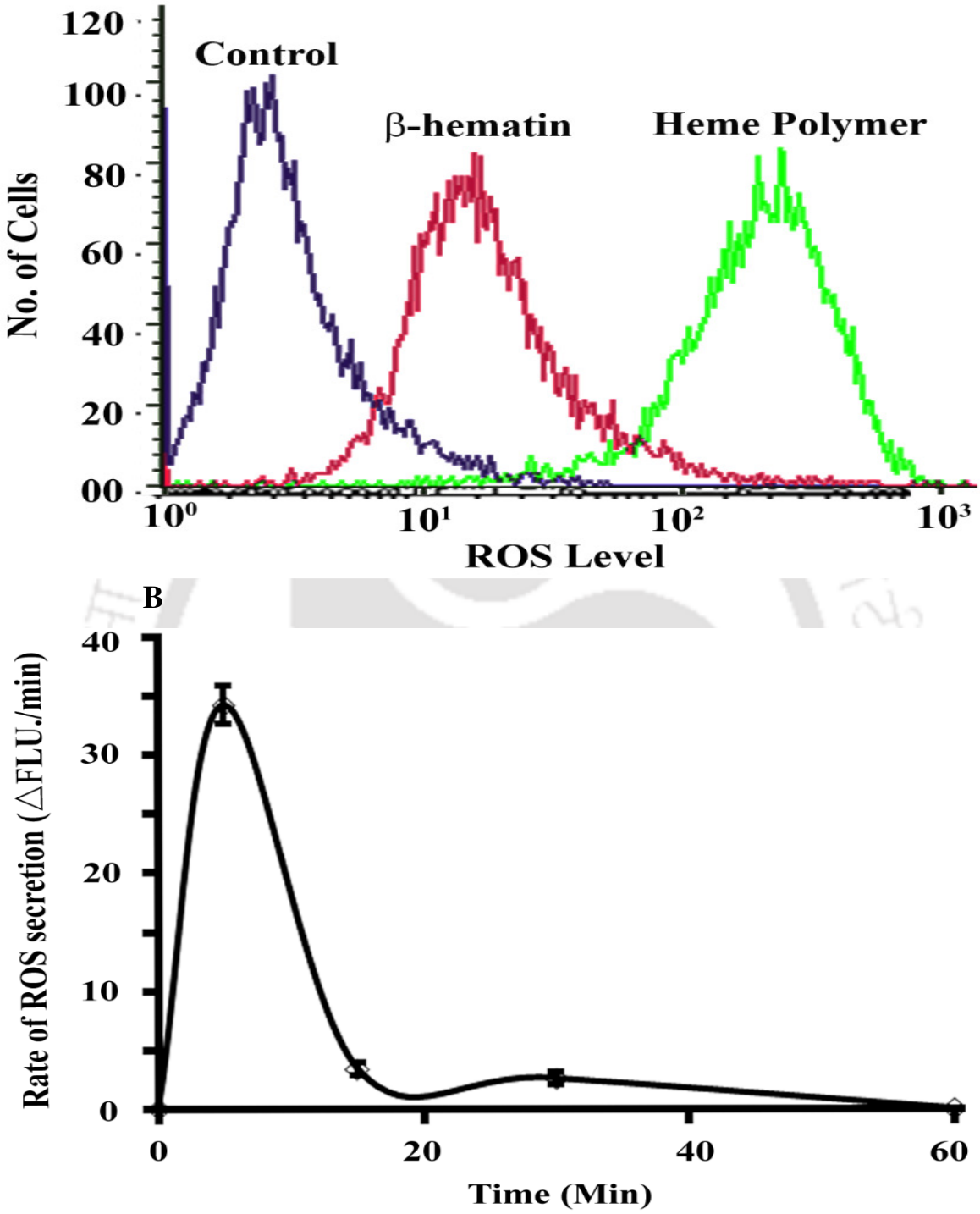

Figure 5.10: Treatment of Macrophage J774A.1 cell with heme polymer causes generation of ROS. (**A)** Cells were left untreated or treated with 20µM of heme polymer or β-hematin and analyzed after 30min by flow cytometry. ROS produced inside the cell was measured by the fluorescence exhibited by fluorescence probe (DCF). **(B)** Cells were treated with 20µM of heme polymer in a total volume of 200µl of PBS and incubated for different time period. 25µl of sample supernatant was taken out at specific time period (0-60min) and DCFH-DA (10µM) was added and fluorescence intensity was measured.

Interestingly, spin trappers TEMPO and PBN abolishes ROS secretion from macrophage stimulated with MetHb+hemin+H_2O_2 clearly indicates the role of MetHb mediated hemin oxidation and hemin free radical generation/HP formation with enhanced ROS production from macrophages (Table 5.4).

Table 5.4. Regulation of ROS secretion from macrophage through MetHb mediated hemin free radicals generation

Stimulation Condition	Extracellular ROS Production (Fold change ± SD)
PBS	1.00
Hemin +MetHb+ H_2O_2	66.12 ± 2.54
Hemin +MetHb+ PBN+ H_2O_2	2.2 ±0.12
Hemin +MetHb+ TEMPO+ H_2O_2	4.3 ±0.15

Macrophage stimulated with MetHb (100µg) + Hemin (20µM) for 1h in presence or absence of TEMPO (100mM) or PBN (1µM) and extracellular ROS was measured as describe in experimental procedures section of chapter II. ROS secreted was expressed as fold change

5.4 Discussion

A unique mechanism of methemoglobin mediated pro-inflammatory effects through the heme polymer formation and its oxidation by inherent peroxidase activity has been explored. There are two possible sources of MetHb in malaria culture supernatant, conversion of hemoglobin into methemoglobin (Fe^{3+}) within infected RBC (due to oxidative stress) or release of MetHb from uninfected RBC after lysis through a process of eryptosis (Cambos and Scorza 2011). Either sources may contaminate the cerebrospinal fluid (CSF) with MetHb to cause oxidative damage due to the formation of 8- iso-pgf2α (Sakamoto, Takaki et al. 2002). The level of MetHb is remarkably elevated (4.5-5.8%) in patients with CM and its level correlates with neurological symptoms or death (Anstey, Hassanali et al. 1996). MetHb and by labile hemin released from it, associated with oxidative damage and inflammation. Brain microenvironment contains MetHb, free hemin and results suggest direct link that MetHb can utilize hemin as a substrate to amplify ROS production and inflammation. MetHb compound II oxidizes free hemin through single electron transfer (reduction of spectral peak at 417 to 406nm) to hemin free radicals (Figure 5.7A). Hemin free radicals react either with each other or other hemin molecule to form HP (Figure 5.5). MetHb synthesized HP has chemical and structural properties similar to synthetic HP, β-hematin (Figure 5.9).

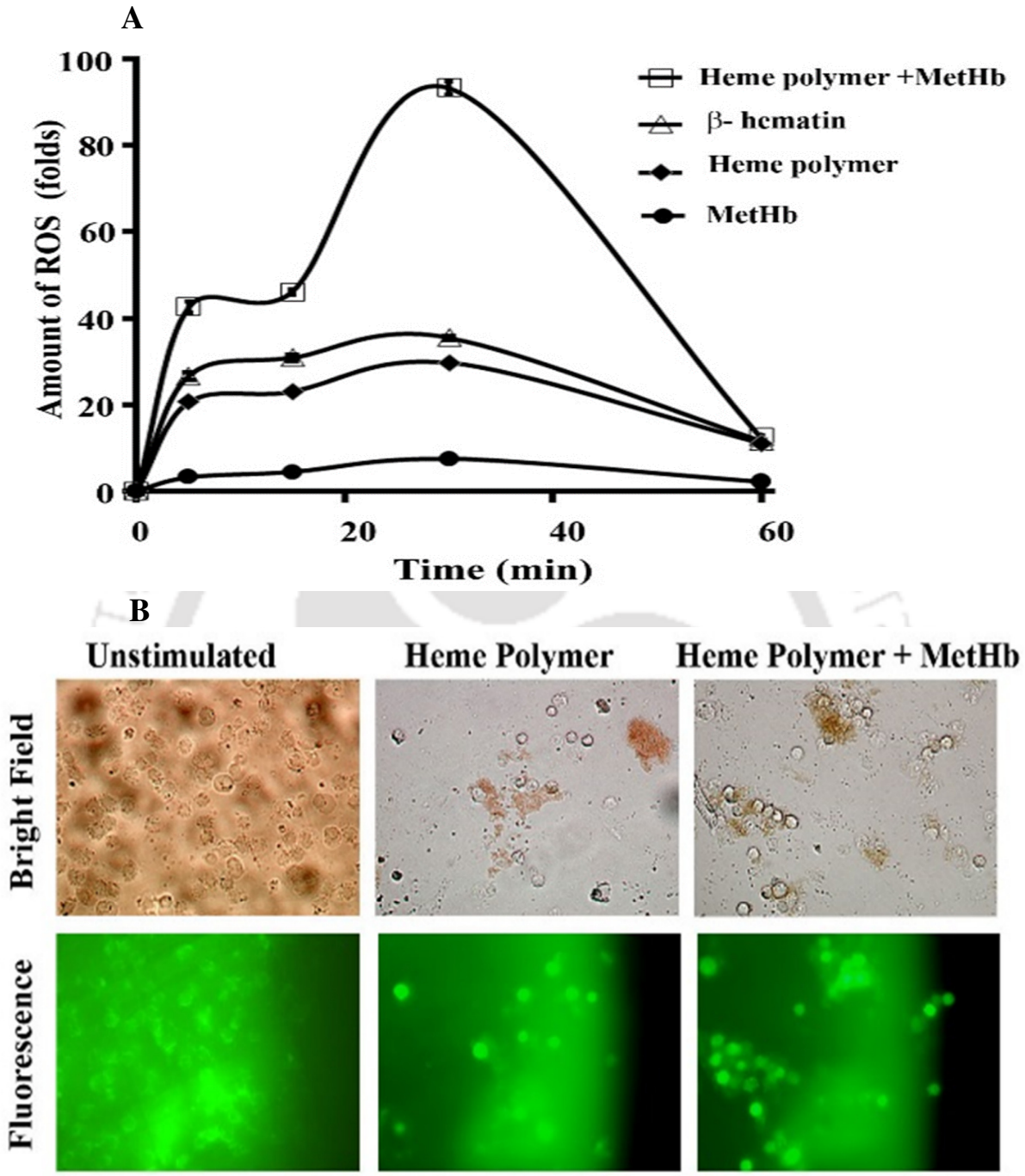

Figure 5.11: Treatment of Macrophage J774A.1 cell with combination of heme polymer and MetHb potentiates ROS generation. (A) Cells were treated with MetHb (100µg), β-hematin (20µM), heme polymer (20µM) or mixture of heme polymer (20µM) with MetHb (100µg) in 200µl of PBS and incubated for different time period. 25µl of culture supernatant was taken out at specific time period (0-60min) and 10µM 2', 7'-dichlorofluorescein diacetate was added and fluorescence intensity was recorded. **(B)** Fluorescence microscopic images of macrophages untreated or treated with heme polymer (20µM) or mixture of heme polymer (20µM) with MetHb (100µg) after 30 min of incubation.

PBN (spin trap) inhibits hemin free radical generation and heme polymerization (Figure 5.7B & 5.8). An extremely high concentration of hemin or H_2O_2 found to be optimal for MetHb mediated heme polymerization, but such a high concentration of H_2O_2 known to catalyze other MetHb mediated reactions (Shiga and Imaizumi 1975). Moreover, over-night incubation of MetHb with H_2O_2 (5mM) does not cause any significant loss of peroxidase activity. Although the exact mechanism of MetHb mediated heme polymerization is not explored, then also based on available literature, it can be speculated that MetHb peroxidase activity is producing hemin free radicals, and a suitable environment is allowing them to form a stable heme polymer. MetHb mediated heme polymerization is remarkably little at pH 7.2, but other hemin binding proteins (such as BSA, hemopexin or PfHRP) present within micro-environment might enhance the heme polymerization to provide a stratum or platform (Sullivan, Gluzman et al. 1996). Such a possibility is intriguing to consider and might be useful to understand the molecular events leading to vesicular blockage during cerebral malaria.

The free radical production (cytotoxic) or scavenging (protective) abilities of methemoglobin, and these properties of enzyme are linked to nature of substrate molecules present within the tissue microenvironment (Deshmukh and Trivedi). In addition, MetHb toxicity causes up regulation of intracellular heme oxygenase-1 (HO-1) level and it may down-regulate ROS level (Roach, Moore et al. 2009). In an intracerebral hemorrhage model, HO-1 expression was found in surrounding tissues, predominately in microglia cells. HO-1 knockout mice exhibit a marked reduction in macrophage activation and ROS production (Chen-Roetling and Regan 2006; Wang and Dore 2007). Brain damage and leaky behavior of the blood brain barrier, linked to micro-hemorrhage and death (apoptosis/necrosis) of surrounding brain tissues. Inflammatory macrophages induce apoptosis or necrosis of stromal and parenchymal cells through production of pro-apoptotic molecules such as oxygen radicals and H_2O_2 (Buehler and D'Agnillo 2010). Hemin free radicals produced as an intermediate might be an effective pro-inflammatory molecule to cause tissue damage and organ dysfunction. Stimulation of the macrophage with HP accumulates a large amount of reactive oxygen species (ROS) within the external microenvironment to cause inflammation (Figure 5.10). Furthermore, the addition of MetHb amplifies the HP mediated stimulatory effects on macrophage to release several fold high ROS into the external micro-environment with an unknown mechanism. Macrophage activation and polarization into distinct subtypes

during malaria and activation of tissue associated macrophages (such as brain or liver) is linked with death of neighboring stromal or parenchymal cells (Buehler and D'Agnillo 2010). In addition, the presence of large amounts of haemozoin within the brain microenvironment and its contribution in inducing brain pathophysiology or stimulating macrophage to release ROS to cause inflammation (Dostert, Guarda et al. 2009). MetHb mediated amplification of haemozoin pro-inflammatory response to produce cytokines or ROS is possible. So far results suggest that MetHb accepts hemin as substrate, but the chances of HP/haemozoin oxidation by MetHb cannot be ruled out. Technical difficulties did not allow studying the heme polymer oxidation mechanism or the identification of a single electron oxidation product of heme polymer in EPR due to its high molecular weight and least solubility in aqueous solvent system.

5.5 Conclusion

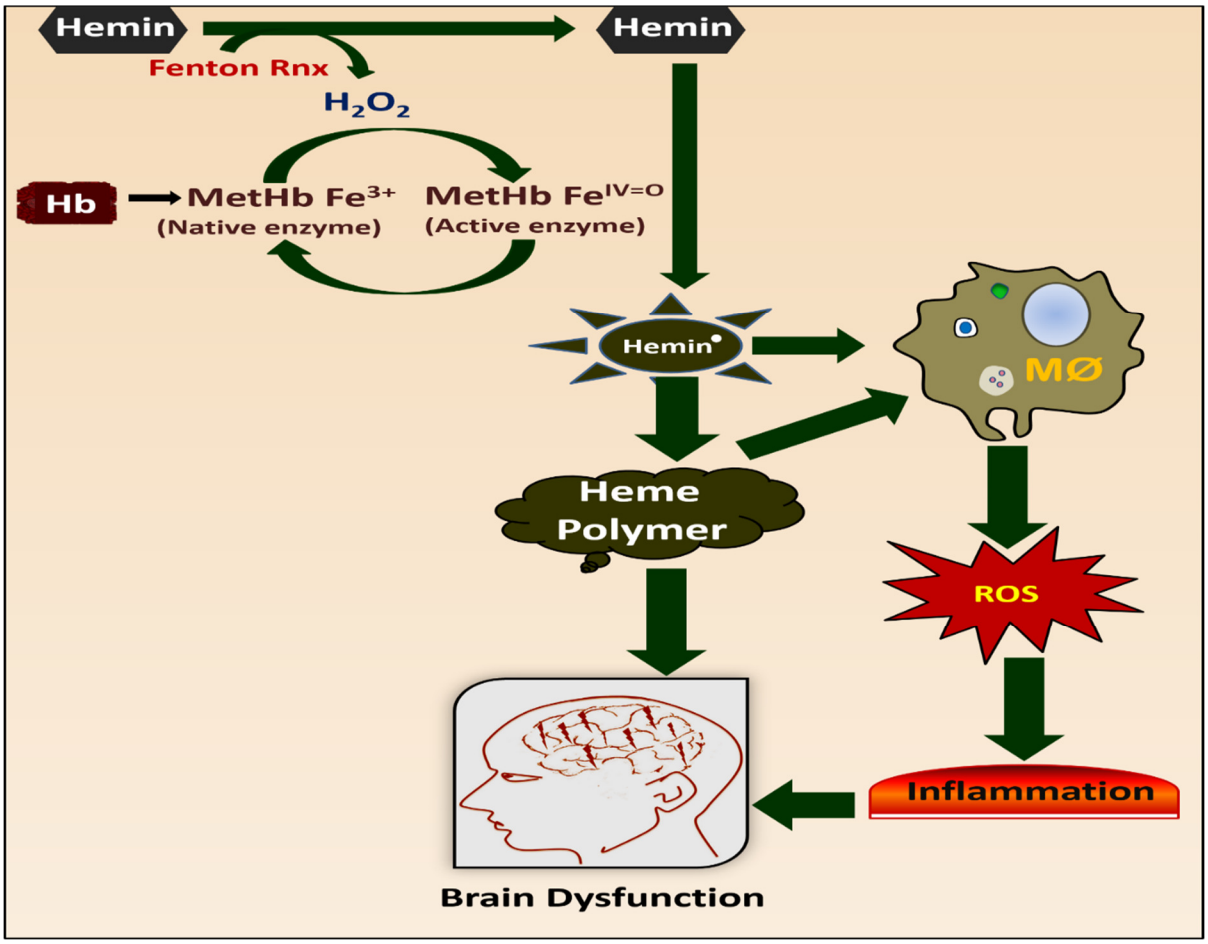

Figure 5.12: Schematic of methemoglobin mediated amplifying pro-inflammatory effect of hemin and heme polymer.

Hemolysis and accumulation of cell free Hb/MetHb in plasma are common with many patho-physiological conditions including malaria. Based on the results of the current work, the molecular events can be divided into 3 steps as outlined in Figure 5.12 (**1**) release and activation of MetHb, (**2**) Hemin oxidation and polymerization, (**3**) Macrophage stimulation, ROS secretion into local microenvironment. MetHb, pro-oxidant molecule causes severe inflammation at the brain site. Hemin or haemozoin (heme polymer) causes inflammation and have a role in vesicular blockage to further aggravate brain dysfunction and damage, but a direct link is established to highlight that a pro-oxidant molecule (methemoglobin) can utilize other pro-oxidant molecule (hemin/heme polymer) to exacerbate the inflammatory potential. In conclusions, MetHb mediated hemin oxidation and subsequent polymerization into hemepolymer might contribute into vesicular blockage and inflammation as observed during cerebral malaria.

Role of LPS Stimulated Macrophages in Maintaining Homeostasis during Hemin Toxicity

6.1 Introduction

Macrophages toll-like receptors (TLRs) are playing crucial role in identifying wide variety of microbial products such as LPS from gram-negative bacteria, antigens from malaria parasite etc. (Takeda, Kaisho et al. 2003; Iwasaki and Medzhitov 2004). Stimulation of TLRs induces the generation and secretion of various factors to the external microenvironment (Roth, Bartocci et al. 1997; Lombardo, Alvarez-Barrientos et al. 2007). In order to maintain homeostasis during initial phases of malaria, TLRs cause secretion of high levels of pro-inflammatory cytokines, which play an important role in controlling parasite growth (Gowda 2007; Franklin, Parroche et al. 2009). In an experimental human malaria model, McCall et al. shown that TLR responses are modulated during *Plasmodium falciparum* infection. It is also known that *Plasmodium falciparum* antigens stimulates TLR-4 to release various proinflammatory cytokines such as TNF-α, IL-1β and IL-6 (McCall, Netea et al. 2007).

It is reported that macrophages activation with cytokines or microbial infection increases their survival and extends the immunological response (Flad, Grage-Griebenow et al. 1999). Classical TLR-4 ligand lipopolysaccharides (LPS) is known to triggers pro-apoptotic or anti-apoptotic program in different cell types. Apoptotic effects are observed in hepatocytes and endothelial cells (Choi, Wong et al. 1998; Hamada, Nishida et al. 1999), whereas anti-apoptotic effects are observed in cardiomyocytes, monocytes, neutrophils, and macrophages (Goyal, Wang et al. 2002; Chao, Shen et al. 2005; Ward, Murray et al. 2005). Bone marrow derived macrophages (BMDM) stimulated with LPS show an increase in viability following TLR-4 signaling via MyD88 dependent pathway (Lombardo, Alvarez-Barrientos et al. 2007).

During malaria, rupture of uninfected and infected RBC releases Hb, MetHb and hemin to create a pro-inflammatory microenvironment (Shio, Kassa et al. 2010). The pro-inflammatory microenvironment is known to show toxicity to various cells and tissue such as RBC, endothelial cells, kidney, liver and brain (Chou and Fitch 1981; Nath, Vercellotti et al. 2001; Chiu, Brittingham et al. 2002; Goldstein, Teng et al. 2003; Higdon, Benavides et al.

2012). These pro-oxidant molecules especially hemin is present in abundant quantity around the macrophages. But, then also macrophages are viable for a longer period of time as compared to other cells (Chua, Brown et al. 2013). They secretes various pro-inflammatory molecules which are responsible for the patho-physiological condition observed during malaria (Coban, Ishii et al. 2007). Macrophages have several hemin detoxification system. The major detoxification system involves heme oxygenase–I (HO-1). It converts hemin into the biliverdin which finally gets converted to bilirubin by biliverdin reductase enzyme (Montellano 2000). But during malaria and other hemolytic disorder phagocytosis is severely been compromised and it may limit the HO-1 mediated hemin detoxification in macrophages. Bio-mineralization of hemin into heme polymer (haemozoin) is an alternate process to reduce hemin toxicity. Hemin to haemozoin conversion machinery is well characterized in malaria parasite and is the major mechanism in parasite to reduce level of toxic free hemin (Nakatani, Ishikawa et al. 2013). In an *in-vitro* system, MetHb or other classical peroxidases catalyzes hemin into the heme polymer (haemozoin) (Trivedi, Chand et al. 2005; Deshmukh and Trivedi 2013).Therefore, we explored whether TLR stimulation (due to the presence of infected RBCs) in macrophages secretes enzymatic activity in the external microenvironment to reduce the level of free hemin through its bio-mineralization into heme polymer (haemozoin).

6.2 Material and methods

6.2.1 Materials

Hemin, N-acetylcysteine (NAC), Quercetin, Thiourea, 2', 7'- dichlorofluorescein diacetate (DCFD-HA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma, St. Louis, MO, USA. Peptone, Yeast extract, Sodium chloride from Hi-media, India. Dimethylsulfoxide (DMSO), Hydrogen peroxide, SDS and Sodium azide, was procured from Merck, Germany. Other reagents and chemicals were of analytical grade purity.

6.2.2 Methods

Cell culture- The detail method for cell culture is described in chapter II (Section 2.2; Macrophage cell culture and treatments; Page number 23).

Heme polymerization assay- Heme polymerization assay was done as described in the chapter II (Section 2.10; Heme polymerization assay; Page number 25).

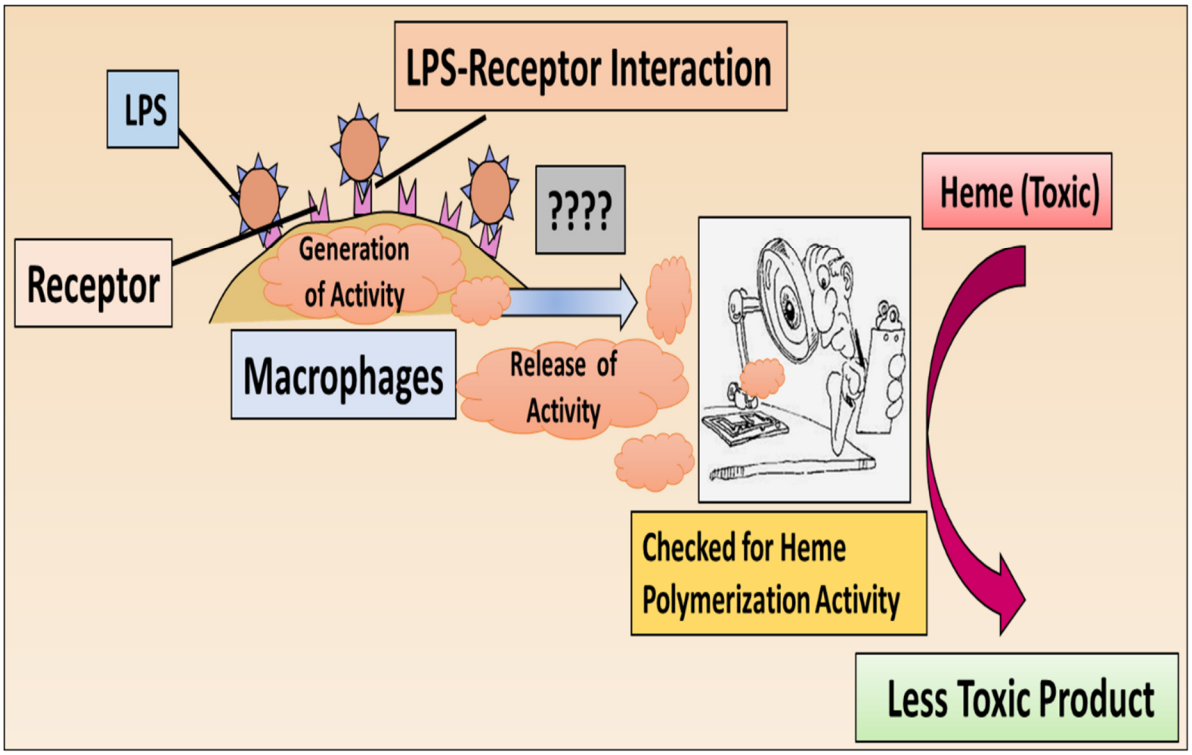

Figure 6.1: Working model for stimulation of macrophages TLRs with its ligand to secrete enzymatic activity in the external microenvironment to reduce the level of free hemin through its bio-mineralization into heme polymer (HP).

Preparation of heat killed bacteria- Bacterial strains were grown in LB medium and heated at 80°C for 30 min in water bath. The heat killed bacteria was centrifuged at 5000xrpm for 5min at 4°C and washed twice with PBS under aseptic condition. The resulting bacterial pellet was resuspended in 10ml of incomplete DMEM medium containing 1% penicillin–streptomycin antibiotic solution. (100 units/ml penicillin and 100 µg/ml streptomycin sulfate)

Measurement of Cellular Viability- Ten thousand cells were seeded overnight in 96-well plate in 0.2ml complete medium. On the day of experiment, cells were treated with different concentration (0-70µM) of hemin or heme polymer (HP) for 10h at 37°C in 5% CO_2 environment. The cellular viability of macrophage cells was measured by MTT reduction assay as described in chapter II (Section 2.3; Cell viability assay; Page number 23). Macrophage treated with incomplete medium was considered as 100% viable and used to

express viability of macrophages. To study the effect of bacteria on the macrophage viability, cells were treated with different amount (1-1000) of heat killed *E.coli* for 12h and viability was measured by MTT assay.

Microscopic observation of macrophages- The morphological changes in untreated or treated macrophage cells as described above were observed under Nikon eclipse TS 100F inverted microscope using 20x and 40x objectives and images were taken with high resolution Nikon L22 camera.

Intracellular ROS measurement- Intracellular ROS was measured with the help of a ROS sensitive fluorescent probe 2′, 7′- dichlorofluorescein diacetate (DCFH-DA) as described in chapter II (Section 2.9; Intracellular ROS measurement; Page number 25).

Macrophage stimulation and secretion protocol (macrophage culture supernatant) - Confluent 10cm^2 dish of J774A.1 macrophages were stimulated for 2h at 37°C with 10ml of heat killed bacterial culture (OD_{600} =1). After stimulation macrophages were gently washed with cell culture grade PBS to remove the bacteria. Ten ml of incomplete medium was added to the cells and allowed the macrophages to secrete factors for another 12h at 37°C in 5% CO_2 environment. The supernatant was collected and filtered with 0.22µm PVDF filter. The filtered culture supernatant is referred as **"macrophage culture supernatant"** and preserved at -20°C for future use.

Preparation of cytoplasm and membrane fraction– After collection of macrophage culture supernatant from unstimulated or LPS stimulated macrophages the cytoplasmic and membrane fraction was collected by incubated them in hypotonic buffer (10mM Tris buffer pH 7.4) for 5min at 4°C. The cell lysate was then passed through a bent 18mm gauge needle for 8-10 times for complete lysis. The lysed cells were centrifuged at 15,000xrpm at 4°C to get supernatant (cytoplasm) and pellet (membrane fraction). Protein concentration for each fraction was measured and expressed as mg/ml.

6.3 Results

6.3.1 Hemin is more toxic than heme polymer (HP) towards macrophages

During malaria and various other hemolytic disorder rupture of RBC increases the hemin load in the serum (Ferreira, Balla et al. 2008). Initially the toxicity of hemin and HP

towards macrophage J774A.1 cells was studied. Treatment of macrophages with different concentration (0-70µM) of hemin or HP for 10h at 37°C reduces the survival of macrophages. On equimolar basis (70µM), hemin shows 93% death compared to the HP which shows 40% death (Figure 6.2A). Hence, free hemin is more toxic than HP towards macrophages.

6.3.2 LPS Stimulation protects macrophages from free hemin toxicity

Malaria infected RBCs and pro-oxidant molecules released from these cells are ligand for TLRs present on macrophages (McCall, Netea et al. 2007; Lu, Yeh et al. 2008; Ropert, Franklin et al. 2008). Stimulation of TLRs induces the generation and secretion of various factors to the external microenvironment (Tushinski and Stanley 1983; Roth, Bartocci et al. 1997; Conte, Holcik et al. 2006; Lombardo, Alvarez-Barrientos et al. 2007). Macrophages unstimulated or stimulated with LPS for 2h (followed by 12h secretion in incomplete medium) were treated with hemin (20µM) at 37°C for 10h and macrophages viability were measured by MTT assay. Result indicate that hemin exposed macrophages show 38 ±4.5% survival whereas LPS stimulated macrophages shows an increase in survival upto 82 ±5.3% (Figure 6.2B). Incomplete medium treated macrophage (control) was considered as 100% viable. The increase in survival of LPS stimulated macrophages gives clue about the cyto-protective mechanism being adopted in these cells towards toxic effects of hemin.

6.3.3 LPS stimulated cells accumulate heme polymer (HP)

The cellular morphology of macrophages (from experimental condition given in 6.3.2 "**LPS Stimulation protects macrophages from free hemin toxicity"**) were observed under light microscope using 40x objective. After 10h, untreated cells look healthy and dividing with intact cellular membrane whereas macrophages treated with 20µM hemin were unhealthy and exhibits cellular shrinkage. Interestingly, LPS stimulated and hemin treated macrophages shows healthy morphology with accumulation of brown color precipitated material in their cytoplasm (Figure 6.2C). The brown color polymeric material was also observed in macrophages treated with HP only. The microscopic observation points out the possibility to convert extracellular hemin into the HP and subsequent internalization into the storage vesicles of macrophages. Hence, data in Figure 6.2 clearly highlights the ability of LPS to provide protection in macrophages against free hemin toxicity.

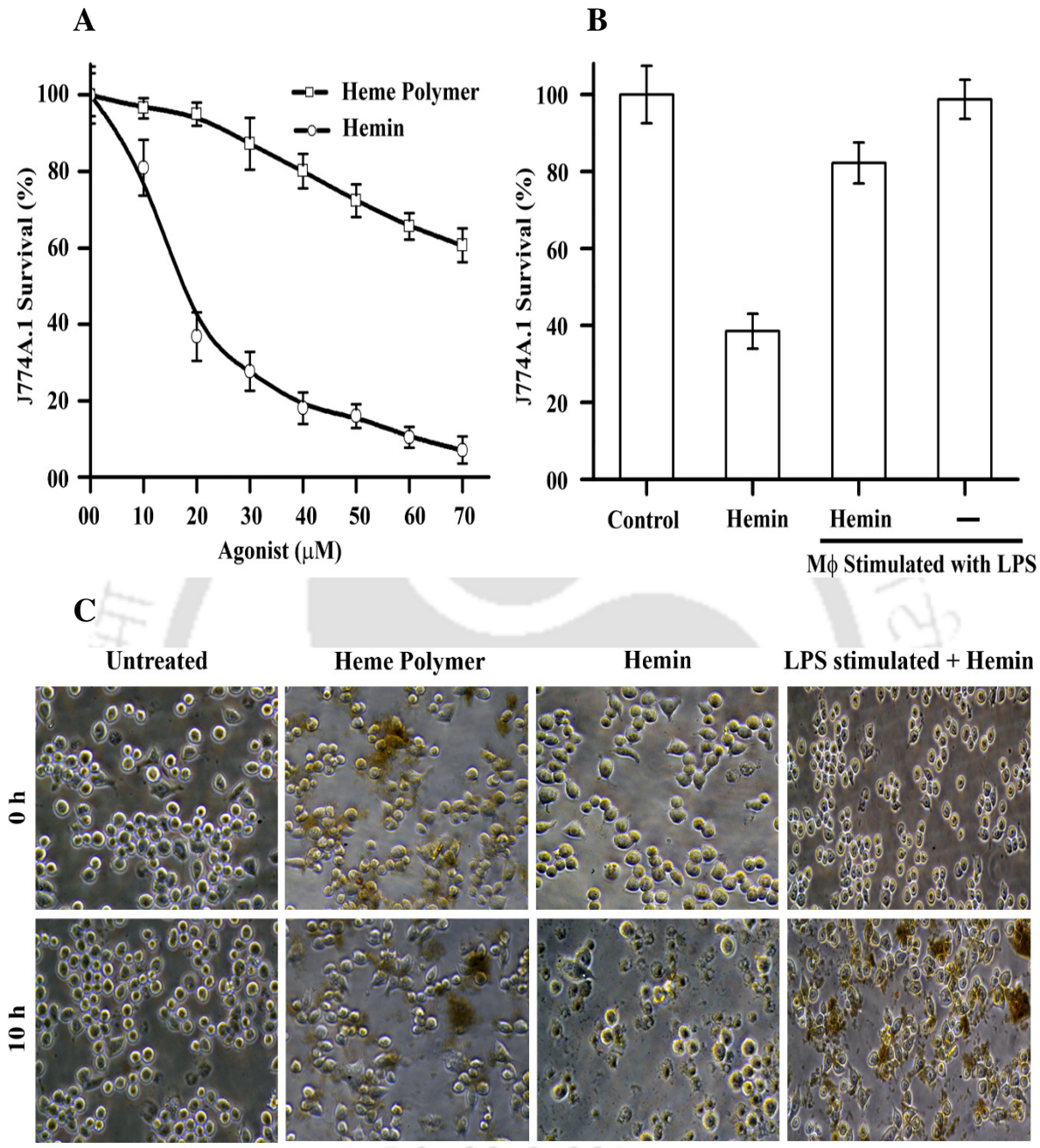

Figure 6.2: Stimulation of macrophage with LPS protect them from hemin toxicity. (A) Assessment of toxicity of hemin or HP (0-70 μM) against macrophages. Cells were treated with different concentration of HP or hemin for 10h at 37℃ and survival was assessed by MTT assay as described in chapter II. **(B)** Macrophages un-stimulated or stimulated with LPS for 2h followed by 12h secretion and treated with hemin (20μM) at 37℃ for 10h and viability were measured by MTT assay. Incomplete medium treated macrophage was considered as 100% viable. Data are the mean ± SD of three independent experiments (n = 3) with triplicate measurements **(C)** Microscopic observation of cellular damage caused by HP or hemin and protection offered by LPS stimulation as described in (A) and (B). Images of 10 fields were captured with a 40x objectives using an inverted microscope TS100F (Nikon, Japan).

6.4.4 LPS stimulation releases unknown factor(s) to catalyze heme polymerization.

Macrophages J774A.1 were either remains unstimulated or stimulated with LPS for 2h at 37°C and macrophage culture supernatant was collected after 12h as described. The culture supernatant was tested for heme polymerization activity (HPA) as described in chapter II (Section 2.10; Heme polymerization assay; Page number 25).The macrophage culture supernatant exhibits HPA in concentration dependent manner (Figure 6.3A). In contrast, macrophages culture supernatant collected from unstimulated or stimulated with LPS at 10°C shows no or very negligible amount of HPA (Figure 6.3A). It is reported that at lower temperature macrophages are not actively releasing activity in response to external stimulus (Salman, Bergman et al. 2000). There are two sources to contribute into the activity present in the macrophage culture supernatant; cytosolic factors from dead macrophages and secretory products from stimulated macrophages. In the past it is reported that macrophages stimulated with *E.coli* reduces their viability (Xaus, Comalada et al. 2000). Macrophages were exposed to different amount of heat killed *E.coli* for 2h and viability was measured. Macrophages did not show any change in viability after treatment with different amount of heat killed bacteria (Figure 6.3B). Different types of bacterial strains (based on LPS level on their cell surface) were used to stimulate macrophages to further confirm the role of LPS to secrete factors. The level of LPS present on bacterial species (Salton and Kim 1996) is used to categories into (1) low LPS, (2) medium LPS and (3) high LPS. It was observed that macrophages stimulated with different strains of bacteria secrete HPA as per the level of LPS present on the bacterial surface. (Figure 6.3C). The specificity of LPS mediated secretion of activity in the macrophage culture supernatant was further evaluated for its localization into different cellular compartments (extracellular, cytoplasm and membrane). Macrophages either remained unstimulated or stimulated with LPS and cytoplasm and membrane fraction (pellet) was prepared as described in "materials and methods" section (**Preparation of cytoplasmic and membrane fraction**). Culture supernatant was collected from unstimulated or LPS stimulated macrophages. The HPA of macrophage culture supernatant, cytoplasm and membrane fraction was determined and expressed as HP formed (µM/mg protein). LPS stimulation is up regulating the content of HPA in the culture supernatant compared to the level in cytoplasm or membrane fraction (Figure 6.3D). Hence, the result given in Figure 6.3

concludes that the macrophages LPS stimulation protect the cells from free hemin toxicity through release of an unknown HPA into the external microenvironment.

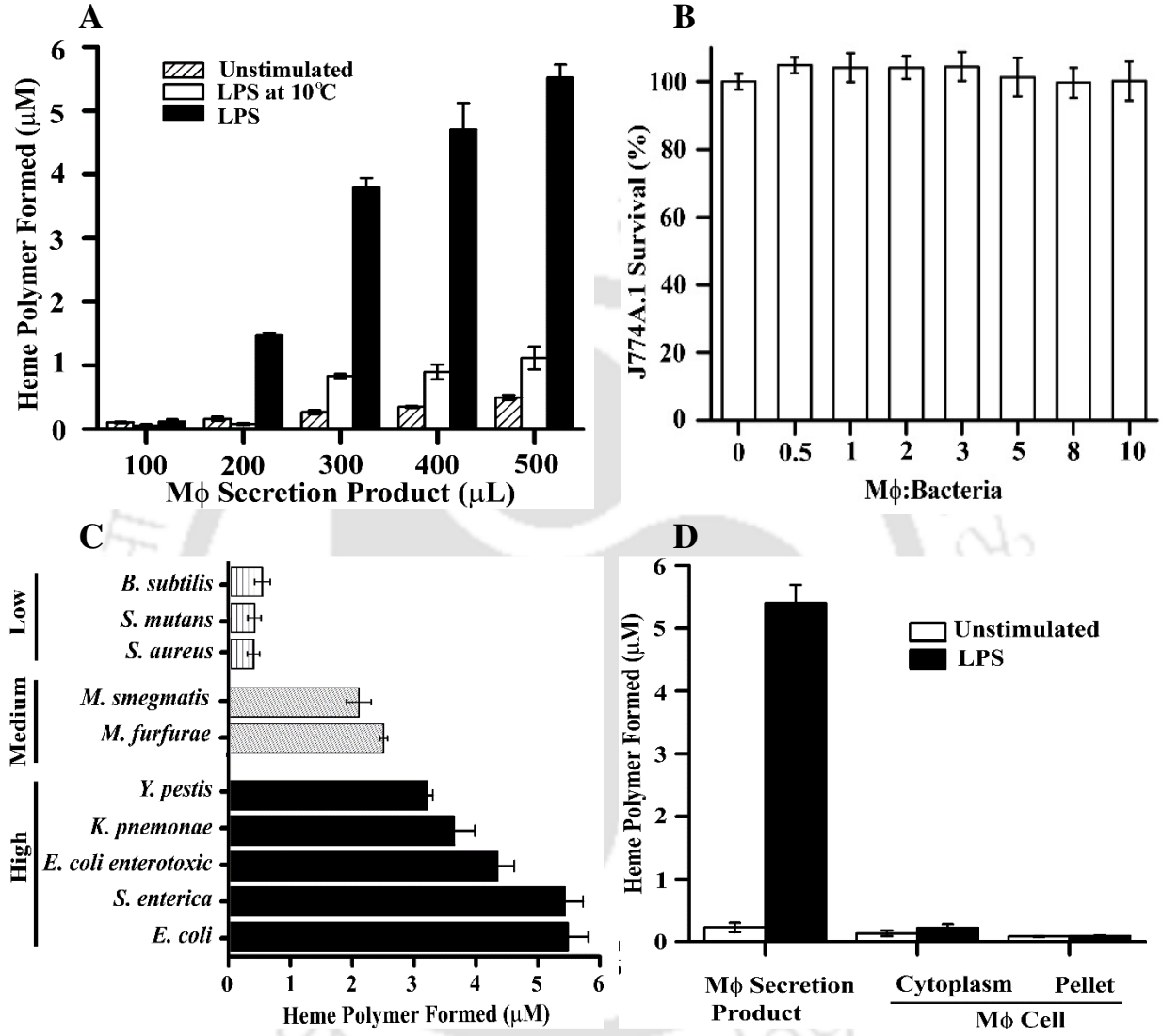

Figure 6.3: Stimulation of macrophage J774A.1with LPS releases an unknown HPA in culture supernatant. (A) Measurement of heme polymerization activity of LPS unstimulated or stimulated macrophages 10℃ and 37℃. Macrophages were stimulated with LPS for 2h at 10℃ and 37℃ and followed by secretion for 12h at 37℃. HP assay was done as described in chapter II. **(B)** Macrophages exposed to different amount of heat killed bacteria (1- 1000) for 2h and kept for secretion for 12h and survival was assessed by MTT assay as described in chapter II. Incomplete medium treated macrophage was considered as 100% viable **(C)** HPA of supernatant obtain with stimulation of different types of bacterial strains. Macrophages were stimulated with different strains of heat killed bacteria for 2h followed by secretion for 12h at 37℃. Supernatants were subjected to HPA as described in chapter II. **(D)** HPA of supernatant, cytoplasm and pellet fraction of unstimulated or LPS stimulated macrophages**.** Cytosol and pellet fraction was obtained by lysis of unstimulated or LPS stimulated macrophages with hypotonic buffer as described in "materials and methods" section. Data are the mean ± SD of experiments with triplicate measurements

6.4.5 Understanding different parameters controlling secretion of HPA in macrophages

Macrophages stimulated with LPS and secretion of HPA is controlled by multiple factors: such as number of ligands, time of stimulation and secretion. Initially, macrophages (MΦ) were stimulated with different amount of heat killed bacteria (1-1000 for each MΦ) for 2h at 37°C and then secretion product (macrophage culture supernatant) was collected after 12h. Macrophages respond to bacteria to secrete HPA in bi-phasic manner, it was proportional to the number of bacteria in the initial phase (upto bacterial number 100) and sharply declined afterwards at high bacteria concentration (Figure 6.4A). In another series of experiment, macrophages were stimulated with heat killed bacteria (100 each MΦ) for different time period (0-2h) and then secretion products were collected after 12h. Macrophages mediated HPA secretion was proportional to the stimulation period upto 1h and then decline afterwards (Figure 6.4B).

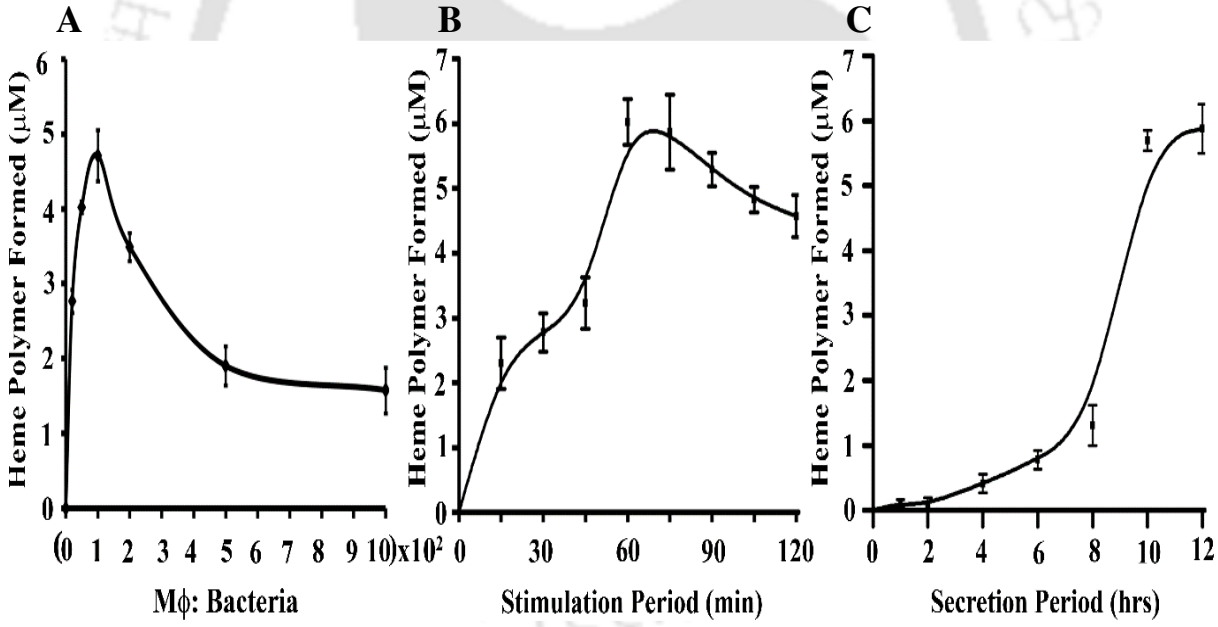

Figure 6.4: Optimization of secretion of activity from the macrophages. (A) Macrophage; LPS interaction is specific in nature. Heme polymerization activity (HPA) of the macrophage culture supernatant obtained by stimulating macrophages with different amount of heat killed bacteria (1-1000) for 2h at 37°C. **(B)** Macrophages stimulated with heat killed bacteria (100 each MΦ) for different time period (0-2h) and then secretion product were collected after 12h **(C)** Macrophages stimulated with heat killed bacteria (100 each MΦ) for 1hr and then secretion product were collected at different time period (0-12h). HP assay was done as described in chapter II. Data are the mean ± SD of experiments with triplicate measurements.

To understand the role of secretion phase in controlling the product recovery, macrophages were stimulated with heat killed bacteria (100 each MΦ) for 1hr and then secretion products

were collected after different time points (0-12h). Macrophages respond to bacteria to secrete HPA proportional to the secretion period and gives highest amount in 12h (Figure 6.4C). In conclusion, macrophage: LPS interaction is specific in nature and ligand concentration (amount of bacteria) as well as stimulation period play crucial role in regulating secretion of HPA.

6.4.6 Intracellular ROS stimulates the secretion of HPA

Macrophages stimulated with LPS generate intracellular ROS to modulate various genes to produce and secrete cytokines and pro-inflammatory factors (Kitamura, Ito et al. 2008). Macrophages were stimulated with different amount of heat killed bacteria (1-200 for each MΦ) for 1hr at 37°C and then intracellular ROS was measured. Intracellular ROS was increasing gradually and reaching maximum at 1:100 (100 bacteria per MΦ) afterwards it sharply declined at high bacterial concentration (Figure 6.5A).

Similarly, intracellular ROS was measured for stimulation and secretion phase to further explored the role of ROS. Macrophages were stimulated with heat killed bacteria (100 each MΦ) for different time point (0-2h) at 37°C and then intracellular ROS was measured. In stimulation phase, it was observed that macrophages give high ROS at 1hr of time period (Figure 6.5B). Similarly ROS was also measured in secretion phase upto 12h and it was observed that the significant high ROS remained throughout this period and it might be driving the production of HPA in the supernatant (Figure 6.5C). A strong correlation (correlation factor, r^2=0.85) between the level of ROS and amount of HPA secreted further support the role of ROS in controlling HPA production (Figure 6.5D).

To further explore the role of intracellular ROS, macrophages were supplied with various antioxidants such as NAC (5mM), quercetin (50µM) and thiourea (200mM). The macrophages pre-incubated with antioxidants and stimulated with heat killed bacteria, amount of HPA was determined in the supernatant. Antioxidant pre-incubation reduces the amount of HPA compared to heat killed bacteria stimulated macrophages (Figure 6.5E). Additional study is required to understand the molecular events downstream to ROS involved in the production of HPA.

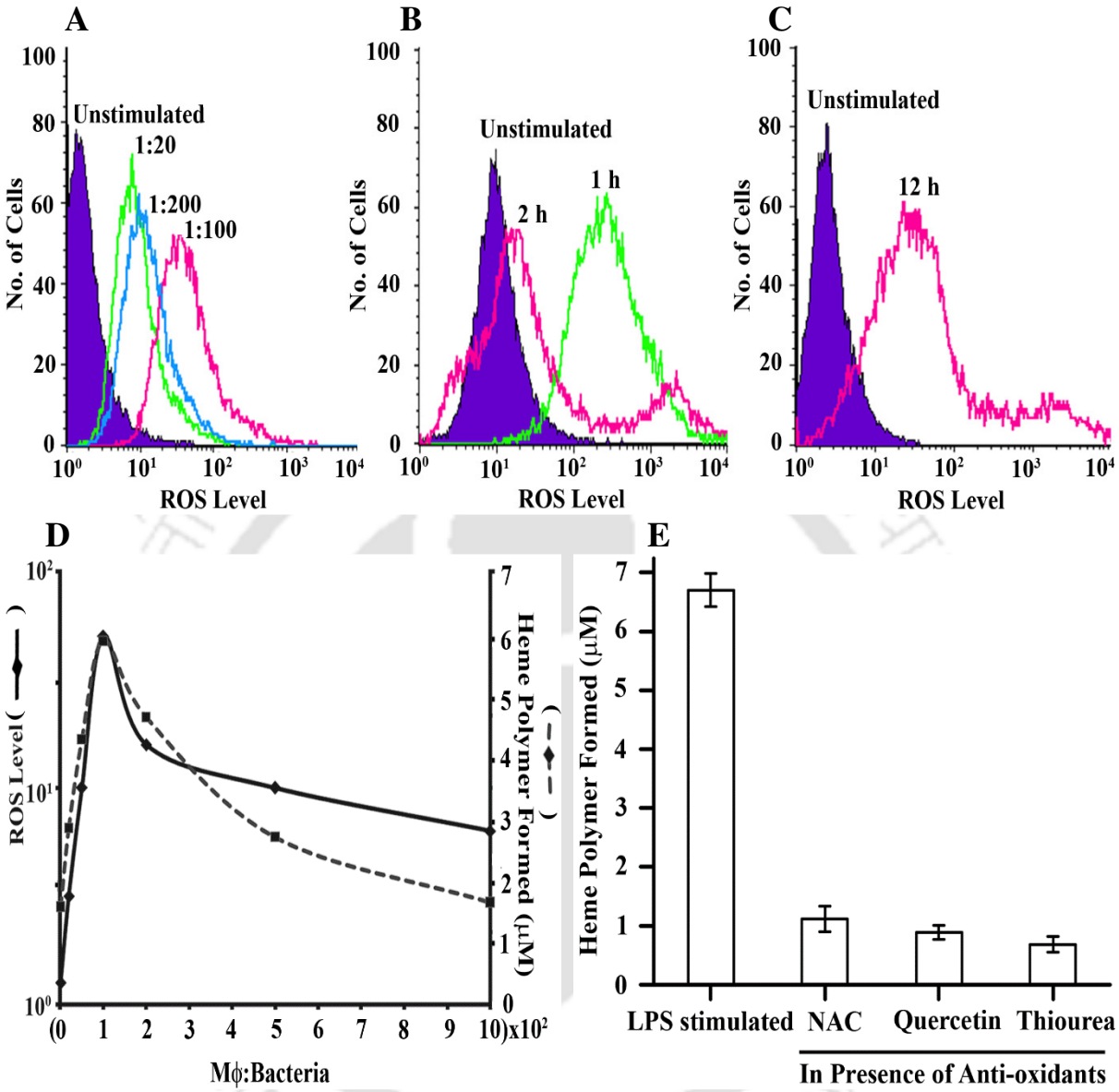

Figure 6.5: Generation intracellular ROS in macrophages stimulates the secretion of HPA in the culture supernatant. (A) Macrophages either unstimulated or stimulated with different amount of heat killed bacteria (1-200 for each MΦ) for 1h at 37℃ and intracellular ROS was measured by ROS probe DCFH-DA. **(B)** Macrophages either unstimulated or stimulated with heat killed bacteria (100 each MΦ) for different time point (0-2h) and intracellular ROS was measured by ROS probe DCFH-DA. **(C)** Macrophages stimulated for 1h with heat killed bacteria (100 each MΦ) and kept for secretion upto 12h and intracellular ROS was measured by ROS probe DCFH-DA. **(D)** Correlation of HP formed and intracellular ROS in macrophages stimulated with different amount of heat killed bacteria for 1h at 37℃. **(E)** Scavenging of ROS with anti-oxidants treatment provides inhibition of HPA. Macrophages either unstimulated or stimulated with heat killed bacteria (100 each MΦ) for 1h in the absence or presence of NAC (5mM), quercetin (50μM) and thiourea (200mM) and allow to secrete for 12h. HPA of the obtained culture supernatant was done as described in chapter II. Data are the mean ± SD of experiments with triplicate measurements.

6.4.7 HPA is proteinase in nature and requires hydrogen peroxide.

To test the biochemical nature of HPA, supernatant containing HPA was treated with proteinase K (PK), SDS (1%), heated upto 90°C for 30min or sodium azide (0.1%) and activity was tested. HPA was polymerizing hemin to form HP but SDS, heat or sodium azide treatment abolishes HPA (Figure 6.6A). It gives strong indication that HPA is proteinous in nature. Interestingly, we found that HPA requires hydrogen peroxide to efficiently catalyze heme polymerization (Figure 6.6B). Classical peroxidase catalyzed reaction requires hydrogen peroxide to generate substrate radicals to form polymeric products (Trivedi, Chand et al. 2005). Addition of guaiacol (peroxidase substrate) in assay mixture reduces the HPA mediated heme polymerization (data not shown) and it indicates that HPA might possess peroxidase like activity but further purification and isolation is required to understand biochemical nature of HPA.

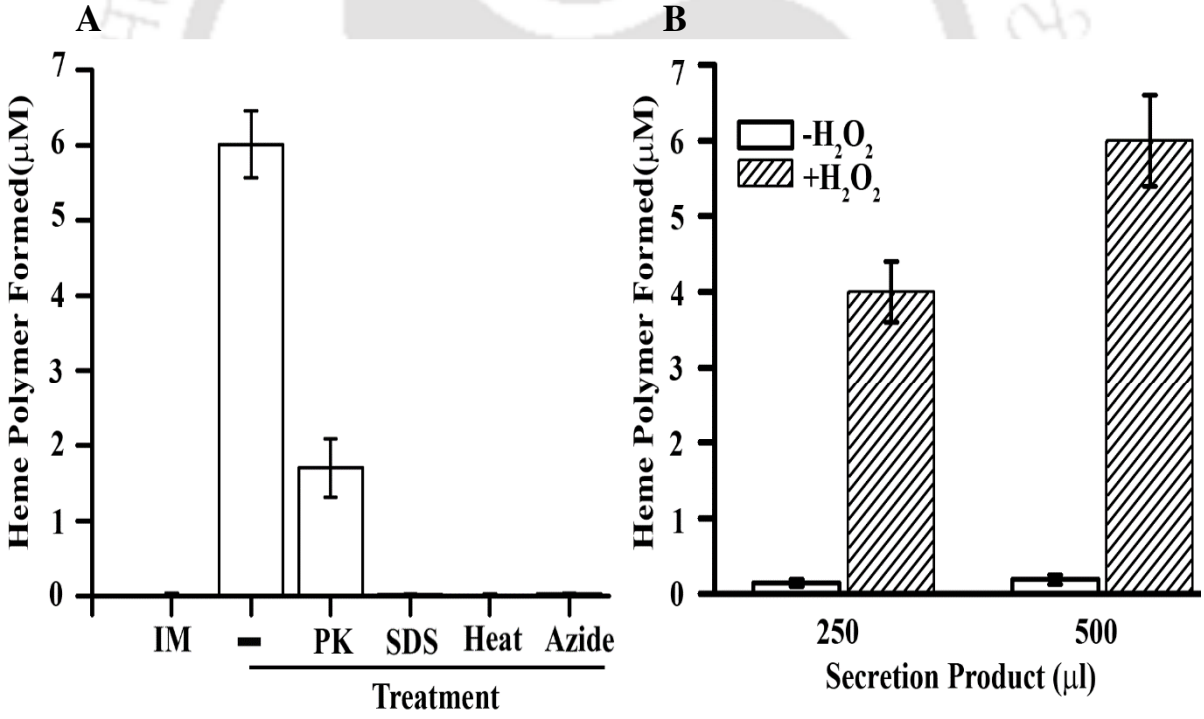

Figure 6.6: Activity is proteinase in nature and sensitivity to hydrogen peroxide. (A) Macrophage culture supernatant was untreated or treated with proteinase K (PK), SDS (1%), heat (90℃ for 30min) or sodium azide (0.1%) and heme polymerization assay was done as described in chapter II. **(B)** Hydrogen peroxide is crucial for HPA. Heme polymerization assay of macrophage culture supernatant was done in presence or absence of hydrogen peroxide (5mM).as described in chapter II Data are the mean ± SD of three independent experiments with triplicate measurement. IM is incomplete medium.

6.4.8 Secretion product protects the macrophages from hemin toxicity

The increase in survival of LPS stimulated macrophages give clue about the cyto-protective mechanism being adopted in these cells towards toxic effects of hemin (Figure 6.2). We further explored whether it is due to the presence HPA in the extracellular milieu or up regulation of pro-survival mechanisms inside the macrophages. Macrophages untreated or treated with 20µM of hemin was incubated with different volume of macrophage secretion product (from LPS stimulated macrophages) in a total reaction volume of 200µl. The reactions were incubated for 10h at 37°C and viability was measured by MTT as described in chapter II (Section 2.3 cell viability assay; Page number 23). Macrophages treated with hemin (20µM) exhibits 39 ± 2.79 % survival whereas hemin treatment in the presence of varying amount of HPA (25µl -100µl) increases the survival in a dose dependent manner and gives viability from 37 ± 3.39 to 75 ± 5.04 %. The change in viability of macrophages in the presence of HPA is statistically significant (p-value, 0.001). Macrophages treated with incomplete medium were taken as 100% survival (Figure 6.7A). Light microscopic observation of macrophages confirms the reversal of cellular damage in the presence of HPA (Figure 6.7B). Moreover, a brownish crystalline matter was observed in cells treated with hemin in the presence of HPA, which is completely absent in hemin treated cells only. Hence data in Figure 6.7 highlights the role of activity in protecting the macrophages from hemin toxicity as observed during malaria.

6.5 Discussion

During malaria and other hemolytic disorders RBC rupture increases hemin burden in the serum and disturb body homeostasis (Ferreira, Balla et al. 2008). Heme is highly pro-oxidant in nature and it induces ROS generation result in lipid peroxidation, DNA damage and protein aggregation, leading to cell death via apoptotic pathway (Jeney, Balla et al. 2002; Kumar and Bandyopadhyay 2005; Glei, Klenow et al. 2006). Hemin stimulates leukocytes and endothelial cells to release proteases and ROS to cause severe tissue damage (Kumar and Bandyopadhyay 2005). The data presented in Figure 6.2A is the first report to provide comparative study of HP and cell free hemin toxicity towards macrophages. It is in agreement to the known literature that haemozoin (hemin bio-mineralization product) is less toxic compared to hemin towards different cell types (Arese and Schwarzer 1997).

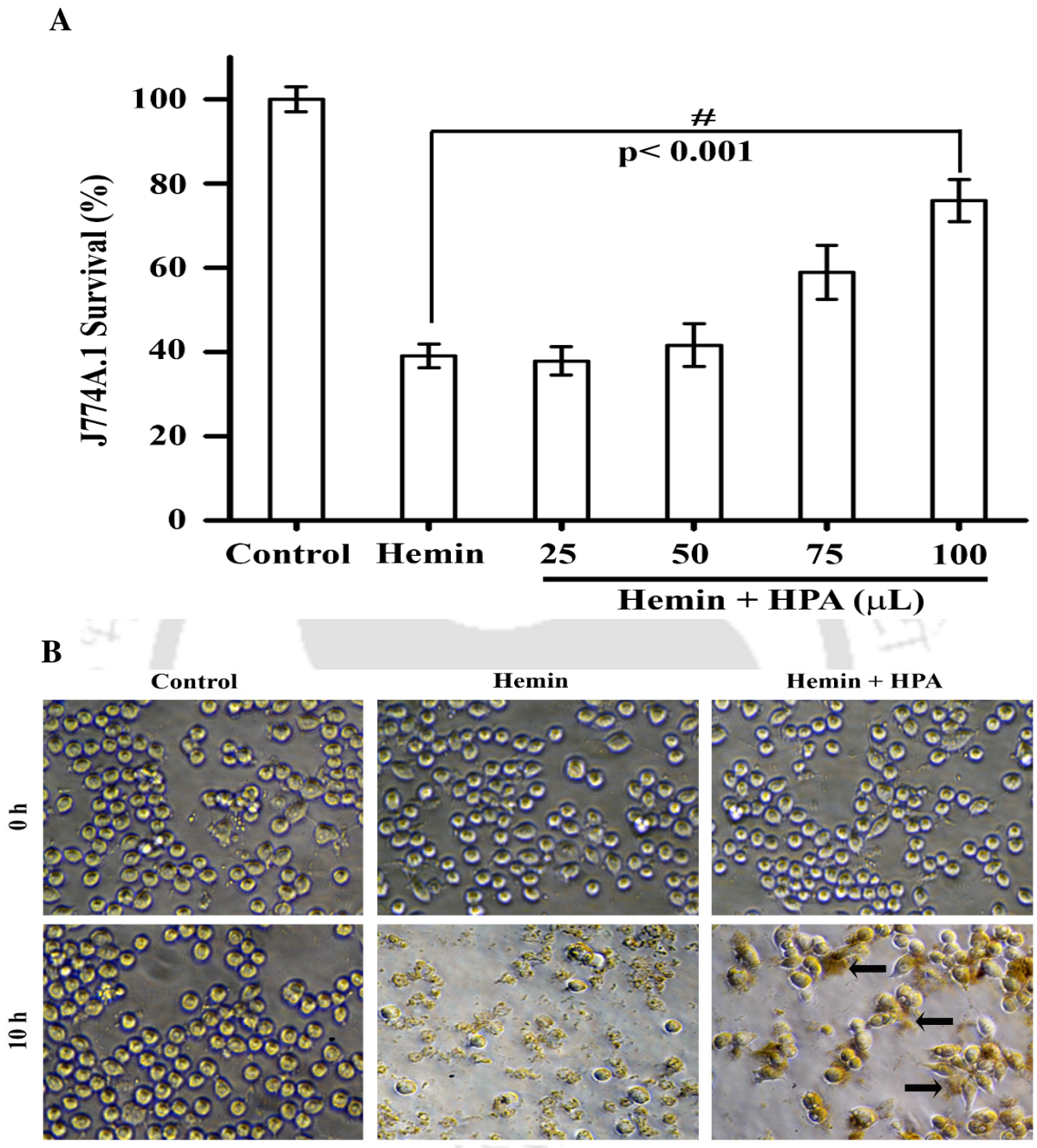

Figure 6.7: HPA protects the macrophages from hemin toxicity. Measurement of toxicity of hemin against macrophage J774A.1 cells in presence or absence of HPA **(A)** Macrophage were treated with hemin (20µM) at 37℃ for 10h in presence or absence of macrophage culture supernatant and survival was assessed by MTT assay as described in chapter II. Incomplete medium treated macrophage was considered as 100% viable and data are the mean ± SD of three independent experiments with triplicate measurements. The pairwise results were analyzed with anova & student t-test and it was statistically significant with #P < 0.001 **(B)** Microscopic observation of untreated (control), and hemin treated macrophages in presence or absence of secretion product. HP formed is denoted by arrows.

Macrophage and monocyte fed with haemozoin are viable but functionally impaired (Schwarzer, Alessio et al. 1998; Giribaldi, Prato et al. 2010).Different types of receptors are known to present on the macrophage membrane to perform immunological function during infection and tissue injury. TLRs represent group of receptors specialized to identify specific molecular patterns of pathogens, malaria infected RBC and malaria products like haemozoin and GPIs (McCall, Netea et al. 2007; Lu, Yeh et al. 2008). Macrophages stimulated with LPS upregulates several genes to acquire resistance against cyto-toxic environment produced by different pathogens (Kitamura, Ito et al. 2008). Macrophages pre-stimulated with LPS show increase in viability towards hemin toxicity (Figure 6.2B). When the supernatant from LPS stimulated macrophages was tested (collected after 12h in incomplete medium), it was observed that secretion product can be able to convert toxic free hemin to less toxic HP (Figure 6.3A). Presence of brown colored precipitated material in macrophage further confirms the conversion of hemin into HP extra-cellularly and its subsequent uptake by the macrophages (Figure 6.2C). Although we didn't identified the brown colored precipitated material or the role of phagocytosis in this process.

It is reported that bacterial infection or LPS exposure can lead to macrophages apoptosis (Xaus, Comalada et al. 2000). But macrophages exposed to different amount of bacteria were ~100% viable ruled out any such possibility (Figure 6.3B). Moreover, pharmacological inhibitors and mice knockout models show that TLR-4 stimulation promotes long-term viability of BMDM macrophages (Lombardo, Alvarez-Barrientos et al. 2007). TLR-4 receptor stimulation with Gram –ve bacteria in macrophages gives maximum HPA (Figure 6.3C). The activity is secreted from macrophages as supported by maximum fraction is being present in the extracellular medium compared to the cytoplasm or membrane (pellet) fraction (Figure 6.3D).The intracellular ROS generated within the macrophages is responsible for the secretion of HPA (Figure 6.5). But it is also reported that macrophages stimulated with MetHb produce multiple ROS spikes to damage antioxidant machinery results into the macrophage apoptosis (Deshmukh and Trivedi 2013). LPS stimulation also produces ROS inside the macrophages to induce apoptosis (Maitra, Singh et al. 2009). However, microarray data shows that macrophages stimulated with LPS cause up regulation of various cytokines and anti-apoptotic factors. These are known to produce inflammatory reactions responsible for providing protection against various invading

pathogens (Rutledge, Jiang et al. 2012). In the biological system peroxidases are known to require H_2O_2 and importantly they can catalyze heme to HP in an *in-vitro* conditions (Trivedi, Chand et al. 2005). In biological system, peroxidases play an important role to maintain homeostasis. They scavenge H_2O_2 from the extracellular space and protect the cells from oxidative damage. Moreover, peroxidases in presence of H_2O_2 catalyzes oxidation of substrate molecules (aromatic or halide) through one-electron oxidation to form a polymeric product (B.C. Saunder 1964). HPA depends on H_2O_2 to catalyze the conversion of free hemin to HP. A detail study is required to understand the molecular details of HPA mediated HP formation and its protective mechanism.

6.6 Conclusion

The present chapter explored the protective mechanism in LPS stimulated macrophages from free hemin toxicity. HPA secreted from stimulated macrophages catalyzes the conversion of free toxic hemin to less toxic HP. The reduction in concentration of free heme increases the viability of macrophages thereby helps in maintaining homeostasis.HPA utilizes H_2O_2 for its activity and may offer protection of host cells against oxidative damage.

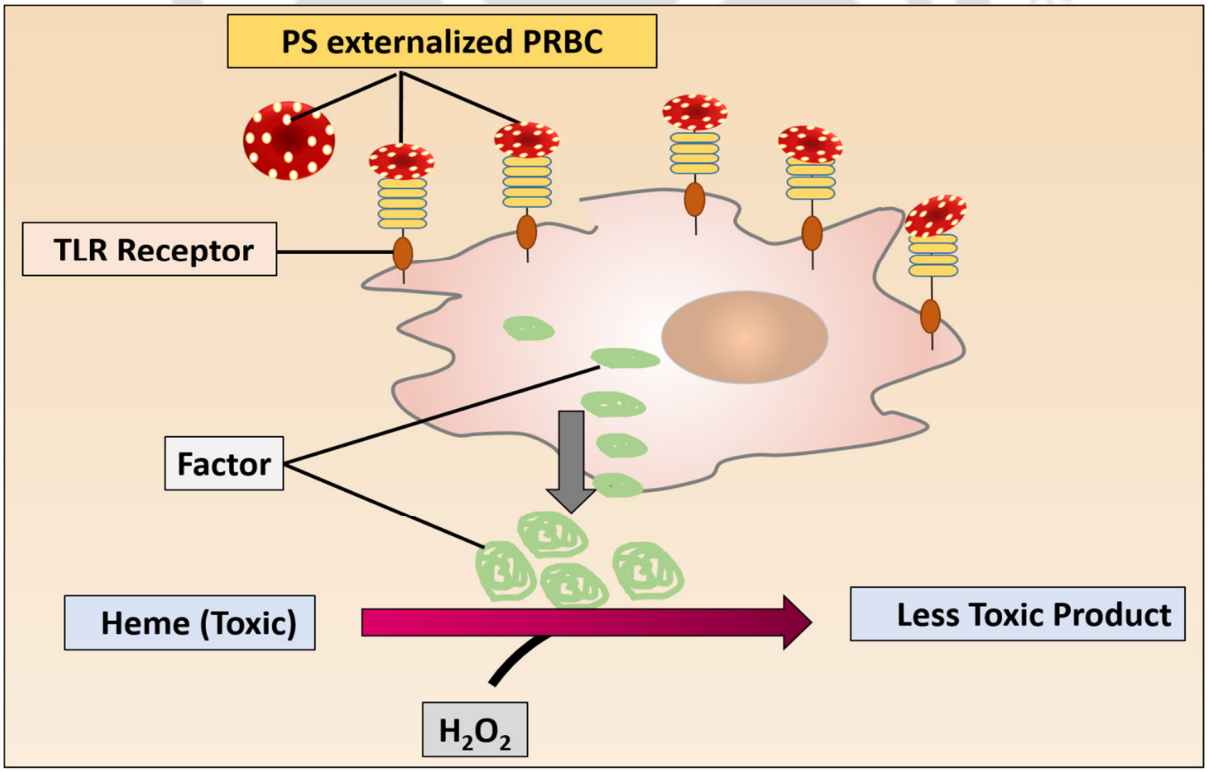

Figure 6.8 Schematic of secretion of activity and its role in neutralizing hemin toxicity towards macrophages.

A schematic model to summarize the current finding is presented in Figure 6.8. Stimulation of macrophages with TLR ligand secretes HPA in the supernatant. Secretion is specific to LPS stimulation and ROS generated inside the macrophages regulates the secretion of HPA. The HPA is proteinaeous in nature and requires hydrogen peroxide for catalyzing heme polymerization. Thus a new mechanism for the hemin detoxification has been explored in the current work.

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