UNIVERSITY OF KWAZULU-NATAL

COMPARISON OF THE EFFECTS OF ORAL AND TRANSDERMAL ADMINSTRATION OF CHLOROQUINE ON SELECTED HAEMATOLOGICAL PARAMETERS AND INFLAMMATORY CYTOKINES IN *P.BERGHEI*- INFECTED MALE SPRAGUE-DAWLEY RATS.

Nontobeko Myllet Gumede

2016

COMPARISON OF THE EFFECTS OF ORAL AND TRANSDERMAL ADMINSTRATION OF CHLOROQUINE ON SELECTED HAEMATOLOGICAL PARAMETERS AND INFLAMMATORY CYTOKINES IN *P.BERGHEI*- INFECTED MALE SPRAGUE-DAWLEY RATS.

By

Nontobeko Myllet Gumede (210552960)

Submitted in fulfilment of the requirements for the degree of Master of Medical Sciences in Human Physiology in the Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, Faculty of Health Sciences

Supervisor: **Dr MV Mabandla**
Discipline of Human Physiology
Faculty of Health Sciences

DECLARATION-UKZN

I, Nontobeko Myllet Gumede hereby declare that the dissertation entitled "**Comparison of the effect of oral and transdermal administration of chloroquine on selected haematological parameters and inflammatory cytokines in *P.berghei*-infected male Spraque-Dawley rats.**" is a result of my own investigation and research. This work has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, I have duly acknowledged in the text.

Student: Nontobeko Myllet Gumede **Signature** [redacted] **Date** 18/03/16

Supervisor: Dr MV Mabandla **Signature** [redacted] **Date** 18/03/16

ACKNOWLEDGEMENT

GLORY BE TO THE HOLY FATHER, AND TO THE SON, AND TO THE HOLY SPIRIT, AS IT WAS IN THE BEGINNING, IS NOW, AND EVER SHALL BE, WORLD WITHOUT END. AMEN

I would like to extend my sincere thanks to the following:

My supervisor Dr MV Mabandla for his input, constructive criticism and guidance throughout the project.

I would like to thank my guardian angel my late mother Elizabeth Thembi Gumede. I am who I am because of you. I am eternally grateful for all that you have done, and all that you have given me. Ngyabonga kakhulu Mam-Msibi, I love you mommy and miss you very much.

I would like to pay my sincere gratitude to my late supervisor, Prof C. T. Musabayane for his valuable advice, and all his teachings. You may be gone but your teachings will live forever in my heart. Rest in peace Prof

To my supportive father, Bernard Gumede, who is always by my side when I needed him the most. Ngiyabonga Mnguni.

To my brothers, Lindokuhle, Sibusiso and Sthembiso. Not forgetting Lindiwe Steven and my nephews. These were my greatest sources of strength. Thank you for your unconditional love, your patience and for providing me with a shoulder to cry on every step of the way.

My thanks are due to my colleagues for their constant support, encouragement and helpful suggestions, (Philile Happiness sibiya, Lebogang Mokotedi, Slindile Hadebe, Nokuphiwa Mfayela and Blessing Mkhwanazi).

I gratefully acknowledge, Miss K. Moodley and Dennis Makhabula, Biomedical Research Unit (BRU) staff, Linda and Dennis for their Technical assistant. Finally, I would like to thank the National Research Foundation (NRF) and the College of Health Sciences for granting me the funds to pursue my studies.

I DEDICATE THIS DISSERTATION TO MY FAMILY OMNGUNI, QWABE, MNGUNI KAYEYEYE. OSIDLABEHLEZI BAKAKHONDLO KAPHAKATHWAYO. NGIPHINDE NGIBONGE NANI BO NDLONDLO, MABUYA BENGABUYI, NDLONDLO ENOPHAPHU EKHANDA, SIWELA.

NGIBONGA ANGIPHEZI…

LIST OF ABBREVIATIONS

ACT	Artemisinin combination therapy
ANOVA	One analysis of variance
API	Annual parasite index
AVP	Arginine vasopressin
AVIDIN-HRP	Avidin-Horseradish Peroxidase
BHT	Butylated hydroxytoluene
BRU	Biomedical Research Unit
b.wt	Body weight
Ca^{+}	Calcium
Cl^{-}	Chloride
CHQ	Chloroquine
CRP	C-reactive protein
DMSO	Dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbant assay
GFR	Glomerular filtration rate
HCT	Haematocrit
HGB	Haemoglobin
IRS	Indoor residual house spraying
IC	Infected control
IL-1	Interleukin-1
IL-1b	Interleukin-Beta
IL-6	Interleukin-6

IL-22	Interleukin
K^+	Potassium
LTD	Limited
LY	Lymphocytes
Kg	Kilogram
µ	Micro
µg	Microgram
µL	Microlitre
MPV	Mean Platelet Volume
MCH	Mean Corpuscular haemoglobin
MO	Monocytes
Mg	Milligram
mmHg	Millimetres of mercury
mmol	Millimole
MDA	Malondialdehyde
NaOH	Sodium hydroxide
NE	Neutrophils
NF-κB	Nuclear factor κB
NIC	Non-infected control
NO	Nitric oxide
OD	Optical density of the standards
pRBC	Parasitized red blood cells
pmol	Picomole
p.o.	per os

RBC	Red blood cell
RDW	Red cell distribution width
SD	Sprague-Dawley
SEM	Standard error of mean
SOD	Superoxide dismutase
SP	Sulphadoxine/pyrimethamine
TNF-α	Tumor necrosis factor-alpha
UK	United Kingdom
UKZN	University of KwaZulu-Natal
USA	United States of America
WBC	White blood cell

TABLE OF CONTENTS

LIST OF TABLES

Page No.

LIST OF FIGURES

Page No.

LIST OF APPENDICES

*Mbatha, B., ***Gumede, N.M.,** *Sibiya, H., *Musabayane C.T.

Evaluation of the efficacy of Syzygium aromaticum-derived oleanolic acid on malaria parasite in *Plasmodium berghei*-infected male Sprague-Dawley rats: effects on blood glucose and renal electrolytes handling. College of Health Sciences Research Symposium 2015, 10-11 September.

ABSTRACT

Introduction

Chloroquine (CHQ), the mainstay antimalarial drug accumulates in organs and alters physiological function. Hypoglycaemia, impairment of kidney function and anaemia are among an array of pathophysiological manifestations caused by malaria infection or oral CHQ treatment. However, it is unclear whether this anaemia is solely due to the parasite or by CHQ. Therefore, there is need to investigate and distinguish between the pathophysiological effects of malaria alone and those of CHQ treatment. The purpose of the current study was to investigate and compare the effects of using oral CHQ treatment or a transdermal CHQ patch in the treatment of malarial infection. To this effect, we evaluated changes in haematological parameters as well as plasma cytokine concentrations in male Sprague-Dawley rats. We also looked at the morphological effects of various visceral organs following malarial infection and subsequent treatment with CHQ. The study duration was 3 weeks divided into pre-treatment (days 0-7), treatment (8-12) and post treatment (13-21) periods. CHQ treatment was either administrated orally (30mg/kg, twice daily) or via a once off CHQ matrix patch (56mg/kg). Oral CHQ treatment reduced red blood cell count, haematocrit, haemoglobin and mean corpuscular haemoglobin in non-infected and infected animals. Topical application increased the above parameters in infected rats. Oral CHQ decreased pro-inflammatory cytokine concentration in infected rats on the day (day 8 of the experiment) of the treatment period in comparison to pre-treatment (baseline) measurements. However, on the last day (day 12) of the treatment period and during the post-treatment period there was an increase in pro-inflammatory cytokine concentration while patch application decreased pro-inflammatory cytokine concentration in infected rats throughout the experimental period. *P.berghei*-infected rats following oral and transdermal CHQ delivery showed mild morphological changes on the liver, heart, kidney and spleen by comparison to infected control animals. In non-infected rats oral CHQ treatment showed adverse morphological effects on the architecture of these organs, while no changes were observed following transdermal CHQ delivery. C-reactive protein is an acute phase protein, a component of innate immune response and is useful in early detection of inflammation. Oral CHQ administration increased CRP concentration. However, CRP concentration was not affected in patch treated animals. The results of the current study have demonstrated that the once off patch application of the CHQ-formulation has no morphological effects when compared to oral administration of CHQ on various organs. The ability of the pectin-CHQ matrix patch to provide slow, sustained CHQ releases into the circulation, avoids drug dumping in various tissue organs therefore circumventing the adverse effects associated with oral administration of CHQ. In addition, our results show that both CHQ and malaria parasite result in the development of anaemia by affecting RBCs and plasma pro-inflammatory cytokines. These findings suggest that transdermal CHQ delivery could therefore be used in conjunction with or as an alternative treatment in the management of malaria.

CHAPTER 1

INTRODUCTION

1.0 Background

Malaria is an acute infectious disease caused by *Plasmodium* parasite and spread by the female anopheles mosquito. Each year an estimated 300 to 500 million clinical cases of malaria occur, resulting in 1.5–2.7 million mortalities annually. According to the World Health Organisation (WHO), there were 214 million new cases of malaria worldwide in 2015 (range 149–303 million) (WHO, 2015). 90% of all malaria deaths occur in sub-Saharan Africa, where a child under five years of age dies every 30 seconds (WHO, 2015). Despite numerous global efforts to control and manage malaria, this infectious disease remains a major health and socioeconomic problem in many low-income countries (Breman *et al.*, 2004). The development of resistance to conventional antimalarial treatment regimes, such as chloroquine (CHQ) and pyrimethamine-sulphadoxine has hampered global malaria control efforts. Therefore, novel approaches that might improve the efficacy of the already existing antimalarial drugs are needed. The WHO has recommended the use of artemisinin combination therapies (ACTs) for management of malaria (WHO, 2008). However, these have proven to be very costly and inaccessible in many low-income countries. Therefore, CHQ has remained the mainstay therapy for malaria prophylaxis and treatment.

The reasonable cost and rapid onset of action of CHQ has resulted in the continuous use of this drug despite the reported resistance. However, over the years CHQ use has decreased due to the reduction of the efficacy of this drug. The decrease in CHQ efficacy is attributed to a number of factors which are associated with the conventional oral route of administration. When administered orally, CHQ undergoes the first-pass metabolism by the liver, where the drug undergoes hepatic degradation thus decreasing its concentration. Consequently, higher initial drug concentrations are required to elicit therapeutic effects. Current research has shown that the high plasma CHQ concentrations following oral administration lead to accumulated deposition of CHQ in the heart, liver, kidney and spleen eliciting adverse effects and organ damage. Hypoglycaemia, cardiovascular disturbance, impaired renal fluid and electrolyte handling are some of the adverse effects associated with oral administration of CHQ. In our laboratory we have formulated oral amidated pectin-CHQ (PC) beads which protect the drug from gastrointestinal degradation and provides a sustained controlled release of CHQ into the bloodstream thereby avoiding administration of higher initial drug doses (Munjeri *et al.*, 1998). Furthermore, we have shown that transdermal delivery of CHQ by amidated pectin matrix patch ameliorates adverse effects associated with oral CHQ administration. Building on these studies, the current study compared the physiological and morphological effects following oral and transdermal

delivery of CHQ in various tissue organs. We envisaged that the sustained, controlled release of CHQ will minimise the deposition of CHQ in tissue organs and therefore avert the adverse effects observed with oral CHQ. Furthermore, malaria infection has been associated with the development of anaemia. However, the causes of anaemia in malaria patients on CHQ treatment are still unclear. Therefore, indicating that there is need to investigate and distinguish between the pathophysiological effects of malaria and CHQ treatment on anaemia. The other objective of the current study was to investigate the effects of CHQ treatment on haematological parameters and plasma cytokines concentrations in an effort to establish the possible mechanisms for the reported malaria associated anaemia.

1.1. Literature review

Malaria remains a disease of devastating global impact, clinical studies have shown that malaria infection cases reach a total of more than 300-800 million every year, the vast majority being children under the age of 5 (Rowe *et al.*, 2006)**.** The *Plasmodium* life cycle has been extensively studied in an effort to manage malaria. There are four species of *Plasmodium* that infect humans, the most deadly of these being *P. falciparum*. The parasite requires two hosts, a female *Anopheles* mosquito and a human. *Plasmodium* parasites are injected into the human blood stream through a bite of an infected female *Anopheles* mosquito (Figure 1) (Sidjanski and Vanderberg, 1997). The sporozoites that are released from the salivary glands of the *Anophele* mosquito quickly migrate to the hepatocytes of the liver. Within the hepatocytes some of these sporozoites remain dormant and some multiply and differentiate, resulting to the production of merozoites. The produced merozoites are then released into the circulatory system, where they infect red blood cells (RBCs) and undergo further multiplication and differentiation, a phenomenon referred to as trophic period, producing trophozoites. The enlargement of these trophozoites results into the production of schizonts. The shizonts that are produced from the erythrocytic stage of the life cycle either further infect more red blood cells or differentiate into sexual forms, macro- or microgametocyte. The gametocytes that are ingested by the mosquito undergo sexual replication and produce zygotes. These zygotes differentiate into ookinetes which undergo meiosis. Some of the ookinetes develop into oocysts after traversing the midgut epithelium. At the later stages of infection, when the oocyst ruptures, only a fraction of the released sporozoites end up in the salivary glands where the life cycle begins again (Figure 1). The erythrocytic stage of the *Plasmodium* life cycle is responsible for most malaria-associated pathologies including fever and anaemia (Haldar and Mohandas, 2009). However, it is unclear whether this anaemia is solely due to the parasite or by treatment with CHQ. There is therefore an imperative need to investigate the possible mechanism for the reported anaemia in malaria patients receiving oral CHQ treatment. The current study, will therefore, investigate and compare the effects of malaria infection and that of CHQ treatment on haematological parameters in an effort to establish the possible mechanism for the reported malaria-associated anaemia.

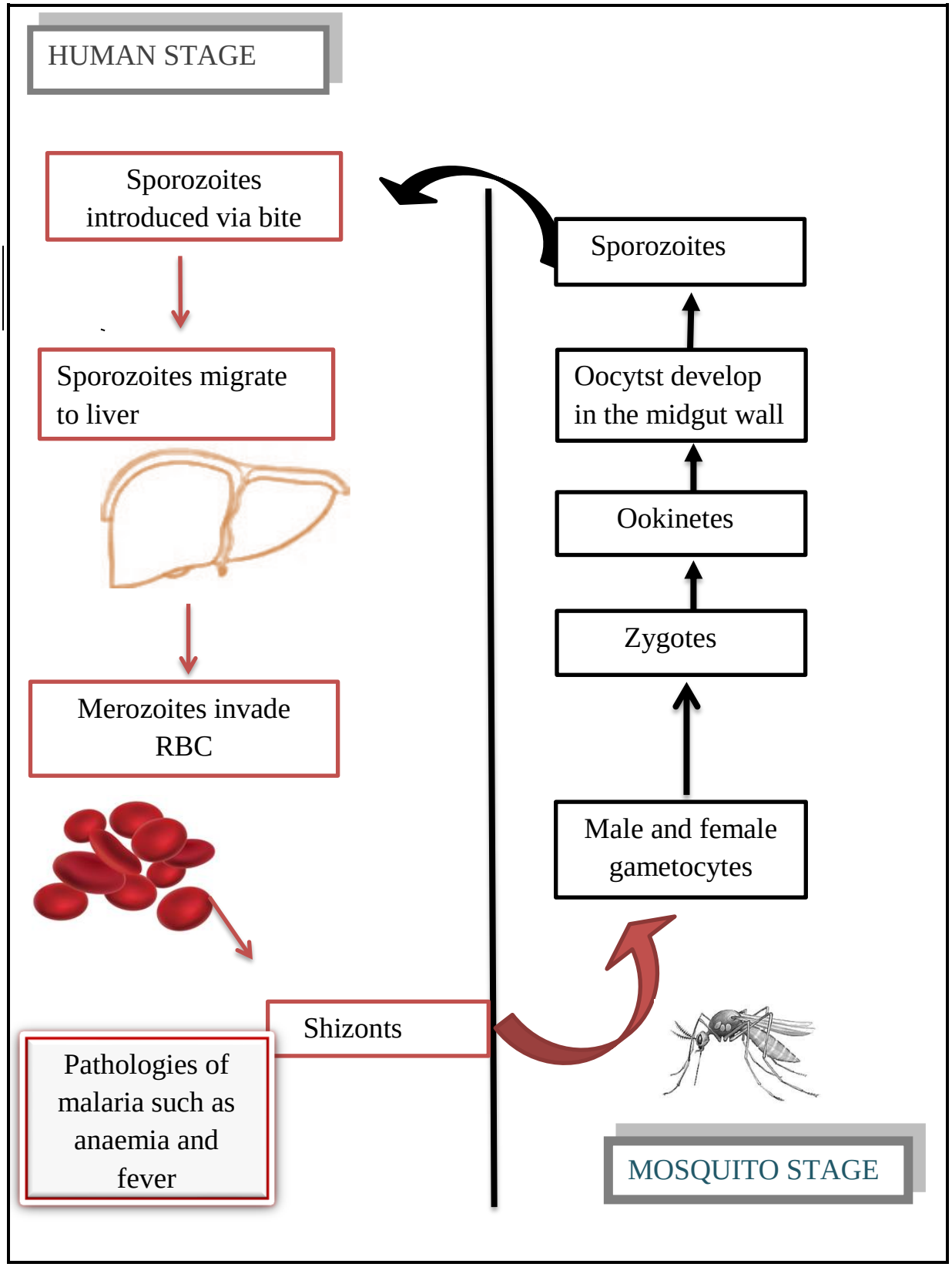

Figure 1: Basic illustration of the *Plasmodium* life cycle. The erythrocytic phase is responsible for clinical manifestations of malaria such as anaemia and fever.

1.2. Malaria complications

Hypoglycaemia, impairment of kidney function and anaemia are among an array of pathophysiological manifestations caused by malaria infection or treatment. This study however, investigated the effects of CHQ treatment on anaemia in *P. berghei*-infected animals. The following section discusses malaria anaemia in detail.

1.2.1. Anaemia

Malaria infection in humans by *Plasmodium* species is associated with a reduction in haemoglobin levels, frequently leading to anaemia (Ekvall, 2003). Anaemia is one of the most severe malaria complications which accounts for a significant number of malaria related mortalities (Chang and Stevenson, 2004). The pathogenesis of anaemia in malaria is multifactorial and incompletely understood. Current literature evidence has shown that anaemia may result from intravascular and extravascular destruction of parasitised and unparasitised erythrocytes, as well as decreased production of erythrocytes (Bjo¨rkman, 2002). Studies have also reported that cytokines are involved in the suppression of erythropoiesis (Faquin *et al.*, 1992). In addition, studies have demonstrated that high serum tumor necrosis factor-alpha (TNF-α) and IL-10 are associated with severe anaemia (Kurtzhals *et al.*, 1998). During malaria infection, there is a high rate of RBC destruction by the parasites without any parallel replacement by erythropoiesis (Phillips and Pasvol, 1992). This destruction is mediated through multiple mechanisms including the rupture of RBC, phagocytosis of parasitized and unparasitised RBCs and hypersplenism. Increased RBC destruction may result from rupture of parasitised red blood cells, which occurs in direct proportion to the percentage parasitaemia and schizontaemia (Bjo¨rkman, 2002). The proliferation and hyperactivity of macrophages within the reticuloendothelial system in response to the destruction of RBCs is responsible for the phagocytosis of both parasitised and unparasitised RBCs. This removal of unparasitised RBCs is believed to be one of the mechanisms resulting into persistent anaemia (Eric *et al.*, 2013). However, recent studies have reported the role of hepcidin in malarial anaemia (Pigeon *et al.*, 2001; Ganz, 2015).

1.2.1.1 Hepcidin

Hepcidin, a peptide hormone produced primarily by the liver, is a major regulator of iron metabolism and is thought to play a central role in the anaemia of chronic inflammation (Pigeon *et al.*, 2001). Hepcidin regulates iron metabolism by binding to the receptors of ferroportin, a transmembrane iron exporter (Nemeth *et al.*, 2004). Ferroportin exports iron into plasma from absorptive enterocytes, macrophages and hepatocytes. Hepcidin-mediated degradation of ferroportin results in decreased iron absorption (Ganz, 2005). Hepcidin therefore acts as an endocrine regulator of total body iron stores and plasma iron concentration (Ganz, 2003). The synthesis of hepcidin is regulated by iron and

erythropoiesis (Weinstein *et al.*, 2002). Hepcidin production is also increased by inflammation, primarily through IL-6 (Nemeth *et al.*, 2004). The presence of the malaria parasite triggers the host's innate immune system. Also, the release of toxins during the *Plasmodium* parasite's life cycle triggers the release of the pro-inflammatory cytokines, TNF-α, IL-6 and IL-22 (Howard *et al.*, 2007). Together with type I interferon, these cytokines stimulate hepcidin transcription through STAT3 signaling (Howard *et al.*, 2007). This hepcidin then blocks the release of iron from enterocytes, hepatocytes, and macrophages, leading to hypoferremia and limited iron availability for erythropoiesis (McDevitt *et al.*, 2004). Suppressed erythropoiesis contributes to malarial anaemia as new red cells are not produced to replace those lost by haemolysis and ingestion by macrophages. Some studies have suggested that CHQ, the drug of choice for this study contains a quionline ring that has been known to act as a hapten, and binds with specific RBC proteins to elicit an immune response leading to the destruction of red blood cells, thus aggravating anaemia (Clark and Cowden, 2003). Furthermore, current literature suggests that the weak base of CHQ accumulates in acidic organelles of the cell, where it blocks acidification (Moss *et al.*, 1992). This alteration in the pH of the endosomal vesicle prevents the release of iron from the transferrin–transferrin receptor complex, ultimately decreasing iron uptake. Although malaria infection has been associated with anaemia for a long time the details on anaemia during malaria infection and CHQ treatment are obscure. In summary, the exact cause of anaemia in CHQ treated malaria patients remains unclear. Therefore, there is need to investigate and distinguish between the pathophysiological effects of malaria alone and those of CHQ treatment. The current study will, therefore, investigate and compare the effects of malaria infection and that of treatment on haematological parameters and pro-inflammatory cytokines concentrations in an effort to establish whether this anaemia is solely due to the parasite or is exacerbated by CHQ treatment.

1.3 Cytokines

Cytokines are small regulatory proteins that are produced by white blood cells and a variety of other cells including those in the nervous system (Hedayat *et al.*, 2010). Previous studies have shown that innate host responses to malaria have been associated with control and clearance of infection (Richards, 1997). Research shows that elevated levels of pro-inflammatory cytokines play a role in the pathogenesis of malaria (Jacobs *et al.*, 1996). In addition current literature shows substantial evidence that severe malaria is correlated with high TNF-α plasma levels, increased production of interferon-GAMMA (IFN-γ) , interleukin-Beta (IL-1b), and decreased production of IL-10 (Lyke *et al.*, 2004). Previous studies have reported the role of pro-inflammatory cytokines in anaemia. TNF-α, IFN- γ, and IL-12 as well as other cytokines have been shown in vitro and in vivo to mediate anaemia (Wickramasinghe and Abdalla, 2000; Means, 2000). In vitro studies have suggested that TNF-α can inhibit the production of erythropoietin (Faquin *et al.*, 1992). Furthermore, several pro-inflammatory cytokines, including IL-1, and IL-6, produced during blood-stage malaria in humans and in mice

(Stevenson and Riley, 2004) inhibit kidney erythropoietin (EPO) production in vitro and in vivo (Jelkmann *et al.*, 1994; Braczkowski *et al.*, 2001). These results provide evidence that not only can cytokines play a role in the pathogenesis of malaria, but they are also involved in malaria anaemia. In this study, pro-inflammatory cytokines were measured to further elucidate the malaria associated anaemia following CHQ treatment.

1.3.1 TNF-α

TNF-α, is a pro-inflammatory cytokine with a wide variety of biological effects. TNF-α is produced by various cell types, including proximal tubules, and endothelial cells, but the primary source of this cytokine is monocytes/macrophages (Jang *et al.*, 2006). It binds two receptors, type 1 and type 2 receptors (Haddad, 2002). The binding of TNF to TNF-R1 triggers a series of intracellular events that ultimately result in the activation of two major transcription factors, nuclear factor κB (NF-κB) and c-Jun (Chen and Goeddel, 2002). These transcription factors are responsible for the inducible expression of genes important for diverse biological processes, including cell growth and death, oncogenesis, inflammatory, and stress responses (Jacobs *et al.*, 1996). Furthermore, TNF-α regulates the production of other cytokines (Aringer and Smolen, 2003). Wenisch *et al*., 1996 showed that *falciparum* malaria patients with renal failure had higher levels of TNF-α (Wenisch *et al.*, 1996) . Literature has shown that when TNF-α is expressed at sufficiently high concentrations, this cytokine produces left ventricular dysfunction, pulmonary oedema, and cardiomyopathy in humans, all of which are seen in patients with advanced heart failure (Seta *et al.*, 1996). In addition current literature shows substantial evidence of the role of this cytokine in the development of anaemia (Clark *et al.*, 2006). Studies have demonstrated that elevated TNF-α level contribute to bone marrow suppression (Clark *et al.*, 1981) and red blood cell destruction (Ulich *et al.*, 1990). TNF-α has also been shown to be capable of reducing the life span of red blood cells (Moldawer *et al.*, 1989), resulting in increased erythrophagocytosis (Taverne *et al.*, 1994). In view of the above effects of TNF-α in aneamia, the current study will measure this pro-inflammatory cytokine following CHQ administration to further assess the effect of CHQ treatment in aneamia.

1.3.2 IL-6

IL-6 belongs to a family of 20 kDa polypeptide cytokines that are secreted from a number of different cells, including fibroblasts, adipocytes, monocytes, and endothelial cells (Stenvinkel and Alvesrand, 2002). IL-6 plays an important role in inflammation and is believed to regulate the early innate defense towards acquired immunity (Yudkin *et al.*, 2000). IL-6 signalling is mediated by two pathways, either by binding to the membrane-bound IL-6 receptor (IL-6r) or by binding to its soluble form of the IL-6r (Mohamed-Ali *et al.*, 1998). Both pathways activate the signal transducing

glycoprotein gp130 followed by activation of gene transcription (Mohamed-Ali *et al.*, 1998). In many cases it mediates its effects in combination with IL-1β and TNF-α (Pang *et al.*, 1994). Depending on the immune response, IL-6 can act both as a pro-inflammatory and as an anti-inflammatory cytokine (Jürgen *et al.*, 2011). During the initiation of inflammation, IL-6 encourages production of acute phase proteins and activation of macrophages as well as recruiting immune cells such as lymphocytes to the site of infection or tissue injury (Jürgen *et al.*, 2011). In the late stage of infection, IL-6 takes a protective role and counteracts the manifestations of certain inflammatory responses by stimulating the development of adaptive immune responses and inducing apoptosis in neutrophils, thereby down-modulating production of reactive oxygen species (ROS) and other toxic compounds and aiding in the resolution of inflammation. Because of the many functions of IL-6 it has been implicated in various inflammatory diseases (Stenvinkel and Alvesrand, 2002). However, of interest to the current study is the effect of this cytokine in anaemia. Accordingly, the current study will measure this pro-inflammatory cytokine following CHQ treatment.

1.4 Effects of CHQ on the immune system

The accumulation of CHQ in lymphocytes and macrophages results in its anti-inflammatory properties, and has led to its clinical use in conditions such as rheumatoid arthritis, lupus erythematosus, and sarcoidosis (Baughman *et al.*, 2003). As mentioned above, pro-inflammatory cytokines are major mediators during infection (Richards, 1997) and elevated levels of these cytokines have been found in systemic shock, autoimmune diseases and chronic inflammatory diseases (Stenvinkel and Alvesrand, 2002) . CHQ, the drug of choice for the current study has been shown to reduce the secretion of pro-inflammatory cytokines (Van den Born *et al.*, 1997). Furthermore, studies have suggested that this inhibitory effect of CHQ on pro-inflammatory cytokine release may be induced by a mechanism related to its weak base properties. Another study reported that CHQ may inhibit TNF-α secretion by disrupting the normal iron metabolism (Picot *et al.*, 1993). Despite the above mentioned anti-inflammatory efforts of CHQ, for the purpose of the current study we investigated the effects of CHQ treatment on pro-inflammatory cytokines in an effort to establish whether malaria anaemia is solely due to the malaria parasite or is exacerbated by CHQ treatment.

1.5 Management of malaria

The use of antimalarial drug has resulted in a significant reduction in malaria-related morbidity and mortality rates (WHO, 2008). Current standard anti-malaria drugs include; artermisinin derivatives, antifolates and quinine related drugs which can be used as monotherapy or in combination. Multidrug resistance of the commonly used anti-malarial agents such as CHQ and sulphadoxine/pyrimethamine combination has been reported in most parts of the world (WHO, 2008). Thus, monotherapy or some of the available combination therapies for malaria are either ineffective or less effective. WHO

recommended the use of combination therapies with artemisinin derivatives (WHO, 2003). Anti-malarial drug combination therapies increase efficacy and shorten duration of treatment thereby increasing compliance (Ogbonna and Unekeb, 2008). The major general limitation of drug combination therapy, however, is the increased cost of the drug and therefore inaccessible in malaria endemic countries. CHQ, therefore, continues to be used in most parts of Africa despite the developed *Plasmodium* resistance. As such, the next section comprehensively discusses the structure, mode of action and delivery routes that are employed for this drug.

1.5.1 Chloroquine

CHQ, a diprotic compound derived from 4-aminoquinoline possesses both blood schizonticidal and gametocytocidal activities against *Plasmodium* parasites (Sullivan *et al.*, 1996). At physiologic pH, CHQ is monoprotonated soluble in lipids and able to traverse cell membranes of the parasitised erythrocyte (Kogstad and Schlesinger, 1986). However, once protonated, the drug becomes membrane impermeable and is trapped in the acidic compartment of the parasite. The *Plasmodium* parasite obtains essential amino acids through the degradation of haemoglobin (Francis *et al.*, 1997). As a result of haemoglobin degradation, a soluble toxic haematin is produced. Haematin is highly toxic to the malaria parasite and is normally detoxified by polymerization into innocuous crystals of haemozoin pigment (Zhang *et al.*, 1999). Studies have also shown that heme can directly attack and may impair intracellular targets including the lipid bilayer, the cytoskeleton, intermediary metabolic enzymes, and DNA (Wagener *et al.*, 2003). Furthermore, there are available reports indicating that high levels of free heme cause severe toxic effects to the kidney, liver, central nervous system and cardiac tissue. As an antimalarial, CHQ acts by inhibiting hemozoin biocrystallization, which gives rise to toxic free heme accumulation (Ginsburg *et al.*, 1998). The accumulation of these free haem moieties within the parasite's food vacuole results in RBC lysis, eventually killing the malaria parasite (Zhang *et al.*, 1999).

CHQ continues to be used as the mainstay treatment in malaria endemic areas, however, current studies have shown that orally delivered CHQ may elicit adverse effects on glucose homeostasis, renal and cardiovascular functions. Alternative methods of CHQ administration such as transdermal delivery have, therefore, been suggested in an effort not only to avoid the bitter taste, but also to modify the dosing schedule, which may improve patient comfort and compliance (Musabayane *et al.*, 2003). We speculate that the use of pectin CHQ matrix patches may eliminate the transiently high initial systemic drug concentrations associated with oral CHQ administration. Furthermore, the sustained controlled slow release of the CHQ from the matrix patch may circumvent drug deposition of CHQ in tissues and hence will minimise the damage caused by oral CHQ in various tissue organs. It is against this background that the current study was designed to compare the morphological effects of oral and transdermal delivery of CHQ on selected tissue organs. Measurement of inflammatory

biomarkers involved in tissue damage could give more information pertaining to the possible adverse effects of oral CHQ treatment on various tissue organs.

1.6 Inflammation and tissue damage

Inflammation is a crucial component of the body's immune system and the first line of defense that takes place during tissue injury (Fujiwara and Kobayashi, 2005). Immune cells such as macrophages, natural killer (NK) cells, neutrophils, mast cells, and dendritic cells (DCs), sense the presence of pathogens and respond by secreting a variety of soluble mediators such as histamine, prostaglandins and cytokines (Alexander, 1994). These pro-inflammatory mediators recruit and activate immune cells to destroy pathogens using various effector mechanisms, such as, phagocytic clearance, production of reactive oxygen and nitrogen species, and secretion of anti-microbial peptides. The whole purpose of the process is to remove the inducing stimulus and to start local tissue recovery. Once the pathogen is cleared or when the tissue has been repaired, the signal that triggered the inflammation needs to be inhibited in order to sustain normal homeostasis. A failure to regulate this suppression may have detrimental consequences for the host, leading to chronic inflammation and in the end severe tissue damage (Fujiwara and Kobayashi, 2005). The next section will discuss the inflammatory marker involved in tissue damage and that will be investigated in this study.

1.6.1 C-reactive protein

C-reactive protein (CRP) is an acute-phase protein that has been used as a marker of acute and chronic inflammation (Pepys and Baltz, 1983). Literature reports that this protein is normally present in trace levels in serum but increases rapidly and dramatically in response to pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α (Gabay and Kushner, 1999). In addition current literature shows substantial evidence that during infectious or inflammatory disease states, CRP levels rise rapidly and stay elevated until the resolution of inflammation (Kolb-Bachofen, 1991). Following activation, CRP undergoes calcium-dependent binding to choline phosphatides expressed on the surface of damaged cells (Ballou and Kushner, 1992). This binding activates the classical complement cascade of the immune system (Nanri *et al.*, 2007). When the inflammation or tissue destruction is resolved, CRP levels decline, making it a useful marker for monitoring disease activity (Jaye and Waites, 1997). Studies have reported that elevated levels of CRP are associated with many pathological manifestations states such as, rheumatoid arthritis (Young *et al.*, 1991), tissue trauma, viral or bacterial infection, hepatitis, and some autoimmune conditions. The ability of CHQ to bind and accumulate in various tissues such as the heart following oral administration is thought to be one of the main factors responsible for the reported CHQ-induced cardiovascular disturbances. Based on the above mentioned mechanisms of CRP, the current study will investigate CRP concentrations following oral and transdermal delivery of CHQ to assess the effect of treatment in tissue damage.

The conventional method for the administration of CHQ has been orally. In the past years this method of drug delivery has been successful in eradicating malaria. However, oral administration of CHQ is associated with a number of disadvantages that have been previously described. Therefore, there is a need of novel CHQ delivery routes, which will enhance the therapeutic efficiency of CHQ, eliminate the inconvenient dosing schedule that is associated with oral administration and mask bitterness of CHQ. This new delivery method should also try and minimize the toxicity of the drug observed in oral administration, by reducing the amount of deposition of the drug in various tissues. A method that has previously been described by Musabayane *et al*., 2003 is the use of amidated pectin hydrogel beads of CHQ which provided a successful method of CHQ delivery as they mask the bitter taste and allowed sustained controlled release of the drug (Musabayane *et al.*, 2003). This method proved successful. As such, this study will be focusing on transdermal delivery as an alternative delivery system of CHQ.

1.7 Transdermal delivery

Transdermal delivery is the delivery of drugs through the skin, using a patch or an ointment. Transdermal drug delivery in general has made an important contribution to medical practice. In our laboratory we have previously shown that transdermal application of pectin hydrogel insulin matrix patches of different insulin concentrations sustain controlled release of insulin into the bloodstream of streptozotocin (STZ)-induced diabetic rats and alleviate a variety of diabetic symptoms (Hadebe *et al.*, 2014). Furthermore, this delivery system allows the drug to escape gastrointestinal degradation as well as the first-pass metabolism by the liver and therefore lower initial drug concentrations are required to bring about therapeutic effects. We have previously shown that oral administration of amidated CHQ beads formulation is able to sustain controlled CHQ release into the blood stream (Munjeri *et al.*, 1998). Building off these previous studies, the current study will compare the morphological effects of CHQ treatment following oral and transdermal delivery in various organs on *P. berghei* infected rats.

1.8 Problem statement and significance

The use of orally administered CHQ remains the mainstay therapy in some parts of Africa despite the developed *Plasmodium* resistance. Evidence in the preceding paragraphs indicates a need for alternative, methods of CHQ administration. The focus of the study was therefore to determine whether transdermal delivery of CHQ in comparison with the conventional delivery methods, that is, oral delivery will minimise the deposition of CHQ in tissue organs and therefore avert the adverse effects of oral CHQ. The current study investigated the morphological effects of transdermal CHQ patch and oral CHQ delivery in the heart, kidney, spleen, and the liver of *plasmodium berghei*-

infected male Sprague-Dawley rats**.** The study also aimed to investigate inflammatory marker such as C-reactive protein; to give more information, pertaining to the possible adverse effects of CHQ treatment on the various organ systems.

Malaria infection has been associated with anaemia for a long time (Haldar and Mohandas, 2009). However, the causes of anaemia associated with malaria patients on CHQ treatment are still unclear, indicating that there is need to investigate and distinguish between the pathophysiological effects of malaria and CHQ treatment on anaemia. Furthermore, severe anaemia in malaria is usually associated with low levels of IL-10 and IL-12 (Clark *et al.*, 2006), and it is believed that these cytokines may have some therapeutic application in severe malarial anaemia. The current study therefore, investigated the effects of CHQ treatment on haematological parameters and plasma cytokine concentrations in an effort to establish the possible mechanisms for the reported malaria associated anaemia.

1.9 Aims/ Objectives of the study

- The objective of this study was to investigate the morphological effects of CHQ treatment in various organs such as the heart, spleen, kidney and liver in *Plasmodium berghei*-infected male Sprague-Dawley rats. Additionally we evaluated the expression of inflammatory markers, following oral and transdermal delivery of CHQ.

- The other objective of the study was to monitor haematological parameters and plasma pro-inflammatory cytokines following CHQ treatment.

2.0 Experimental design

The physiological and morphological effects following oral and transdermal delivery of CHQ in various tissue organs were assessed in separate groups of non-infected and *P.berghei*-infected male Sprague-Dawley rats (n=6 in each group) over a period of 21 days. The experimental design is summarised in (Figure 2) below.

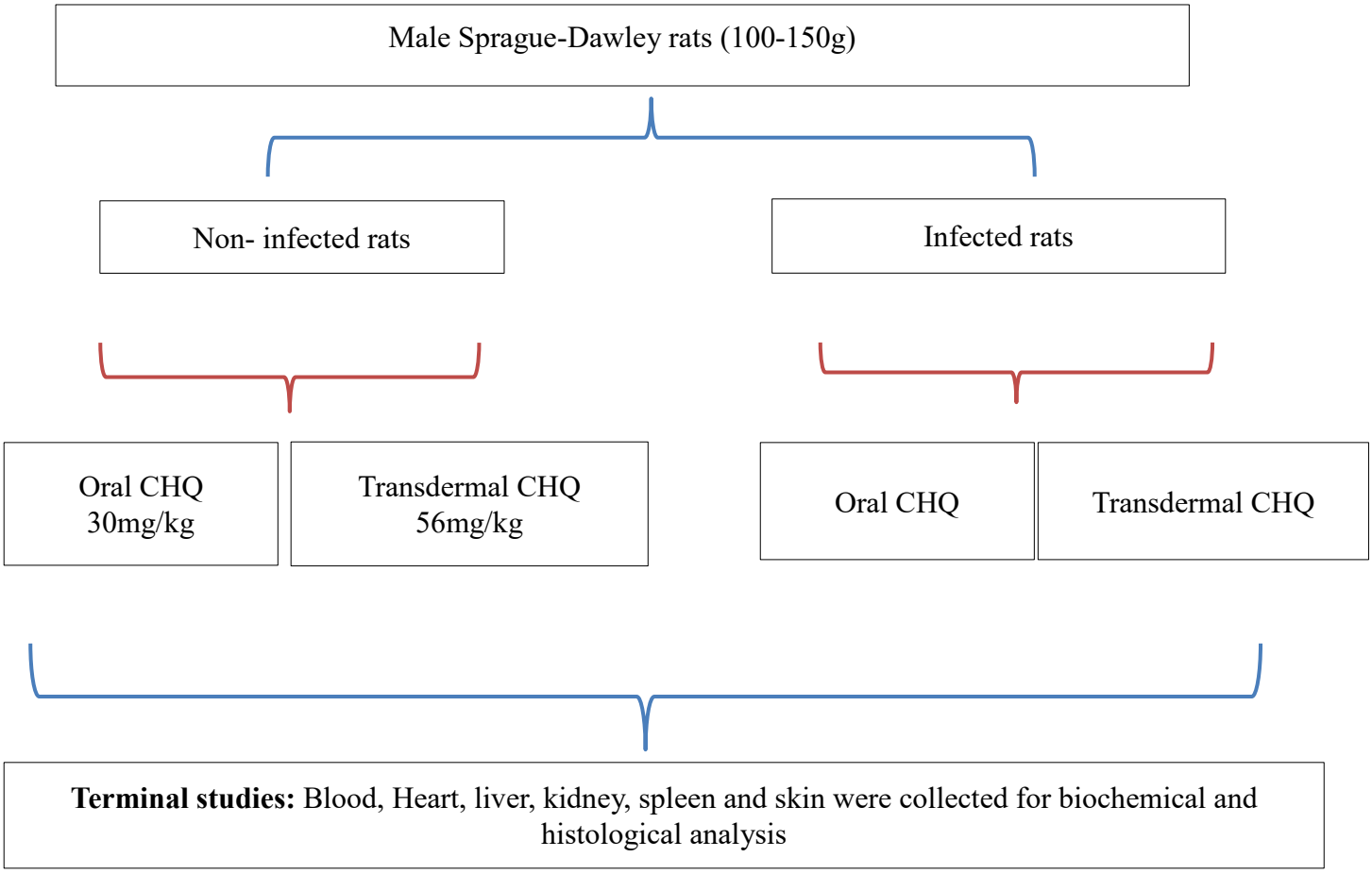

Figure 2: Flow diagram showing the summary of the experimental design. The animals were divided into separate groups malaria infected and non-infected rats (90-120g body weight). The CHQ-treated animals were further be divided to those treated with oral CHQ and those treated with topically applied CHQ in a form of a patch.

2.1 Data analysis

All data were expressed as mean±standard error of the means (SEM). GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA) was used for all statistical analyses. One way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test was used to establish statistical comparison between various groups. Values of $p < 0.05$ were taken to imply statistical significance

This manuscript will be submitted to Malaria Journal. The manuscript has been written according to the Journal guidelines

Oral and transdermal administration effects of chloroquine on selected haematological parameters & pro-inflammatory cytokine concentration in malaria infected rats

Gumede, NM., and Mabandla, MV

School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of Kwa-Zulu-Natal, Private BagX54001, University of Kwa-Zulu-Natal, South Africa.

Correspondence: Dr MV Mabandla

Discipline of Human Physiology

School of Medical Sciences

University of Kwa-Zulu-Natal

Private BagX54001

Durban 4000

South Africa

Phone: 0312608602

Fax: 0312607123

E-mail: mabandlam@ukzn.ac.za

1.0 Abstract

Malaria infection is associated with the development of anaemia. However, the causes of anaemia in malaria patients treated with chloroquine (CHQ) are still unclear. In this study, we evaluated haematological parameters in an effort to establish the mechanism by which malaria associated anaemia occurs. We also investigated the effect of CHQ treatment on plasma cytokines in *P.berghei*-infected animals. Haematological parameters and pro-inflammatory cytokines were monitored in non-infected and infected rats following oral administration of CHQ (30 mg/kg) or topical application of the pectin CHQ matrix patch (56 mg/kg) over a period of 21days. Oral CHQ treatment reduced red blood cell count, haematocrit, haemoglobin and mean corpuscular haemoglobin in non-infected and infected animals. Topical application increased the above parameters in infected rats. Oral CHQ decreased pro-inflammatory cytokine concentration in infected rats on the day (day 8 of the experiment) of the treatment period in comparison to pre-treatment (baseline) measurements. However, on the last day (day 12) of the treatment period and during the post-treatment period there was an increase in pro-inflammatory cytokine concentration with oral treatment while patch application decreased pro-inflammatory cytokine concentration in infected rats throughout the experimental period. We conclude that malaria infection as well as oral CHQ treatment result in the development of anaemia, an effect not present following topical treatment. Oral treatment exacerbated the inflammatory response to infection while topical application of the patch ameliorated this response. The current study therefore demonstrated the feasibility of the use of the pectin CHQ matrix patch in the management of malaria.

Key words: Malaria, chloroquine, aneamia, haemoglobin, pro-inflammatory cytokines, lymphocytes

1.1 Introduction

Malaria is a major global health problem, with an estimated 300 to 500 million clinical cases occurring annually and 1.5 to 2.7 million reported deaths, predominantly in children living in Sub-Saharan Africa (WHO, 2015). Iron deficiency is a common pathophysiological manifestation caused by malaria infection (Dreyfuss *et al.*, 2000). In malaria, the destruction of parasitised and normal erythrocytes, as well as ineffective erythropoiesis contributes almost equally to anaemia (Bjo¨rkman, 2002). However, recent data suggests that these mechanisms alone or in combination do not adequately explain the anaemia associated with malaria infection (Ganz, 2015). Hepcidin is a peptide hormone that regulates iron metabolism and is thought to play a central role in the anaemia associated with chronic inflammation (Drakesmith and Prentice, 2012). During malaria infection, the presence of malaria parasite triggers the activation of the host's innate immune system to release pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1 (IL-1) and interleukin-22, which stimulate hepcidin release (Howard *et al.*, 2007). It has been suggested that the quinolone ring of Chloroquine (CHQ) and other quinine related drugs bind with specific red blood cell (RBC) proteins to elicit an immune response leading to the destruction of these RBCs, thus enhancing the anaemia condition (Clark and Cowden, 2003). Furthermore, it has also been shown that CHQ accumulates in the acidic organelles of the cell where it blocks acidification (Moss *et al.*, 1992). This alteration in the pH of the endosomal vesicle prevents the release of iron from the transferrin–transferrin receptor complex, ultimately decreasing iron uptake via this pathway (Moss *et al.*, 1992). In our laboratory we have reported that oral CHQ treatment reduced the haematocrit of both malaria infected and non-infected animals however, topical application of the pectin CHQ matrix patch increased haematocrit of *P.berghei*-infected rats but did not alter the haematocrit of non-infected animals (Sibiya, 2013). This study however did not elucidate the mechanisms by which the haematocrit was reduced in the oral CHQ treated animals, hence the aim of the current study was to investigate whether oral or transdermal treatment with CHQ attenuates changes in haematological parameters and pro-inflammatory cytokine concentration in *P.berghei*-infected rats.

2.0 Materials and methods

2.1 Drugs and chemicals

Chloroquine diphosphate (CHQ) was sourced from Sigma-Aldrich Chemical Company, St Louis, Missouri, United States of America. All other chemicals which were of analytical grade quality were purchased from standard commercial suppliers.

2.2 Animals

40 male Sprague-Dawley rats (90-120 g) bred and housed in the Biomedical Resource Unit of the University of KwaZulu-Natal Westville campus were used in the study. The animals were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), CO_2 content of <5000 p.p.m., relative humidity of 55 ± 5 %, and illumination (12 h light/dark cycle) and noise levels of <65 decibels. The animals had free access to standard rat chow (Meadows feeds, Pietermaritzburg, South Africa) and water. All animal experimentation was reviewed and approved by the University of KwaZulu-Natal's Animal Ethics Research Committee (References 034/14 Animal and 034/15 Animal).

2.3. Experimental design

Short-term effects of oral CHQ treatment and a transdermally applied pectin-formulation on red blood cell (RBC) count, haematocrit (HCT), haemoglobin (HGB), red cell distribution width (RDW), mean Platelet volume (MPV), mean corpuscular haemoglobin (MCH), white blood cell (WBC) count, neutrophils (NE), lymphocytes (LY) monocytes (MO) and plasma pro-inflammatory cytokine (TNF-α, IL-1β and IL-6) concentration were monitored in non-infected and *P. berghei*-infected male Sprague-Dawley rats (n= 6 in each group). Studies were carried out over a 3 week period divided into pre-treatment (days 0-7), treatment (days 8-12) and post treatment (days 13-21) periods. During the 5 day treatment period (days 8-12), non-infected and *P. berghei*-infected animals either received an oral dose of CHQ (30 mg/kg, p.o.) by means of a ball-tipped18-gauge gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa) attached to a 1mL syringe twice (Naumann *et al.*, 2009) daily at 9h00 and 17h00 or topical application of the pectin CHQ matrix patch (56 mg/kg). The animals were individually housed in Makrolon polycarbonate metabolic cages (Techniplasts, Labotec, South Africa) at the Biomedical Resource Unit, University of KwaZulu-Natal.

2.4 Malaria infection

Malaria was induced in male Sprague-Dawley rats (90-120g) by a single intra-peritoneal injection of *P.berghei* (10^5 parasitised RBC) (Gumede *et al.*, 2003). The *P berghei* parasite was supplied by Professor Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). Successful malaria induction was confirmed by microscopic examination of Giemsa stained thin smears of the tail blood. Percentage parasitaemia ranging from 15-20% was considered as a stable malaria state before commencing any experimental procedures.

2.5 Chloroquine administration

During the 5 day treatment period (days 8-12) non-infected and *P. berghei*-infected animals were given CHQ (30 mg/kg, p.o.) orally by means of a ball-tipped 18-gauge gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa) attached to a 1 mL syringe twice daily at 9h00 and 17h00 or a pectin CHQ matrix patch. For transdermal application of the pectin-CHQ matrix patch, 56 mg/kg CHQ was administered once for the duration of treatment period. On the first day of treatment, the pectin CHQ matrix patches were applied at 9h00. Prior to treatment, the rats were shaved on their dorsal region for the application of the pectin CHQ matrix patch. The patch was secured in place with adhesive hydrofilm (BSN Medical, Pinetown, South Africa).

2.6 Terminal studies

A separate group of non-infected and infected rats treated with CHQ (30 mg/kg, p.o.) or the pectin-CHQ patch (56 mg/kg) (n=6 per group) were sacrificed by cardiac puncture during the pre-treatment (day 7), treatment (days 8 and 12) and the post-treatment period (day 21) for measurements of haematological parameters and pro-inflammatory cytokines. On the days the assessments were made, the animals were anaesthetized in an anaesthetic chamber with 100 mg/kg of isofor inhalation anaesthetic for 3 min. Blood samples were immediately used to measure RBCs, HCT, HGB, WBC count, NE, LY, MO, MCH, RDW, and MPV.

2.7 Measurement of blood parameters

Blood was collected by cardiac puncture into pre cooled heparinised tube and placed on ice. The blood samples were then immediately analysed for RBCs, HCT, HGB, WBC count, NE, LY, MO, MCH, RDW, and MPV using an automated cell counter (Coulter Electronics, Luton, Bedfordshire, UK) with standard calibration according to the manufacturer's instruction.

2.8 Cytokine assay

Cytokine analysis was done using the Bio-Plex Pro cytokine assay. This assay enables the quantification of multiple protein biomarkers in a single well of a 96-well plate. The kit consisted of a 96 well plate, premixed coupled magnetic beads, detection antibodies, standards, assay buffer, wash buffer, detection antibody diluent, streptavidin-PE, standard diluent and sample diluent. Bio-Plex Pro assays are essentially immunoassays formatted on magnetic beads. The assay principle is similar to that of a sandwich ELISA. Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Data from the reactions are acquired using a Bio-Plex software presents data as median fluorescence intensity (MFI) as well as concentration (pg/ml). The concentration of analyte bound to each bead is proportional to the MFI of reporter signal.

The assay procedure was as follows, each well was prewet using 100 µL of Bio-Plex assay buffer thereafter, the buffer was removed by vacuum filtration. This was followed by adding (50 µL) of diluted couple beads into each well. The plate was washed 2 times with (100 µL) of the wash buffer. This was followed by adding (50 µL) of the standard, samples and blank into each well. The plate was covered with sealing tape and protected from light with aluminium foil and incubated on a shaker at 850 ± 50rpm for 1 hour. With 10 minutes left in the incubation 20x detection antibodies were vortexed from 50 seconds. The dilution antibodies were prepared by adding (2.850 µL) of detection antibodies diluent to (150 µL) detection antibodies. After the incubation, the plate was washed three times with a (100 µL) wash buffer. This was followed by adding (25 µL) of detection antibodies in each well. The plate was then incubated on a shaker for 30 minutes at room temperature. With 10 min left a 100x SA-PE was prepared by adding (5.940 µL) of assay buffer and 60ul of 100x SA-PE. Following incubation, the wash process as previously described was repeated 3 times. SA-PE (50ul) was then added in each well and incubated for 10 minutes. Following incubation the plate was washed 3 times with (100 µL) wash buffer. To resuspend the beads for plate reading, (125 µL) of assay buffer was added each well. The plate was resealed and shaked at 850±50rpm for 30 seconds. Afterwards the plate was ready for reading in Bio-Plex manager software (version 6.0).

2.9 Data analysis

GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA) was used for all statistical analyses. One way analysis of variance (ANOVA) followed by Tukey-Kramer multiple

comparison post hoc test was used to establish statistical comparison between various groups. All data were expressed as means ± standard error of means (SEM). Values of $p < 0.05$ were taken to imply statistical significance.

3.0 RESULTS

3.1 Effects of CHQ treatment on Red blood cells, haematocrit and haemoglobin

The effects of oral CHQ (30 mg/kg) and a once off topical application of the pectin-CHQ patch (56 mg/kg) treatment on non-infected and *P.berghei*-infected rats were assessed for factors that may be responsible for the development of anaemia. Table 1 shows the RBC, HCT and HGB, concentrations of non-infected and *P.berghei*-infected rats throughout the 21 day experimental period.

A decrease in RBC, HCT and HGB was observed in *P.berghei*-infected control (IC) rats when compared to non-infected control (NIC) animals *(NIC vs IC, $P<0.05$, Table 1). Oral CHQ administration decreased RBC, HCT and HGB in non-infected animals when compared to pre-treatment control (NIC) animals $^{\#}$ (NIC vs NIO, $p<0.05$, Table 1). *P.berghei*-infected rats treated with oral CHQ decreased RBC, HCT and HGB compared to non-infected animals $^{@}$(NIO vs IO $p<0.05$, Table 1). Topical application of the pectin-CHQ patch had no effect on the above parameters in non-infected animals (NIP) when with pre-treated control animals (NIC). However, a significant increase in RBC, HCT and HGB values was observed in *P.berghei*-infected animals following treatment with the pectin-CHQ matrix patch compared to infected control animals $^{\alpha}$ (IC vs IP, $p<0.05$, Table 1).

Table1: Comparison of the effect of twice daily oral CHQ (30 mg) treatment or a once off topical application of the pectin CHQ matrix patch (56 mg), on red blood cell (RBC), haematocrit (HCT) and haemoglobin (HGB) in non-infected and *P.berghei*-infected animals. Values are presented as means ± SEM, where columns represent means (n=6 in each group).

Treatment			**Time(days)**	**RBC($10^{6/}$ul)**	**HCT(%)**	**HGB(g/dl)**
Oral	**Non-infected**	Pre-treatment (**NIC)**	7	7.90 ± 0.2	44.00 ± 1.8	14.5 ± 0.3
		Treatment **(NIO)**	8	7.30 ± 0.2$^{\#}$	39.30 ± 1.7$^{\#}$	14.1 ± 0.5$^{\#}$
			12	7.28 ± 0.1	38.32 ± 0.7	13.59 ± 0.1
		Post-treatment	21	7.49 ± 0.7	37.69 ± 0.3	10.90 ± 1.2
	***P.berghei*-infected**	Pre-treatment **(IC)**	7	5.99 ±0.3*	36.60 ± 0.9*	10.03 ± 0.6*
		Treatment **(IO)**	8	5.63 ± 0.1$^{@}$	37.20 ± 0.7$^{@}$	9.70 ± 0.4$^{@}$
			12	5.77 ± 0.4$^{@}$	36.08 ± 0.6$^{@}$	8.55 ± 0.3@
		Post-treatment	21	5.23± 0.4	34.67 ± 0.4	9.50 ± 0.5
Patch	**Non-infected**	Pre-treatment (**NIP)**	7	7.85 ± 0.3	40.60 ±0.9	15.02 ± 0.3
		Treatment	8	7.18 ± 0.1	37.90 ± 0.7	12.96 ± 0.2
			12	7.88 ±0.1	38.65 ± 0.6	12.59 ± 0.5
		Post-treatment	21	7.87 ± 0.1	45.50 ± 0.6	11.61 ± 0.2
	***P.berghei*-infected**	Pre-treatment **(IC)**	7	4.94 ± 0.45	38.01 ± 0.1	9.42 ± 0.2
		Treatment **(IP)**	8	5.46 ± 0.4 $^{\alpha}$	44.8 ± 0.5 $^{\alpha}$	14.57±0.2 $^{\alpha}$
			12	5.58 ± 0.3 $^{\alpha}$	42.63 ± 0.9 $^{\alpha}$	14.43±0.3 $^{\alpha}$
		Post-treatment	21	5.57 ± 0.4	43.45 ±0.6	15.46±0.5

* $p<0.05$ compared to non-infected animals (NIC)

$^{\#}$ $p<0.05$ compared to NIC

$^{@}$ $p<0.05$ compared to non-infected orally treated animal (NIO)

$^{\alpha}$ $P<0.05$ compared to infected control animals (IC)

3.2 RDW, MPV and MCH

The effects of CHQ treatment on RDW, MCH and MPV were measured on samples collected from non-infected and *P.berghei*-infected rats treated with either oral CHQ or the pectin-CHQ matrix patch. RDW, MCH and MPV were measured from samples collected during pre-treatment period (day 7), treatment period (days 8 and 12) and post-treatment period (day 21).

Table 2 represents RDW, MCH and MPV of both non-infected and *P.berghei*-infected groups treated with either oral CHQ or pectin-CHQ patch. RDW, MCH and MPV of untreated *P.berghei*-infected control (IC) rats decreased by comparison to non-infected control (NIC) *(NIC vs IC, $P<0.05$, Table 3). Oral CHQ administration decreased RDW, MCH and MPV in non-infected animals when compared to pre-treatment control (NIC) animals $^{\#}$ (NIC vs NIO, $p<0.05$, Table 2). Administration of oral CHQ in *P.berghei*-infected animals decreased RDW, MCH and MPV during the treatment period by comparison with non-infected rats $^{@}$(NIO vs IO, $P<0.05$, Table 2). A once-off topical application of the pectin-CHQ patch had no effect on RDW, MCH and MPV in the non-infected group (NIP) by comparison with the pre-treatment control values (NIC). However, once-off topical application of the pectin-CHQ patch increased RDW MCH, and MPV of *P. berghei*-infected rats by comparison to infected control animals $^{\alpha}$ (IC vs IP, $P<0.05$, Table 2).

Table 2: Comparison of the effect of twice daily oral CHQ (30 mg) treatment with a once off topical application of the pectin CHQ matrix patch (56 mg), on red cell distribution width (RDW), Mean Platelet Volume (MPV), Mean Corpuscular haemoglobin (MCH) in non- infected and *P.berghei*-infected animals.

Treatment			**Time (days)**	**RDW(%)**	**MPV(FL)**	**MCH(pg)**
Oral	**Non-infected**	Pre-treatment **(NIC)**	7	15.44 ± 0.2	6.90 ± 0.2	18.35 ± 0.3
		Treatment **(NIO)**	8	15.20 ± 0.4	6.85 ± 0.3	19.30 ± 0.1
			12	13.41 ± 0.1	7.06 ± 0.2	18.66 ± 0.3
		Post-treatment	21	14.52 ± 0.4	6.53 ± 0.3	19.85 ± 0.9
	***P.berghei*-infected**	Pre-treatment **(IC)**	7	13.2 ± 0.2*****	6.23 ± 0.1*****	16.74 ± 0.3*****
		Treatment **(IO)**	8	13.4 ± 0.6**@**	5.23 ± 1.1@	17.22 ± 0.7@
			12	11.4 ± 1.2@	5.75± 0.4@	16.46 ± 1.2@
		Post-treatment	21	14.8 ± 1.55	6.60 ± 0.2	18.81 ± 0.8
Patch	**Non-infected**	Pre-treatment **(NIP)**	7	16.1 ± 0.2	7.51 ± 0.3	19.13 ± 0.2
		Treatment	8	15.56 ± 0.1	6.10 ± 0.5	18.05 ± 0.2
			12	15.60 ± 0.3	6.60 ± 0.1	15.97 ± 0.3
		Post-treatment	21	14.48 ± 0.4	7.10 ± 0.8	14.75 ± 0.4
	***P.berghei*-infected**	Pre-treatment **(IC)**	7	14.61 ± 0.3	5.65 ± 0.4	19.06 ± 0.5
		Treatment **(IP)**	8	17.00±0.4 α	8.87±0.1 α	26.68 ± 0.4 α
			12	17.81±0.1 α	8.25±0.1 α	25.86 ± 0.4 α
		Post-treatment	21	15.40±0.1	7.40 ± 0.1	27.75 ± 0.4

* $p<0.05$ compared to non-infected animals (NIC)

@ $p<0.05$ compared to non-infected orally treated animal (NIO)

α $P<0.05$ compared to infected control animals (IC)

3.3. The effects of CHQ treatment on leukocyte

To evaluate the effects of oral CHQ and pectin-CHQ matrix patch treatment on leukocyte concentration. WBC (white blood cells), neutrophils (NE), lymphocytes(LY) and monocytes (MO) were measured on samples collected from non-infected and *P. berghei*-infected rats treated with either oral CHQ or the pectin-CHQ matrix patch. The samples were collected during pre-treatment (day 7), treatment (day 8 and 12) period and post-treatment (day 21) period.

P. berghei-infected control rats (IC) exhibited a significant ($p<0.05$) increase in WBC count, LY, NE and MO by comparison with the non-infected control (NIC) animals *(NIC vs IO, $p<0.05$, Table 3). No effects were observed in the WBC count, LY, NE, and MO counts following administration of CHQ in non-infected groups by comparison with pre-treatment control animals (NIC). Oral CHQ treatment of *P.berghei*-infected animals increased plasma WBC, LY, and NE during the treatment period and post-treatment period by comparison to non-infected animals $^{@}$ (NIO vs IO, $p<0.05$, Table 3). Topical application of the pectin-CHQ matrix patch had no effect on the above parameters in the non-infected animals in comparison to non-infected control animals. The Topical application of the pectin-CHQ matrix decreased WBC count, LY, NE, and MO of *P.berghei*–infected animals in comparison to the infected control animals(IC) $^{\alpha}$(IC vs IP, $p<0.05$, Table 3).

Table 3: Comparison of the effects of twice daily oral CHQ (30 mg) treatment with topically application of the pectin CHQ matrix patch (56 mg) on WBC (white blood cells), neutrophils (NE), lymphocytes(LY) and monocytes (MO) in non-infected and *P. berghei*-infected animals.

Treatment			**Time (days)**	**WBC($10^{3/}$ul)**	**LY($10^{3/}$ul)**	**MO($10^{3/}$ul)**	**NE($10^{3/}$ul)**
Oral	**Non-infected**	Pre-treatment **(NIC)**	**7**	6.02 ± 0.5	1.23 ± 0.2	0.15 ± 0.3	5.23 ± 0.3
		Treatment **(NIO)**	**8**	4.03 ± 0.5	1.25 ± 0.4	0.14 ± 0.1	5.33 ± 0.1
			12	4.83 ± 1.2	2.05 ± 0.7	0.16 ± 0.1	5.40 ± 0.2
		Post-treatment	**21**	6.83 ± 0.4	2.90 ± 0.1	0.19 ±0.1	6.10 ± 0.9
	***P.berghei*-infected**	Pre-treatment **(IC)**	**7**	11.36 ± 0.6*	6.23 ±0.5*	2.23 ± 0.4*	9.23±0.63*
		Treatment **(IO)**	**8**	8.93 ± 0.2$^{@}$	4.84±0.1$^{@}$	0.13 ± 0.1	7.71±0.2$^{@}$
			12	6.20 ± 0.5$^{@}$	3.56±0.2$^{@}$	0.15 ± 0.1	7.41±0.1$^{@}$
		Post-treatment	**21**	8.50 ± 1.0$^{@}$	4.78±0.6$^{@}$	0.17 ± 0.3	8.14±0.1$^{@}$
Patch	**Non-infected**	Pre-treatment **(NIC)**	**7**	6.13 ± 0.5	1.25 ± 0.3	0.19 ± 0.3	5.33 ± 0.4
		Treatment	**8**	5.16 ± 0.6	1.21 ± 0.2	0.13 ± 0.2	5.29 ± 0.1
			12	5.13 ± 0.2	2.22 ± 0.3	0.15 ± 0.1	5.62 ± 0.1
		Post-treatment	**21**	5.60 ± 1.8	2.97 ± 1.2	0.18 ± 0.2	4.98 ± 0.3
	***P.berghei*-infected**	Pre-treatment **(IC)**	**7**	10.45 ± 0.5	2.32±0.5	2.87 ±0.5	9.53±0.6
		Treatment **(IP)**	**8**	5.25 ± 0.2$^{\alpha}$	1.65±0.5$^{\alpha}$	0.39 ± 0.3	5.52 ± 0.3$^{\alpha}$
			12	4.83 ± 0.3$^{\alpha}$	1.79 ±0.2$^{\alpha}$	0.25 ± 0.5	5.22 ± 0.5$^{\alpha}$
		Post-treatment	**21**	4.11 ± 0.4	2.62 ± 0.1	0.15 ± 0.5	4.35 ± 0.2

* $p<0.05$ compared to non-infected animals (NIC)

$^{@}$ $p<0.05$ compared to non-infected orally treated animal (NIO)

$^{\alpha}$ $P<0.05$ compared to infected control animals (IC)

3.4 Effect of CHQ treatment on plasma cytokines

To further elucidate the possible mechanisms associated with aneamia, the current study measured plasma pro-inflammatory cytokine concentration in non-infected and infected rats. Plasma samples were collected during pre-treatment (day 7), treatment (day 8 and 12) and post-treatment (day 21) periods. The effect of CHQ on TNF-α, IL-1β and IL-6 was evaluated in non-infected and *P.berghei*-infected male Sprague-Dawleys rats treated with either oral CHQ or an topical application of the dermal patch. Plasma samples that were collected on day 7 served as our control (C).

The infected control animals had a higher TNF-α concentration when compared to the non-infected animals $^{\alpha}$(NIO vs IC day 8, $p<0.05$, Figure1). Administration of CHQ decreased TNF-α concentration in *P.berghei*-infected rats on day 8 of treatment period in comparison to baseline (day 7) (C) * (C vs IO day 8, $p<0.05$, Figure 1B). However, oral CHQ increased TNF-α concentration on day 12 of treatment period and during the post-treatment period * (C vs IO day 12 &21, $p<0.05$, Figure 1B). Topical application of the pectin-CHQ matrix patch decreased TNF-α concentrations of *P.berghei*-infected animals throughout the 21 day period in comparison to control $^{\#}$ (C vs IP day 8, 12 & 21, $p<0.05$, Figure 1B).

In the non-infected animals, oral CHQ administration resulted in higher TNF-α concentration when compared to the CHQ-patch treated animals $^{@}$ (NIO vs NIP day 12 & 21,$p<0.05$, Figure 1A). Similar results were seen in the infected animals $^{@}$ (IO vs IP day 12 & 21,$p<0.05$, Figure 1B).

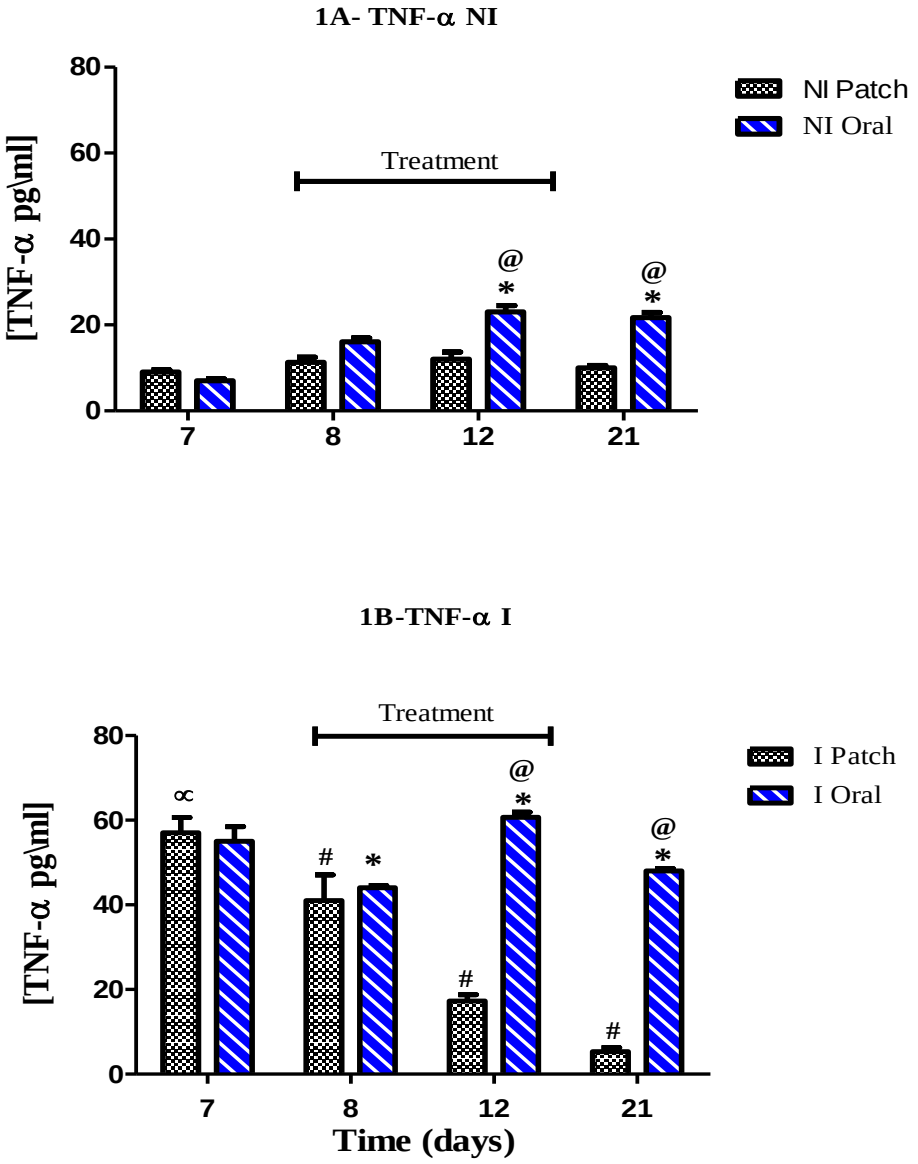

Figure 1: TNF-α concentration profiles of non-infected (NI) and *P. berghei*-infected rats (I) treated with oral CHQ or a pectin-CHQ matrix.

$^{\alpha}$ $p<0.05$ non-infected control compared to infected control

*$p<0.05$ orally treated animal compared to baseline

$^{\#}p<0.05$ CHQ-patch treated animals compared to baseline (day 7)

$^{@}p<0.05$ in comparison to pectin-CHQ patch

Figure 2 shows the effects of CHQ treatment on IL-1β concentration in non-infected and *P. berghei*-infected rats. The infected control animals had a higher IL-1 β concentration when compared to the non-infected animals [α](NIO vs IC day 8, $p<0.05$, Figure2). Administration of oral CHQ decreased IL-1β concentration in *P.berghei*-infected rats on day 8 of treatment period in comparison to baseline (day 7) (C) * (C vs IO day 8, $p<0.05$, Figure 2B). However, oral CHQ increased IL-1β concentration on day 12 of treatment period and during the post-treatment period * (C vs IO day 12 &21, $p<0.05$, Figure 2B). Topical application of the pectin-CHQ matrix patch decreased IL-1β concentrations of *P.berghei*-infected throughout the 21 day period when comparison to control [#] (C vs IP day 8, 12 &21, $p<0.05$, Figure 2B).

In the non-infected animals, oral CHQ administration resulted in higher IL-1β concentration when compared to the CHQ-patch treated animals [@] (NIO vs NIP day 12&21, $p<0.05$, Figure 2A). Similar results were seen in the *P.berghei*-infected animals [@] (IO vs IP day 12&21, $p<0.05$, Figure 2B).

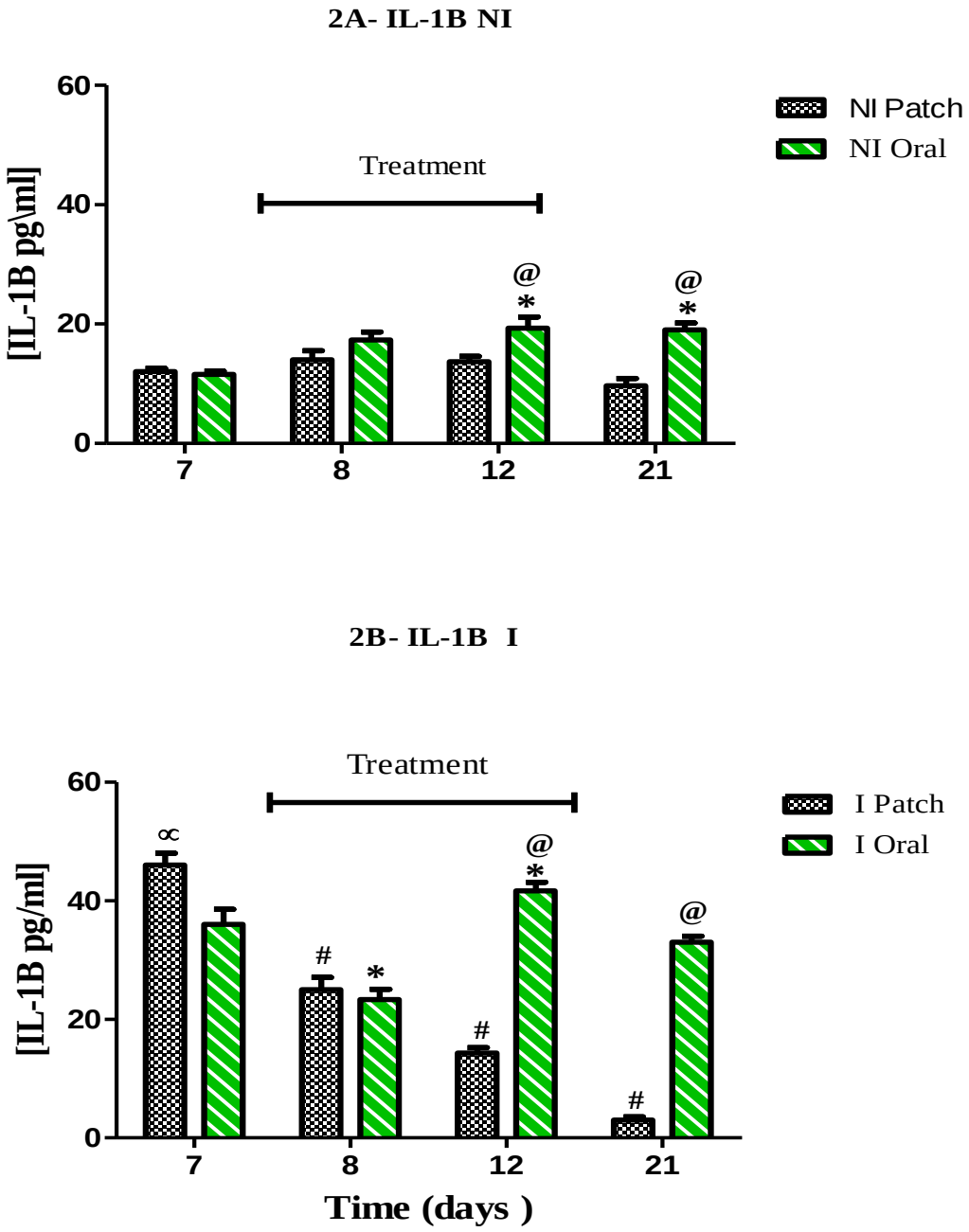

Figure 2: IL-1β concentration profiles of non-infected (NI) and *P.berghei*-infected rats (I) treated with oral CHQ or pectin-CHQ matrix patch.

$^{\alpha}$ $p<0.05$ non-infected control compared to infected control

*$p<0.05$ orally treated animal compared to baseline

$^{\#}p<0.05$ CHQ-patch treated animals compared to baseline (day 7)

$^{@}p<0.05$ in comparison to pectin-CHQ patch

Figure 3 shows IL-6 concentration during the pre-treatment, treatment and post-treatment period of animals treated with oral CHQ or topical application of the pectin CHQ matrix patch. The infected control animals had a higher IL-6 concentration when compared to the non-infected animals $^{\alpha}$(NIO vs IC day 8, $p<0.05$, Figure3). Oral administration of CHQ decreased IL-6 concentration in *P.berghei*-infected rats on day 8 of treatment period in comparison to baseline (day 7) (C)* (C vs IO day 8, $p<0.05$, Figure 3B). However, oral administration of CHQ increased IL-6 concentration on day 12 of treatment period and during the post-treatment period * (C vs IO day 12 &21, $p<0.05$, Figure3B). Topical application of the pectin-CHQ matrix patch decreased IL-6 concentrations of *P.berghei*-infected animals throughout the 21 day period when comparison to control $^{\#}$ (C vs IP day 8, 12 &21, $p<0.05$, Figure 3B).

In the infected animals, oral CHQ administration resulted in higher IL-6 concentration when compared to the CHQ-patch treated animals $^{@}$ (IO vs IP day 12&21, $p<0.05$, Figure 3B).

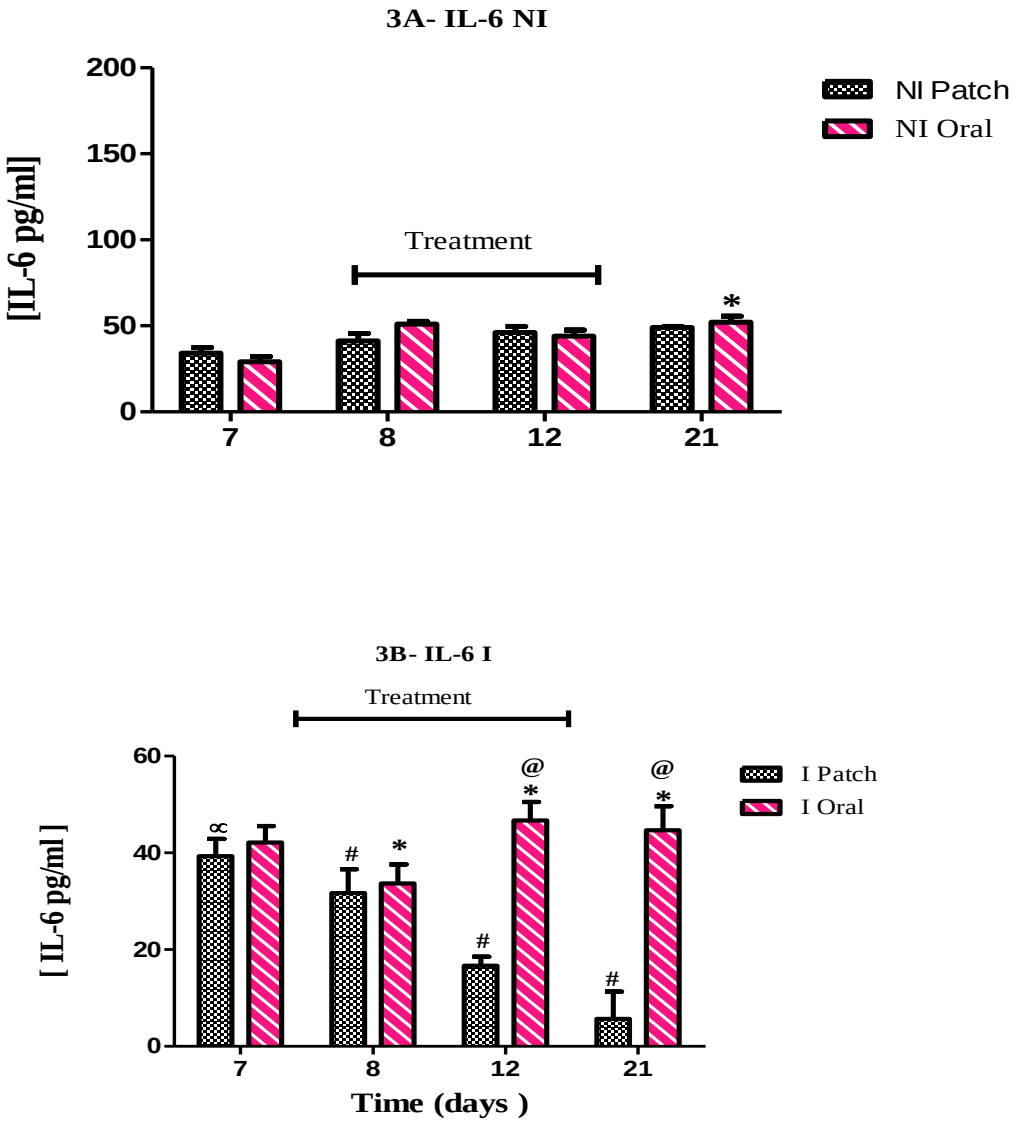

Figure 3: IL-6 concentration profiles of non-infected (NI) and *P. berghei*-infected rats (I) treated with a pectin-CHQ matrix patch or oral CHQ.

$^{\alpha}$ $p<0.05$ non-infected control compared to infected control

*$p<0.05$ orally treated animal compared to baseline

$^{\#}p<0.05$ CHQ-patch treated animals compared to baseline (day 7)

$^{@}p<0.05$ in comparison to pectin-CHQ patch

4.0 Discussion

Anaemia is one of the most severe malaria complications which accounts for a significant number of malaria related deaths (Phillips and Pasvol, 1992). The current study investigated the effects of CHQ treatment on haematological parameters and plasma pro-inflammatory cytokine concentrations in an effort to establish the possible mechanisms for the reported malaria associated anaemia. We also we evaluated the effects of using a topical application of a CHQ-pectin patch when compared to the standard treatment of using oral CHQ.

The results of the present study showed a reduction in the red blood cell, haematocrit, haemoglobin and mean corpuscular haemoglobin concentration of malaria infected animals when compared to the non-infected group of animals. Studies have reported various mechanisms that contribute to aneamia during malaria infection. These changes could be attributed to the uncontrolled destruction of RBC's by the *plasmodium* parasite during their life cycle (Bjo¨rkman, 2002). Increased RBC destruction may result from rupture of parasitized red blood cells, which occurs in direct proportion to the percentage parasitaemia and schizontaemia (Bjo¨rkman, 2002). The proliferation and hyperactivity of macrophages within the reticuloendothelial system in response to the destruction of RBCs is responsible for the phagocytosis of both parasitized and unparasitized RBCs (Phillips and Pasvol, 1992). This removal of unparasitized RBCs is believed to be one of the mechanisms resulting in persistent anaemia (Eric *et al.*, 2013). In addition, studies have reported the increased production of reactive oxygen species (ROS) in malaria. This increased ROS concentration has been reported to cause anaemia (Chirico and Pialoux, 2012). Hence, we believe that malaria induced oxidative stress also plays a role in aneamia observed during the malaria infection.

Oral CHQ treatment reduced red blood cells, red cell distribution width, haematocrit, haemoglobin and mean corpuscular haemoglobin concentration, in both non-infected and malaria infected animals. Our findings correlated with a previous study which reported that CHQ decreased RBCs and HCT (Omotosho *et al.*, 2014). Furthermore, Mbajiorgu *et al*., 2006 reported that CHQ treatment caused a reduction in RBCs, HGB and MCH (Mbajiorgu *et al.*, 2006). Clark and Cowden., 2003 attributed these changes to the ability of CHQ to bind to infected RBC proteins and elicit an immune response, eventually causing destruction of RBC, thus aggravating anaemia. Furthermore, literature suggests that the weak base of CHQ accumulates in acidic organelles of the cell, where it blocks acidification (Moss *et al.*, 1992). This alteration in the pH of the endosomal vesicle prevents the release of iron from the transferrin–transferrin receptor complex, ultimately decreasing iron uptake. In the non-infected animals, topical application of pectin CHQ matrix patch did not alter RBC, HCT, HGB and MCH however a significant increase in RBC, HCT, HGB and MCH values was observed in *P.berghei*-infected rats. We speculate that this ability of the pectin-CHQ patch to increase RBC, HCT, HGB and MCH is partly attributed to the pectin-CHQ patch's ability to eradicate the malaria parasites

from the circulation thereby halting the destructions of RBCs. We also speculate that the pectin CHQ matrix patch's effect in not altering RBC, HCT, HGB and MCH in non-infected animals might be attributed to the controlled, sustained release of CHQ into systemic circulation therefore circumventing the toxic effects of oral CHQ delivery.

The current study also compared the effects of CHQ treatment on platelet count. Platelets play an important role in inflammatory and proliferative events (Cerletti *et al.,* 2012). Mean platelet volume (MPV) is the measurement of the average size of platelets in blood, and provides information about platelet activation in the body (Jakubowski *et al.*, 1983). An increase in MPV has been documented in patients with stroke, diabetes mellitus (DM) and malaria (Kumar, 2006). Previous reports indicate that interleukin 6 (IL-6) stimulates megakaryocytopoiesis in the bone marrow, increasing platelet numbers in the circulation (Williams *et al.*, 1992). Results obtained from this study on platelet counts showed a decrease in MPV in *P.berghei*-infected animals treated with oral CHQ. Findings from the current study are in correlation with a previous study which reported a decrease in platelet count following CHQ treatment (Omotosho *et al.*, 2014). Our results indicate that treatment with oral CHQ may lead to bleeding problems (Vinik *et al.*, 2001). Topical application of the pectin-CHQ patch, increased MPV concentrations in *P.berghei*-infected rats. The findings of the current study indicate that the pectin-CHQ patch application may ameliorate the malaria associated aneamia and could be a better alternative in the management of malaria.

WBCs function mainly to fight infection, defend the body by phagocytosis against invasion by foreign organisms. Accordingly, the study investigated the effects of malaria and CHQ treatment on WBC count. We showed that the *P.berghei*-infected group had higher WBC count compared to the non-infected control group. We speculate that this increase in WBC count in *P.berghei*-infected rats was due to an immune response against the malaria infection. Following treatment our results showed higher WBC count in oral CHQ treated rats compared to non-infected animals. These results confirm findings of a previous study by Mbajiorgu *et al.*, 2006 which showed that oral CHQ treatment significantly increased eosinophil, monocyte and neutrophil count (Mbajiorgu *et al.*, 2006). CHQ is deposition in various visceral organs following oral administration probably causing adverse effects in the liver, kidney and heart (Chaturvedi and Chaturvedi, 2011). Furthermore, the antimalarial activity of CHQ results in the generation of toxic CHQ haeme complexes as well as accumulation of free haem moieties (Zhang *et al.*, 1999). Studies have shown that the free haeme undergoes oxygenation resulting in the formation of oxygen radicals (Francis and Goldberg, 1997). Although, the effect of CHQ on oxidative stress were not measured in the current study, we speculate that this increase in oxidative stress during treatment with oral CHQ may be partly responsible for this increase in WBC observed in the current study. An application of the pectin-CHQ matrix patch had no effects on the above parameters in the non-infected and *P.berghei*–infected animals in comparison to pre-treatment

values. This data shows that the use of pectin-CHQ matrix patch in malaria endemic areas may lead to increased population immunity and decreased susceptibility to opportunistic infections and hence could be a better alternative for malaria management.

The role of cytokines in the development of aneamia is not well understood. Pro-inflammatory cytokines, such as TNF-α and IL-10, are believed to play a role in the development of aneamia (Kurtzhals *et al.*, 1998). In vitro studies have suggested that TNF-α can inhibit the production of erythropoietin (Faquin *et al.*, 1992). Therefore, to further elucidate the causes of malaria associated aneamia, the current study measured plasma pro-inflammatory cytokines. Oral administration of CHQ decreased pro-inflammatory cytokine concentration in *P.berghei*-infected rats on day 8 of the treatment period in comparison to baseline. These results confirm findings that have shown an inhibitory effect of CHQ on pro-inflammatory cytokine concentration (Van den Born *et al.*, 1997). In addition, CHQ has been shown to inhibit TNF-α release in macrophages through the inhibition of TNF-α mRNA synthesis (Weber and Levitz, 2000). Furthermore Jang and colleagues showed that CHQ also interferes with macrophage function by blocking the conversion of cell-associated TNF-α to mature protein, and reduces the levels of IL-1β and IL-6 mRNA by altering their stability in a pH-dependent manner (Jang *et al.*, 2006). However, on day 12 of the treatment period and during the post-treatment period there was an increase in pro-inflammatory cytokine concentration while topical application of the patch decreased pro-inflammatory cytokines in *P.berghei*-infected rats during the treatment period and post-treatment period. We speculate that this increase in pro-inflammatory cytokines following treatment with oral CHQ may be attributed to the increased deposition of CHQ in tissues such as the heart, liver, spleen and kidney. This hypothesis is further supported by pharmacokinetic studies of CHQ which have shown rapid CHQ absorption following oral CHQ administration of this drug (Gustafsson *et al.*, 1987). CHQ is a potent autophagic drug that may lead to cellular degradation of hepatocytes in the liver (Abraham *et al.*, 1968). CHQ accumulates especially in the Kupffer cells of the liver with resultant lysosomal damage including overloading of the liver lysosomes with non-digestible material, and an increase in their size and number (Schneider *et al.*, 1997). Furthermore, it has been indicated that about 51% of CHQ is cleared by the kidney unchanged (McChesney *et al.*, 1976). However, a small fraction of it accumulates in epithelia cells of the kidneys (Gustafsson *et al.*, 1983). In addition, some of the administered CHQ is deposited in the heart (Baguet and Fabre, 1999) where CHQ exerts antiarrythmic actions to increase heart rate (Dondo and Mubagwa, 1990). It is speculated that the increase in heart rate may alter perfusion pressure of the kidney, and amend renal haemodynamics as well as electrolyte handling (Dondo and Mubagwa, 1990).We speculate that the deposition of CHQ in various tissue organs following oral CHQ administration may elicit an inflammatory response or tissue destruction hence pro-inflammatory cytokine concentration were measured in this study.

In addition, it has been shown that the release of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1 and IL-22, stimulate hepcidin release (Howard *et al.*, 2007). Hepcidin blocks the release of iron from enterocytes, hepatocytes, and macrophages, leading to hypoferremia and limited iron availability for erythropoiesis (McDevitt *et al.*, 2004). This suppressed erythropoiesis contributes to malarial anaemia as new red cells are not produced to replace those lost by haemolysis (Armitage *et al.*, 2009). These observations are of considerable importance as we speculate that the ability of pro-inflammatory cytokines to increase secretion of hepcidin might be one of the mechanisms that oral CHQ may aggravate aneamia. Topical administration of CHQ decreased pro-inflammatory cytokine concentration in *P.berghei*-infected rats during the treatment and the post-treatment period. In addition, the concentration of TNF-α, IL-1β and IL-6 of non-infected animals remained unchanged during treatment period in comparison to baseline. TNF-α, IFN-γ, and IL-12 as well as other cytokines have been shown in vitro and in vivo to promote the development of anaemia (Wickramasinghe and Abdalla, 2000; Means, 2000). In vitro studies have suggested that TNF-α can inhibit the production of erythropoietin (Faquin *et al.*, 1992). Furthermore, several pro-inflammatory cytokines, including IL-1, and IL-6, produced during blood-stage malaria in humans and in mice (Stevenson and Riley, 2004) inhibit kidney EPO production in vitro and in vivo (Jelkmann *et al.*, 1994; Braczkowski *et al.*, 2001). Based on the above effects of pro-inflammatory cytokines in aneamia we, therefore, speculate that the ability of pectin-CHQ matrix patch to decrease pro-inflammatory cytokine concentration could provide a new approach to modify the anaemia associated with malaria.

Conclusion

Oral administration of CHQ and malaria parasite results in the development of anaemia by affecting RBCs and plasma pro-inflammatory cytokines. The current study has therefore demonstrated the feasibility of the use of pectin CHQ matrix patch for topical application of CHQ in the management of malaria.

References

1 Abraham R, Hendy R and Grasso P. Formation of myeloid bodies in rat liver lysosomes after chloroquine administration. *Exp Mol Path;* 1968; **9**: 212-229.

2 Armitage A, Pinches R, Eddowes L, Newbold C and Drakesmith H. *Plasmodium falciparum* infected erythrocytes induce hepcidin (HAMP) mRNA synthesis by peripheral blood mononuclear cells. *British Journal of Haematology;* 2009; **147**: 769–771.

3 Baguet J and Fabre F. Chloroquine cardiomyopathy with conduction disorders *Heart;* 1999; **81**: 221-223.

4 Bjo¨rkman A. Malaria associated anaemia, drug resistance and antimalarial combination therapy. *International Journal for Parasitology;* 2002; **32**: 1637–1643.

5 Braczkowski R, Romanawsky W, Danikiewicz A, Muc-Wierzgon M, Blazelonis A and Zubelewicz B. Decrease of erythropoietin level by human recombinant tumour necrosis factor alpha in patients with advanced cancer. *J Biol Regul Homeost;* 2001; **15**: 366–369.

6 Chaturvedi P and Chaturvedi M. Effect of African potato (Hypoxis hemerocallidea) extract on oxidative stress induced by chloroquine in Albino rats. *African Journal of Food, Agriculture, Nutrition and Development;* 2011; **11**: 76-89.

7 Chirico E and Pialoux V. Role of oxidative stress in the pathogenesis of sickle cell disease. *International Union of Biochemistry and Molecular Biology Life* 2012; **64**: 72-80.

8 Clark I and Cowden W. The pathophysiology of *P. falciparum* malaria. *Pharmacology and Therapeutics;* 2003; **99**: 221-260.

9 Dondo F and Mubagwa K. Chloroquine interacts with muscarinic receptors and inhibits cholinergic effects in the heart. *African Journal of Medical Science;* 1990; **19**: 237-243.

10 Drakesmith H and Prentice A. Hepcidin and the iron-infection axis. *338;* 2012; **Science**: 768-772.

11 Dreyfuss M, Stoltzfus R, Shrestha J, Pradhan E, LeClerq S, Khatry S *et al.* Hookworms, Malaria and Vitamin A deficiency contribute to anemia and Iron deficiency among pregnant women in the plains of Nepal. *J Nutr;* 2000; **130**: 2527-2536.

12 Eric M, Kevin M and Laird J. Determinants of Anemia among Preschool Children in Rural, Western Kenya. *Am J Trop Med Hyg;* 2013; **88**: 757–764.

13 Faquin W, Schneider T and Goldberg M. Effect of inflammatory cytokines on hypoxia-induced erythropoietin production. *Blood;* 1992; **79**: 1987–1994.

14 Francis S and Goldberg D. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annual Review of Microbiology;* 1997; **51**: 97-123.

15 Ganz T. Hepcidin and the Global Burden of Iron Deficiency. *Clinical Chemistry;* 2015; **61**: 577-578

16 Gasparyan A, Ayvazyan L, Mikhailidis D and Kitas G. Mean platelet volume: a link between thrombosis and inflammation? *Curr Pharm Des;* 2011; **17**: 47-58.

17 Gumede B, Folbb P and Ryffela B. Oral artesunate prevents *Plasmodium berghei* ANKA infection in mice. *Parasitology International;* 2003; **52**: 53-59.

18 Gustafsson L, Walker O, Alvan G, Beermann B, Estevez F, Gleisner L *et al.* Disposition of chloroquine in man after single intravenous and oral doses. *British Journal of Clinical Pharmacology* 1983; **15**: 471-479.

19 Gustafsson L, Lindstrom B, Grahnen A and Alvan G. Chloroquine excretion following malaria prophylaxis. *British Journal of Clinical Pharmacology;* 1987; **24**: 221-224.

20 Howard C, McKakpo U, Quakyi I, Bosompem K, Addison E and Sun K. Relationship of hepcidin with parasitemia and anaemia among patients with uncomplicated *Plasmodium falciparum* malaria in Ghana. *Blood;* 2007; **121**: 3016-3022.

21 Jakubowski JA, Thompson C, Vaillancourt R, Valeri C and Deykin D. Arachidonic acid metabolism by platelets of differing size. *British Journal of Haematology;* 1983; **53**: 503-551.

22 Jang C, Choi J, Byun M and Jue D. Chloroquine inhibits production of TNF-alpha, IL-1beta and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes. *Rheumatology;* 2006; **45**: 703-710.

23 Jelkmann W, Fandrey J, Frede S and Pagel H. Inhibition of erythropoietin production by cytokines. Implications for the anemia involved in inflammatory states. *Ann NY Acad Sci;* 1994; **718**: 300–309.

24 Kumar A. Thrombocytopenia- an indicator of acute vivax malaria. *Indian J Pathol Microbiol;* 2006; **49**: 505-508.

25 Kurtzhals J, Adabayeri V, Goka B, Akanmori B, OliverCommey J, Nkrumah F *et al.* Low plasma concentrations of interleukin 10 in severe malarial anemia compared with cerebral and uncomplicated malaria. *Lancet;* 1998; **351**: 1768–1772.

26 Mbajiorgu E, Aire T, Volk W, Albert M and Debusho L. Haematological profile of male rats treated with ethanol and/ or Chloroquine and fed normal or low Protein diet. *The Internet Journal of Hematology;* 2006; **3**: 1-11.

27 McChesney E, Banks W and Fabian R. Tissue distribution of chloroquine, hydroxychloroquine, and desethylchloroquine in the rat. *Toxicology and Applied Pharmacology* 1976; **10**: 501-513.

28 McDevitt M, Xie J and Gordeuk V. The anemia of malaria infection: role of inflammatory cytokines. *Current Hematology;* 2004; **3**: 97–106.

29 Means R. The anaemia of infection. *Baillie´re's Best Pract Res Clin Haematol;* 2000; **13**: 151–162.

30 Moss D, Fargion S, Fracanzani A, Levi S, Cappellini M, Arosio P *et al*. Functional role of the ferritin receptors of human liver, hepatoma, lymphoid and erythroid cells. *J Inorg Biochem;* 1992; **47**: 219–227.

31 Naumann B, PA W, Sarangapani R, Hu S, Dixit R and Sargent E. Investigations of the use of bioavailability data to adjust occupational exposure limits for active pharmaceutical ingredients. *Toxicological Sciences;* 2009; **112**: 196-210.

32 Omotosho O, Adebiyi M and Oyeyemi M. Comparative study of the haematology and serum biochemistry of male Wistar Rats treated with Chloroquine and Artesunate. *J Phys Pharm* 2014; **4**: 413-419.

33 Phillips R and Pasvol G. Anaemia of *Plasmodium falciparum* malaria *Baillière's Clinical Haematology* 1992; **5**: 315–330.

34 Schneider P, Korolenko T and Busch U. A review of drug induced lysosomal disorders of the liver in man and laboratory animals. *Microsc Res Tech;* 1997; **36**: 253-275.

35 Sibiya H. Evaluation of the efficacy of transdermal delivery of chloroquine on *Plasmodium berghei*-infected male sprague-dawley rats: Effects on blood glucose and renal electrolyte handling.MMeD thejsis thesis, University of KwaZulu Natal. 2013.

36 Stevenson M and Riley E. Innate immunity to malaria. *Nature Rev Immunol;* 2004; **4**: 169–180.

37 Van den Born B, Dijikmans B, Rooij H, Lecessi S and Verweij C. Chloroquine and Hyroxy-chloroquine equally effect Tumour Necrosis Factor Alpha, Interleukin 6 and Interferon Gamma by peripheral nuclear cells. *Journal of Rheumatology;* 1997; **24**: 55-60.

38 Weber S and Levitz S. Chloroquine interferes with lipopolysaccharide-induced TNF-alpha gene expression by a non lysosomotropic mechanism. *J Immunol;* 2000; **165**: 1534-1540.

39 WHO. World malaria report 2015 2015.

40 Wickramasinghe S and Abdalla S. Blood and bone marrow changes in malaria. *Baillie ´re's Best Pract Res Clin Haematol;* 2000; **13**: 277–299.

41 Zhang J, Krugliak M and Ginsburg H. The fate of ferriprotorphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. *Molecular and Biochemical Parasitology;* 1999; **99**: 129–141.

This manuscript will be submitted to Malaria journal. The manuscript has been written according to the Journal guidelines

Identifying morphological changes in visceral organs following oral or transdermal administration of chloroquine in *P.berghei*-infected rats

Gumede, NM., and Mabandla, MV

School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of Kwa-Zulu-Natal, Private BagX54001, University of Kwa-Zulu-Natal, South Africa.

Correspondence: Dr MV Mabandla

Discipline of Human Physiology

School of Medical Sciences

University of Kwa-Zulu-Natal

Private BagX54001

Durban 4000

South Africa

Phone: 0312608602

Fax: 0312607123

E-mail: mabandlam@ukzn.ac.za

1.0 Abstract

Oral chloroquine (CHQ) administration has been shown to affect normal functioning of visceral organs. These effects are partly ascribed to transiently high plasma CHQ concentration following administration. The controlled release of CHQ administrated transdermally may minimise the deposition and damage that may be caused by oral CHQ administration in visceral organs thus averting the adverse effects associated with oral CHQ treatment. Accordingly, the current study compared the morphological effects of oral CHQ and transdermal CHQ patch delivery in the heart, kidney, spleen, and the liver of *plasmodium berghei*-infected rats**.** We also investigated the plasma concentration of C-reactive protein (CRP). The study duration was 3 weeks divided into pre-treatment (days 0-7), treatment (8-12) and post treatment (13-21) periods. CHQ treatment was either administrated orally (30mg/kg, twice daily) or via a once off CHQ matrix patch (56mg/kg). a Skin sample (patch treated rats) as well as liver, kidney, spleen and the heart were collected from all experimental animals**.** *P.berghei*-infected rats following oral and transdermal CHQ delivery showed mild morphological effects on the liver, heart, kidney and spleen by comparison to infected control animals. In non-infected rats oral CHQ treatment showed adverse morphological effects on the architecture of these organs, while no changes were observed following transdermal CHQ delivery. Oral CHQ administration increased CRP concentration. However, CRP concentration was not affected in patch treated animals. Therefore, our transdermal formulation minimised the challenges of dose dumping associated with oral CHQ delivery. This suggests that the CHQ patch has the potential to avert complications associated with oral CHQ administration.

Keywords: *Plasmodium berghei*, malaria, transdermal patch, morphology, chloroquine, c-reactive protein

1.1 Introduction

The use of orally administered Chloroquine (CHQ) remains the mainstay therapy in some parts of Africa despite the developed *Plasmodium* resistance (WHO, 2006). The challenge with oral CHQ administration is that it involves the administration of 4 tablets upon presentation of the disease, 2 tablets 6-8 hours later and 2 tablets for two consecutive days (Munjeri *et al.,* 1998). Additionally, the bitter taste of CHQ elicits non-compliance in a majority of patients resulting in treatment non-compliance, which is believed to lead to therapeutic failure and a growing number of CHQ-resistant strains of *P. falciparum* in Sub-Saharan Africa (Musabayane *et al.,* 1993; White, 2004). In addition, when administered orally, CHQ is susceptible to gastrointestinal degradation as well as first-pass metabolism by the liver; as a result high initial CHQ concentrations are required to bring therapeutic effects (Gustafsson *et al.,* 1983). However, the presence of high CHQ plasma concentrations following oral administration leads to the accumulation of CHQ in epithelial cells of various organs such as the heart, kidney, spleen, adrenal glands and the liver thus disrupting function (Gustafsson *et al.,* 1983). Reports suggest that CHQ accumulates in the kidney (Cooper and Magwere, 2008), heart (Baguet and Fabre, 1999) and adrenal glands (Gustafsson *et al.*, 1987) altering the physiological functions of these organs. Furthermore, prolonged administration can produce dermatologial effects as well as other severe effects that include cerebral disorders, irreversible retinopathy and maculopathy (Cooper and Magwere, 2008). Therefore, intense usage of oral CHQ in areas where malaria is endemic means that information is needed about its effects on organs where the drug accumulates so as to gain insight into the impact of the long-term administration of this drug. C-reactive protein (CRP) is an acute-phase protein that has been used as a sensitive systemic marker of inflammation and tissue damage (Pepys and Baltz, 1983). Therefore, the current study investigated CRP in order to assess tissue damage following CHQ administration. Alternative methods of CHQ administration such as transdermal delivery have been shown to provide sustained controlled release of CHQ in the bloodstream (Munjeri *et al.*, 1998). In this study we compared the morphological effects of oral CHQ and transdermal CHQ patch delivery in various organs of *plasmodium berghei*-infected male Sprague-Dawley rats. The study also investigated the change in the concentration of C-reactive protein following treatment of the infected animals.

2.0 Materials and methods

2.1 Drugs and chemicals

Chloroquine diphosphate (CHQ) was sourced from Sigma-Aldrich Chemical Company, St Louis, Missouri, United States of America. All other chemicals which were of analytical grade quality were purchased from standard commercial suppliers.

2.2 Animals

40 male Sprague-Dawley rats (90-120 g) bred and housed in the Biomedical Resource Unit of the University of KwaZulu-Natal Westville campus were used in the study. The animals were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), CO2 content of <5000 p.p.m., relative humidity of 55 ± 5 %, A 12 h light/dark cycle (lights on at 6 am) and noise levels of <65 decibels. The animals had free access to standard rat chow (Meadows feeds, Pietermaritzburg, South Africa) and water. All animal experimentation was reviewed and approved by the University of KwaZulu-Natal's Animal Ethics Research Committee (References: 034/14 Animal and 034/15 Animal).

2.3 Experimental design

2.3.1 Effects of oral and transdermal CHQ treatments on various visceral organs

The effects of oral CHQ (30mg/kg) treatment and a once off topical application of the pectin CHQ matrix patch (56mg/kg) on the heart, kidney, liver and spleen were investigated in non-infected and infected rats. The studies were carried out over a period of 3 weeks divided into pre-treatment (days 0-7), treatment (days 8-12) and post treatment (days 13-21) periods. The animals were housed individually in Makrolon polycarbonate metabolic cages (Techniplasts, Labotec, South Africa) at the Biomedical Resource Unit, University of KwaZulu- Natal. During the treatment period, the orally treated group were given CHQ diphosphate solution CHQ (30mg/kg), twice daily, 8 hours apart by means of a ball-tipped, 18-gauge gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa) attached to a 1 ml syringe, for 5 consecutive days. For transdermal application of CHQ, the animals were treated with pectin CHQ matrix patches containing CHQ (56mg/kg). Prior to treatment, the rats were shaved on the dorsal neck region in preparation for the application of the pectin CHQ matrix patch. On the first day of the treatment period, A pectin CHQ matrix patch was applied at 9h00. The patch was secured in place with adhesive hydrofilm (BSN Medical, Pinetown, South Africa). Parasite density was monitored daily at 9h00, in all groups of *P berghei*-infected animals. Thin tail-blood films stained by Giemsa method as previously described by Gumede *et al*., (2003) were examined by microscopic examination. To assess the effect of CHQ treatment on various

visceral organs, the animals were sacrificed during pre-treatment (day 7), treatment (days 8 and 12) and post-treatment period (day 21).

2.4. Methods

2.4.1. Preparation of the pectin-CHQ matrix patches

Pectin-CHQ hydrogel matrix patches were prepared using a well-established protocol in our laboratory (Musabayane *et al.*, 2003) with slight modifications. Briefly, patches were prepared by dissolving (5g) CHQ and pectin (4.4g) in separate beakers containing 110 mL de-ionised water. The beakers were then placed in a water bath at 37ºC and agitated at 38 x G using a mixer (Heidolph instruments GmbH & Co. KG, Schwabach, Germany) for 15 minutes**.** Following agitation, DMSO (3.3 mL) was added and mixed for 5 minutes. Subsequently, vitamin E (1.65 mL) and eucalyptus oil (1.65 mL) were added to the mixture. Following 1½ h of further mixing and agitation an aliquot of the mixture (11 mL) was transferred to a petri dish and frozen at -5^{o}C for 18 hours. After freezing, a 2% CaCl2 solution (1.5mL) was added on top of the frozen pectin and then left to stand at room temperature.

2.4.2. Induction of malaria

A CHQ susceptible strain of *P. berghei* was used for the induction of malaria. The *P. berghei* parasite was kindly supplied by Professor Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). Malaria was induced in male Sprague-Dawley rats via a single intra-peritoneal injection of *P. berghei* (10^5 parasitized red blood cells) (Gumede *et al.*, 2003). Successful malaria induction was confirmed by microscopic examination of Giemsa stained thin smears of the tail blood. Percentage parasitaemia ranging from 15 - 20% was considered as a stable malaria state.

2.5. Sacrifice and removal of skin

All animals were sacrificed by placing each in an anaesthetic chamber filled with 100 mg/kg of halothane vapour for 3 minutes. Thereafter, skin and subcutaneous tissues around the dorsal region of neck where the patch was applied was dissected and removed. Liver, kidney, spleen and the heart were also removed and stored in 10% neutral buffered formaldehyde until further analysis.

2.6. Histological studies

2.6.1 Haematoxylin and Eosin analysis

The heart, liver, kidney and spleen of non-infected non-treated controls, *P berghei*-infected non-treated rats and *P berghei*-infected CHQ treated rats were placed in 10% neutral buffered formaldehyde solution to determine whether there was any morphological damage in the organs following oral and transdermal delivery of CHQ. The organs were dehydrated in an ascending grade of 70%, 90% as well as 100% ethanol. Thereafter, the organs were placed in xylene and placed in a suitable container of melted paraffin wax until completely infiltrated. Using a rotary microtome (Robert-Bosch-Straße, Walldorf, Baden-Württemberg, Germany), blocks of tissue were sectioned at 3-5µm thickness. Subsequently, the sections were stained with haematoxylin and eosin (H and E). The tissue sections were then rehydrated in decreasing concentrations of 100%, 70% and 50% ethanol for 3 minutes after which they were placed in deionized water until ready for staining. The sections were then cleared in xylene until they were ready for permanent mounting with applicator drop DPX mounting glue directly over the tissue section. The processed sections were viewed and captured using a Leica Scanner, SCN400 and Slide Path Gateway LAN software for analysis (Leica Microsystems CMS, Wetzlar, Germany).

2.7 C-Reactive Protein assay

2.7.1. Principle

Serum C-Reactive protein (CRP) concentrations were measured using rat CRP ELISA kit. The kit consisted of a 96 well plate coated with an antibody specific to CRP, biotinylated detection antibody, concentrated Avidin-Horseradish Peroxidase (AVIDIN-HRP) conjugate, wash buffer, substrate reagent and a stop solution. This ELISA kit was formulated for the Sandwich-ELISA method. Briefly, standards and samples were added to wells coated with an antibody specific to rat CPR. A biotinylated detection antibody specific for rat CPR and AVIDIN-HRP conjugate was added to each microplate well before incubation at 37°C for 1 hour. This was followed by the addition of the substrate solution to each well. When the colour changed, the reaction was stopped. The enzyme-substrate reaction was stopped by the addition of a sulphuric acid solution after which the colour turned yellow. The endpoint was read spectrophotometrically on a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 405 nm while the optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of rat CPR. The concentration of rat CPR in the samples was measured by extrapolating the OD of the samples from the standard curve.

2.7.2 Protocol

Each standard or sample was analysed in duplicate. For the standards, 100 µL of the following concentrations (50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng/mL) was added into each well. Similarly, the same volume (100 µL) of the sample was added into the remaining wells. The plate was sealed, mixed gently and incubated at 37°C for 90 minutes. Following incubation, the reaction volume was emptied by inverting the plate and tapped gently against a clean absorbent paper. Biotinylated Detection Ab (100µL) was added into each well and then aspirated. The plate was resealed and tapped gently to ensure thorough mixing. This was followed by incubation for 1 hour at 37°C. After the incubation, the liquid in the wells was dumped. Wash buffer (350µL) was added into each well after which it was aspirated. The wash process was repeated 3 times. After the final wash, the plates were inverted on absorbent paper so as to remove excess liquid. This was followed by adding the ARVIDIN-HRP conjugate (100µL) into each well. The plate was then incubated for 1 hour at 37°C. Following incubation, the wash process as previously described was repeated 5 times. Substrate solution (90µL) was added to each well after which the plate was covered with a new plate sealer and tin foil (to protect from light), incubated at 37°C for 15 minutes or until the colour change reaction was complete. The reaction was stopped by adding the stop solution (50µL) as soon as the change in colour became noticeable. The absorbance was read at 450nm using a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany).

2.8 Data analysis

GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA) was used for all statistical analyses. One-way analysis of variance (ANOVA) followed by the Turkey-Kramer multiple comparison post hoc test was used to assess CRP concentrations in the various groups. All data was expressed as means ± standard error of means (SEM). Values of $p < 0.05$ were taken to imply statistical significance.

3.0. Results

3.1. Liver histology

Liver histology was assessed in non-infected non-treated, non-infected treated, infected non-treated, infected treated with oral CHQ or transdermal patch.

Figure 1A shows the H and E stained sections of liver sections of the untreated non-infected control group. Figure 1A revealed normal hepatic architecture with sinusoidal radiating (S) from the central vein (CV). The hepatocytes appeared polyhedral in shape with well-defined boundaries and acidophilic cytoplasm. Each cell exhibited a round vesicular, centrally located nucleus. Some binucleated cells were also seen. Compared to the untreated non-infected control rats (Figure 1A), the *P. berghei* infected non-treated group (Figure 1B) showed severe hyperplastic kupffer cells (KC), portal tract inflammation (PCI) and deposition of haemozoin pigment (HP). Treatment with CHQ infected group (Figure 1C, orally treated and Figure 1D, transdermally treated) showed moderate hyperplastic kupffer cells, portal tract inflammation and deposition of haemozoin pigment when compared to the untreated infected control rats (Figure 1B). Photomicrograph 2 represents the liver of non-infected rats treated with either oral CHQ or transdermal patch. Oral CHQ treated rats (Figure 2A) showed enlarged sinusoidal area and portal tract inflammation, while pectin-CHQ patch (Figure 2B) showed normal architecture of the liver (12 × 200µm).

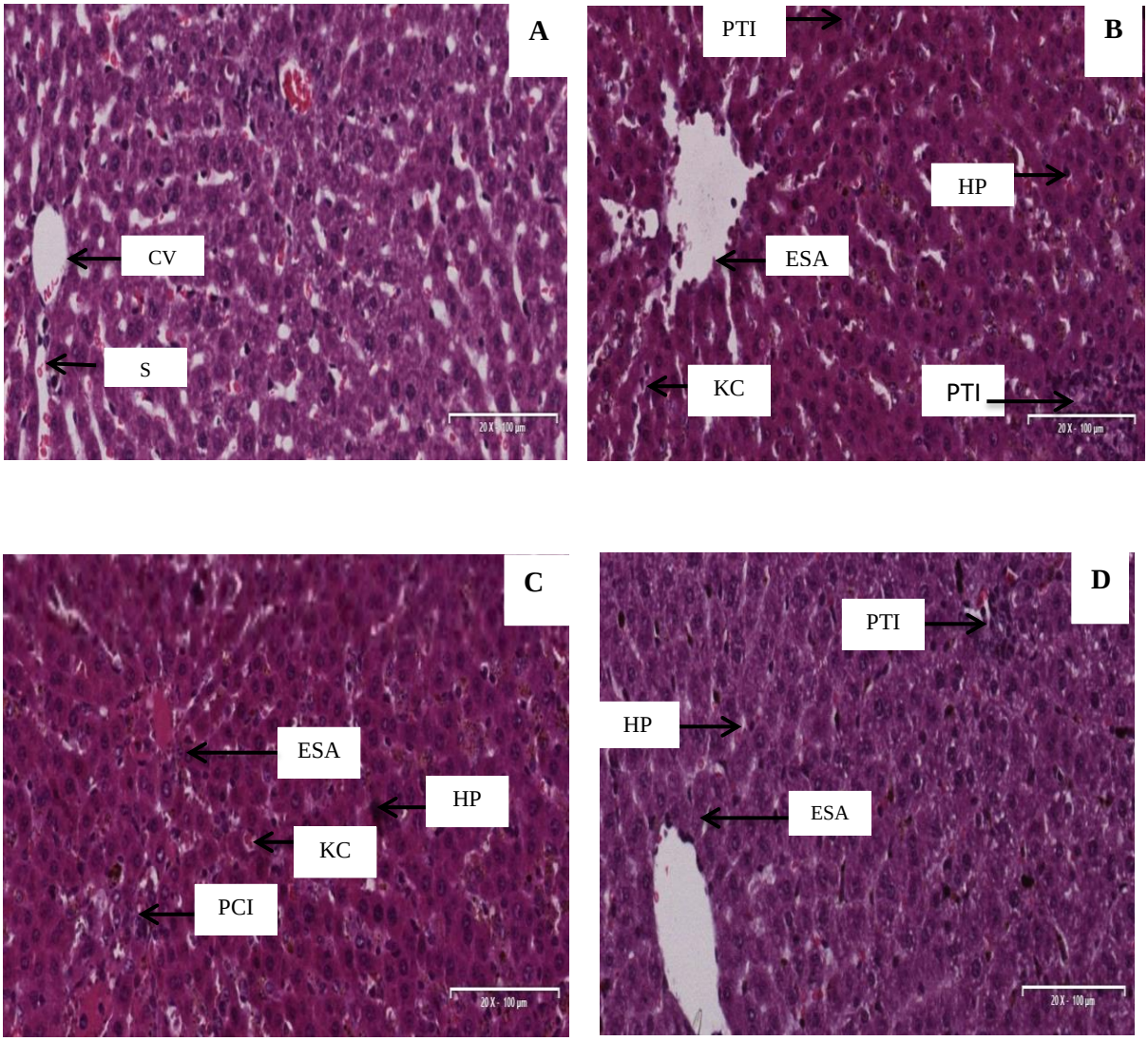

Figure 1: H and E microphotographs illustrating the morphology of the liver in non-infected and *P-berghei*-infected rats treated or untreated. Photomicrograph (1A) represents the normal architecture of liver sections of the non-infected non-treated group showing normal central vein (CV) and sinusoid (S). Photomicrograph (1B) shows liver tissues from the infected non-treated rats showing enlarged sinusoidal area (ESA), haemozoin pigment (HP), hyperplastic kupffer cells (KC), and portal tract inflammation (PTI). Photomicrographs (1C) show moderate hyperplastic kupffer cells, portal tract inflammation and deposition of haemozoin pigment following oral CHQ treatment. Photomicrographs (1D, transdermal) shows mild deposition of haemozoin and mild portal tract inflammation and deposition of haemozoin pigment (20 × 200µm).

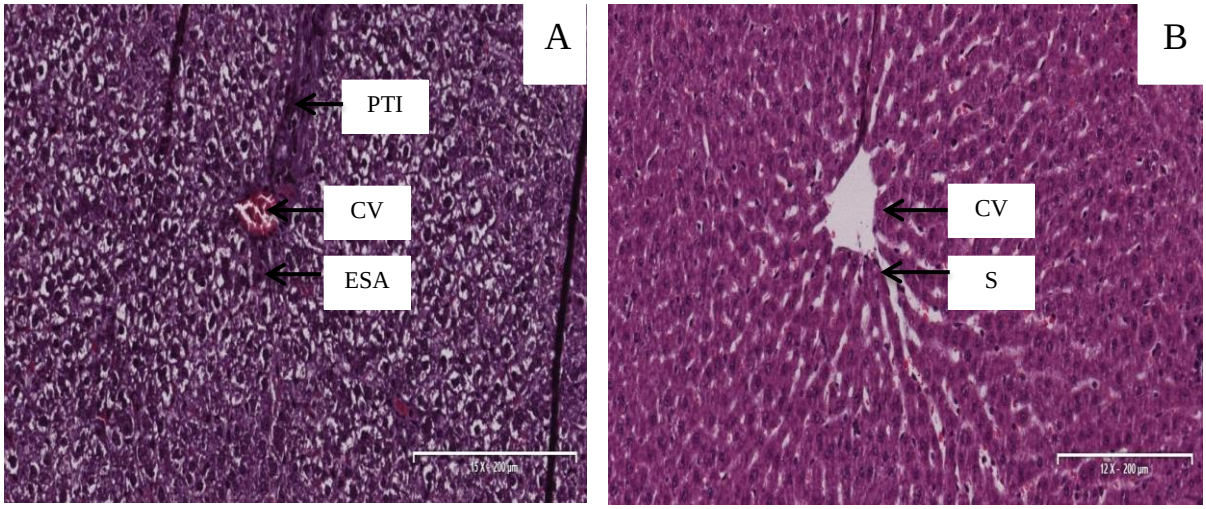

Figure 2: H and E microphotographs illustrating the morphology of the liver of non-infected treated rats. Photomicrograph 2A shows liver tissue from the non-infected rat treated with oral CHQ. Showing central vein, enlarged sinusoidal area and portal tract inflammation (12 × 200µm). Photomicrograph 2B illustrates the effect of transdermally delivered CHQ on the morphology of the liver. Figure 2B represents normal central vein with sinusoidal radiating to central vein (Mag 15×200 µm).

3.2. Kidney histology

Kidney histology was assessed in non-infected non-treated, non-infected treated, Infected non-treated, infected treated with oral CHQ or transdermal patch.

Figure 3A shows the normal section of the kidney of the non-treated group. It illustrates normal glomerular basement membrane (NGBM), nuclei (N) and squamous cells (SC). Compared to the untreated non-infected control rats (Figure 3A), *P.berghei*- infected rats (Figure 3B) showed thickened glomerular basement membrane (TGBM) and thickened basement membrane of the Bowmans capsule (BC). Treatment with oral CHQ (Figure 3C) and CHQ dermal patches (Figure 3D) however, attenuated these features when compared with the untreated *P.berghei*-infected rats (Figure 3B). Photomicrograph 4 represents the kidney of non-infected rats treated with either oral CHQ or transdermal patch. Oral CHQ treated rats showed thickened glomerular basement membrane (Figure 4A) when compared to pectin-CHQ patch (Figure 4B) treated rats.

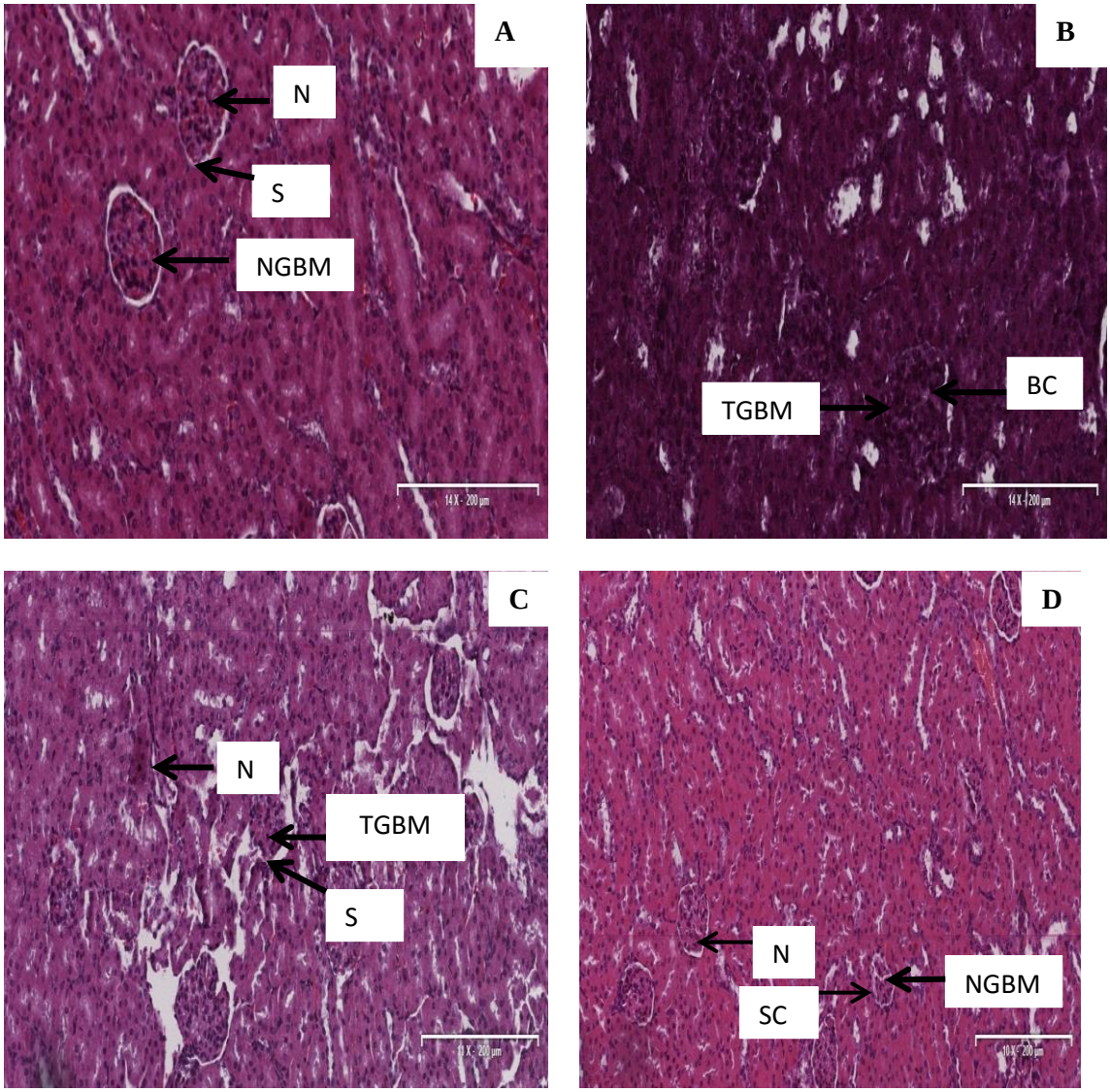

Figure 3: H and E photomicrographs illustrating the effects of malaria parasite and CHQ treatment on the morphology of the kidney. Photomicrograph (3A) represents the normal glomerulus of the untreated non-infected rat kidney section showing normal glomerular basement membrane (GBM), nuclei (N) and squamous cells (SC). Photomicrograph (3B) represents the injured glomerulus of *P berghei* -infected rat showing thickened glomerular basement membrane (TGBM) and thickened basement membrane of the Bowmans capsule (BC). Treatment with CHQ (Figure 3C, orally treated and Figure 3D, transdermally treated) however, attenuated these features (Mag 14 × 200 µm).

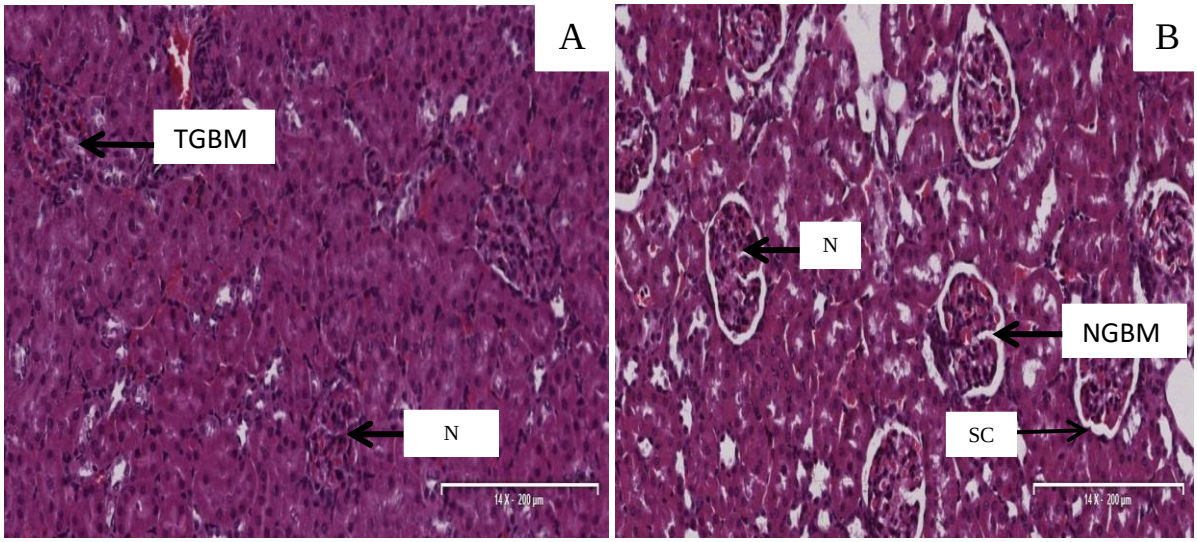

Figure 4: H and E photomicrographs illustrating the effects of transdermally delivered and oral CHQ on the morphology of the kidney in non-infected rats**.** Photomicrograph **(A)** represents the injured glomerulus of the oral CHQ treated rat showing thickened glomerular basement membrane (TGBM). Photomicrograph **(B)** represents normal glomerular basement membrane (NGMB), nuclei (N) and squamous cells (SC).

3.3. Heart histology

Heart histology was assessed in non-infected non-treated, non-infected treated, infected non-treated, infected treated with oral CHQ or transdermal patch rats.

Figure 5A illustrates the normal heart tissue of non-infected non-treated animals. Compared to the untreated non-infected control rats (Figure 5A), *P. berghei*-infected rats (Figure 5B) showed severe hypertrophy of cardiomyocytes (HCM). Both oral CHQ administration and a once-off topical application of the pectin-CHQ patch showed moderate hypertrophy of cardiomyocytes (Figure 5C, orally treated and Figure 5D, transdermally treated). Photomicrograph 6 represents the heart of non-infected rats treated with either oral CHQ or transdermal patch. Oral CHQ treated rats showed moderate hypertrophy of cardiomyocytes (Figure 6A), while Pectin-CHQ patch showed normal heart tissue (Figure 6B) compared oral CHQ (Figure 6A).

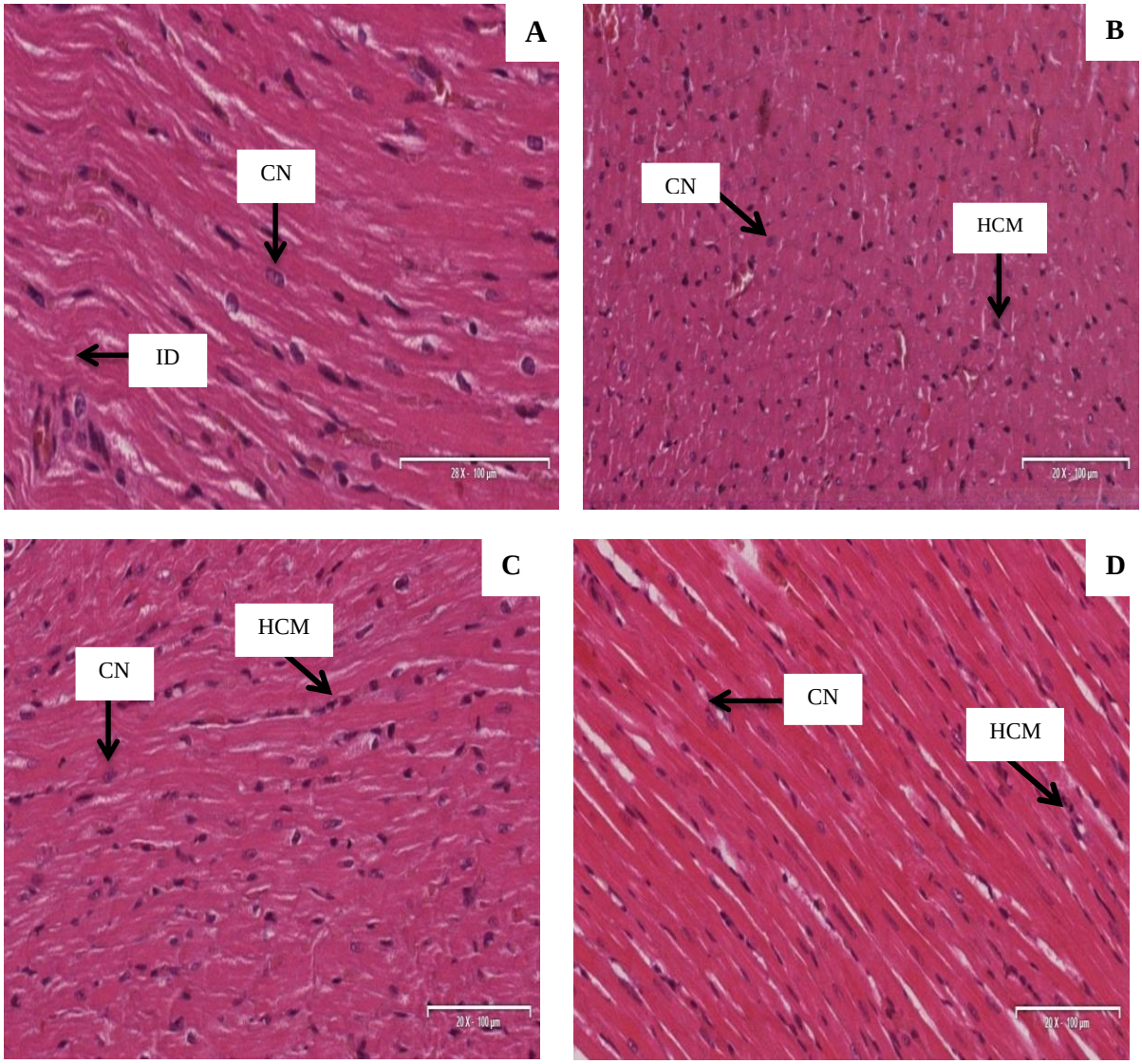

Figure 5: Photomicrograph (A) represents the normal heart of non-infected non-treated control rats. showing normal myocardial fibers, joined to each other by intercalated discs (ID), and having central nuclei (CN). Photomicrograph (5B) illustrates severe hypertrophy of cardiomyocytes (HCM) of *P. berghe*i-infected rats. Photomicrograph (5C and D) shows moderate hypertrophy of cardiomyocytes following oral CHQ (5C) and pectin CHQ matrix patch (5D) (20× 200µm).

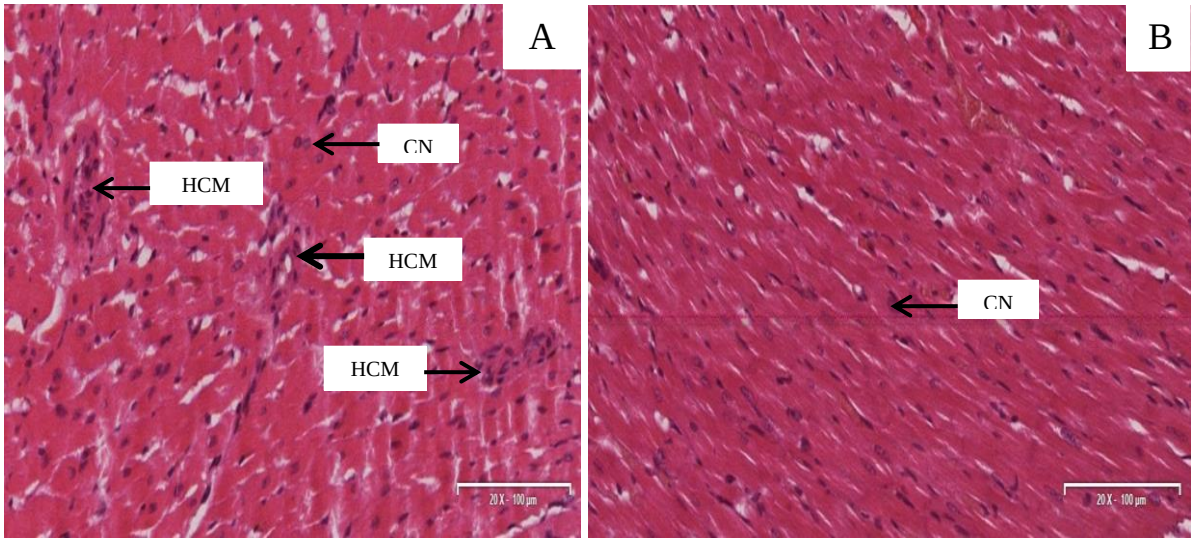

Figure 6: Figures 6 show the heart of the non-infected treated rats. Oral treated non-infected rats (Figure 6A) showed moderate hypertrophy of cardiomyocytes when compared to the non-infected pectin CHQ patch treated rats (Figure 6B). Photomicrograph 6B shows normal myocardial fibers having central nuclei of pectin CHQ patch treated rats (20× 200µm).

3.4 Spleen histology

Spleen histology was assessed in non-infected non-treated, non-infected treated, infected non-treated , infected treated with oral CHQ or transdermal patch treated rats.

Figure 7A show the structure of the untreated non-infected spleen composed of white pulp (WP) and red pulps (RP) surrounded by a capsule of dense connective tissue. Compared to the controls (Figure 7A), *P.berghei*-infected rats (Figure 7B) showed hyperplasia of the WP (HWP) and deposition of malaria pigment (HP). Photomicrograph (7C) shows *P.berghei*-infected oral CHQ treated group and photomicrograph (7D) transdermal patch treated group. In both groups WP and RP show better architecture and hence less damage than in figure 7B. Photomicrograph 8 represents the spleen of non-infected rats treated with either oral CHQ or transdermal patch. Oral treated rats (Figure 8A) showed red pulp disorganization when compared to pectin-CHQ patch treated rats (Figure 8B).

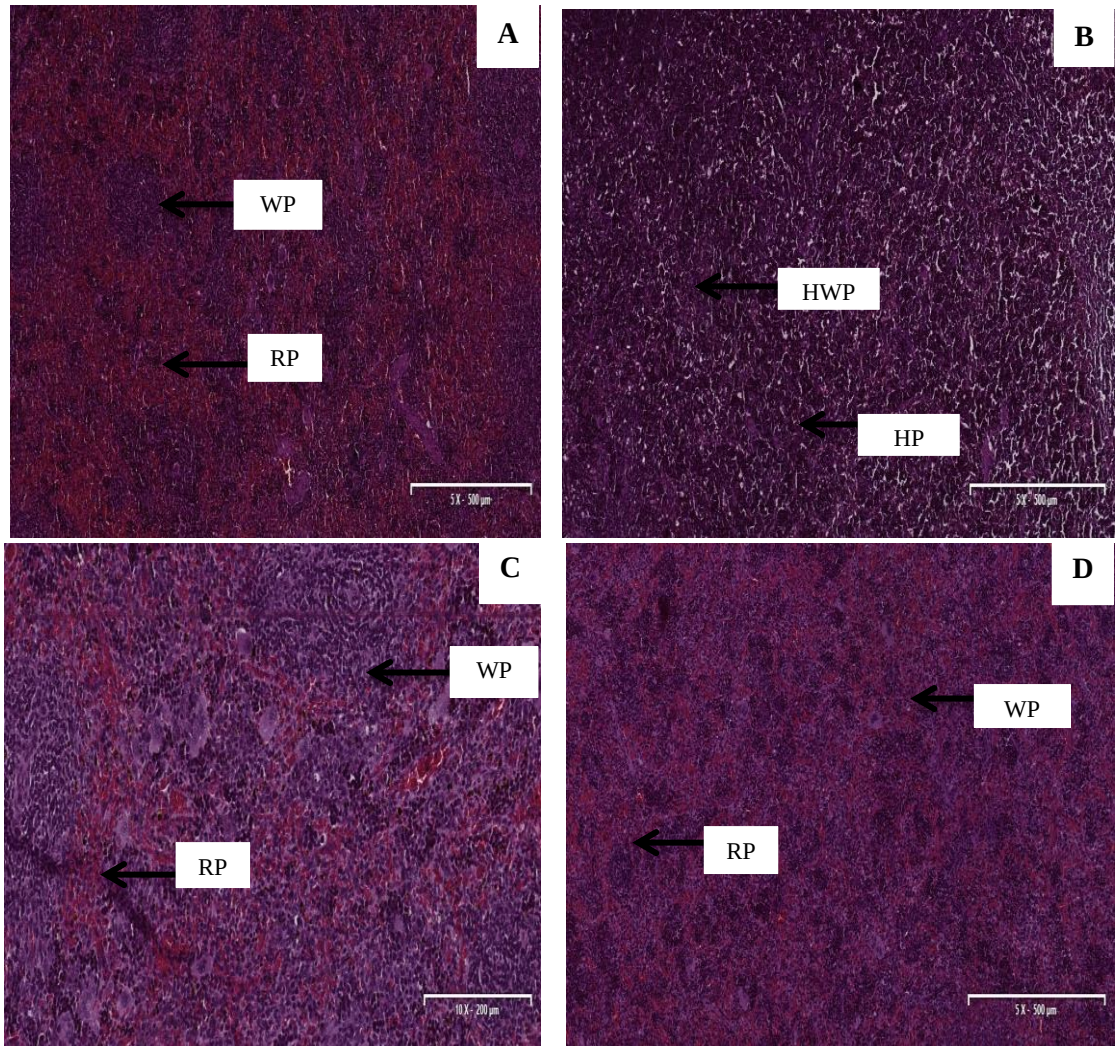

Figure 7: Photomicrograph (7A) represents the normal spleen of the control animals showing distinct white pulp (WP), embedded in a red matrix called the red pulps (RP), making up the bulk of the organ. As shown in photomicrograph (7B), there is an apparent enlargement of white pulp areas

preceding the disappearance of white and red pulp segregation. Following treatment with CHQ (Figure 7C, orally treated and Figure 7D, transdermally treated), the spleen had almost regained its normal structure and organization of white and red pulps (Mag 5× 200µm).

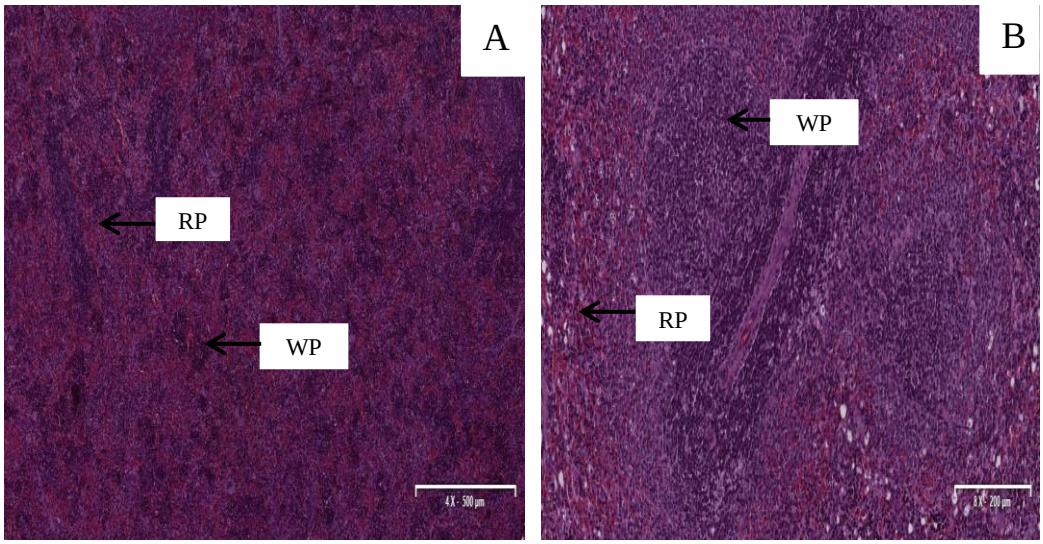

Figure 8: H and E microphotographs illustrating the morphology of the spleen of non-infected treated rats. Photomicrograph (8A) illustrates red pulp disorganization following oral CHQ treatment (Mag 4× 200µm). Photomicrograph (8B) represents the spleen of the non-infected pectin CHQ patch treated rats showing distinct white pulp, embedded in a red matrix called the red pulps.

3.5 Effects of CHQ dermal patch on the skin

Figure 9 shows the H and E skin stained sections of untreated non-infected control and *P. berghei*-infected treated with once-off topical application of the pectin CHQ matrix patch. Figure 9A represents intact secretory ducts (ISD), uninjured stratum basale (USB) and intact sebaceous glands (ISG) of the non-infected control animals. Photomicrograph B represents intact secretory ducts (ISD), uninjured stratum basale (USB) and intact sebaceous glands (ISG) of the *P. berghei* infected treated with once-off topical application of the pectin CHQ matrix patch. Compared to the non-infected control animals (Figure A), neither inflammation nor necrosis was detected in the skin as the photomicrographs revealed preserved epidermis and dermis after the treatment period.

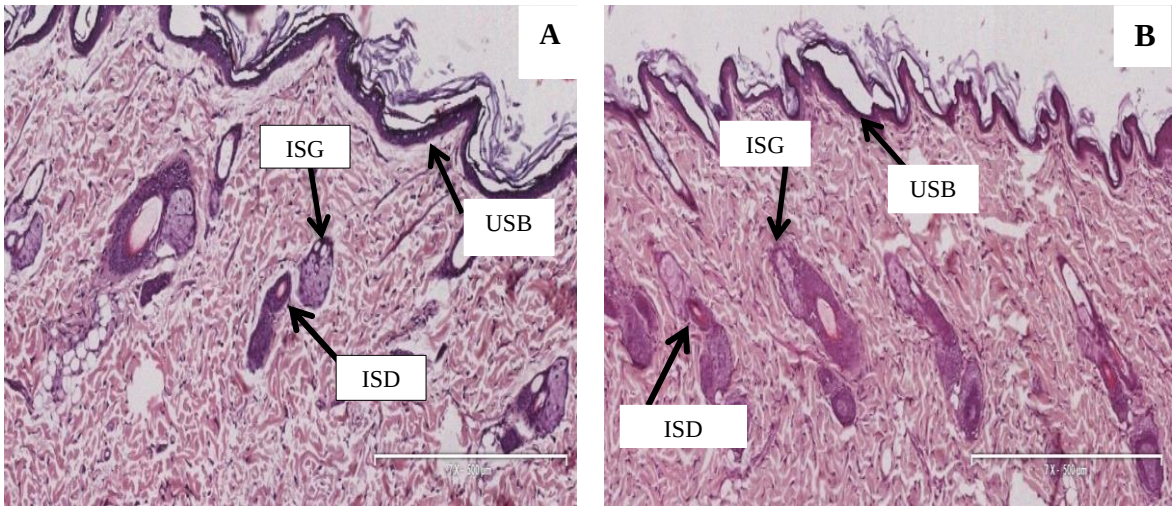

Figure 9: H and E stains illustrating the effects of CHQ-containing dermal patches on the morphology of the skin. Picture (9A) represents intact secretory ducts (ISD), uninjured stratum basale (USB) and intact sebaceous glands (ISG) of the non-infected control animals (Mag 7×500 µm). Picture B represents intact secretory ducts, uninjured stratum basale and intact sebaceous glands of the *P. berghei* infected treated with once-off topical application of the pectin CHQ matrix patch (Mag 8×500 µm).

3.6. Hepatic, heart, kidney and spleen mass

The heart mass of non-infected (NIC) control animals did not differ significantly throughout the experimental period (Table 1). The hepatic mass of non-infected animals treated with oral CHQ and pectin matrix patch did not differ significantly throughout the experimental period. There was a significant increase in hepatic mass infected groups (IC) of animals treated with both CHQ and transdermal delivery $^{\#*}$(NIC vs IO and IP, $P<0.05$, Table 1). There were no significant differences in the kidney mass of both non-infected and infected control animals, as well as the treated groups as shown in Table 1. We observed an increase in the size of the spleen of *P-berghei*-infected group when compared to our control $^{@}$ (NIC vs IC, $P<0.05$, Table 1). This increase was reversed by treatment with oral and transdermal CHQ $^{\#*}$ (NIC vs IO and IP, $P<0.05$, Table 1).

Table 1: Heart, spleen,hepatic and kidney masses of non-infected and *P-berghei*-infected groups of animals treated twice daily with oral CHQ or the pectin CHQ matrix patch, for baseline/pre-treatment (day 0-7), treatment (day 8-12) and post-treatment (day 13-21) periods.

Protocol	Parameter	Control		Oral CHQ		Patch	
		Non infected	*P. berghei* infected	Non infected	*P. berghei* infected	Non infected	*P. berghei* infected
Pre-treatment	Heart mass (g/100g b.wt)	0.45± 0.01	0.42± 0.03	0.44±0.05	0.81± 0.02	0.54± 0.03	0.57 ± 0.01
	Spleen mass (g/100g b.wt)	0.59± 0.80	1.70±0.36[@]	0.33±0.01	2.81± 0.15*	0.35 ± 0.02	2.94± 0.01[#]
	Liver mass (g/100g b.wt)	3.83± 0.08	6.72 ± 0.32	3.75±0.35	8.31± 0.05*	3.68± 0.13	7.88± 0.01[#]
	Kidney mass (g/100g b.wt)	0.80± 0.02	0.89 ± 0.01	0.41±0.03	0.83 ± 0.02	0.41 ± 0.02	0.74 ± 0.01
Treatment	Heart mass (g/100g b.wt)	0.43± 0.02	0.51± 0.07	0.54±0.07	0.94± 0.03	0.53±0.01	0.59± 0.01
	Spleen mass (g/100g b.wt)	0.51± 0.30	2.47±0.07[@]	0.47±0.07	1.38 ±0.01*	0.46±0.05	1.50 ±0.01
	Liver mass (g/100g b.wt)	3.71± 0.08	8.63± 0.40	4.94±0.38	9.51± 0.10*	4.01±0.33	7.44 ± 0.01[#]
	Kidney mass (g/100g b.wt)	0.89± 0.04	1.01 ± 0.40	0.45±0.04	0.89 ± 0.01	0.47±0.03	0.74 ± 0.01
Post-treatment	Heart mass (g/100g b.wt)	0.47± 0.07	N/A	0.52±0.54	1.04± 0.02	0.58±0.03	0.53±0.01
	Spleen mass (g/100g b.wt)	0.39± 0.10	N/A	0.41±0.48	1.01± 0.03	0.44±0.02	0.87 ± 0.1
	Liver mass (g/100g b.wt)	3.82± 0.05	N/A	4.44±0.06	6.48±0.06*	4.35±0.44	7.15± 0.03[#]
	Kidney mass (g/100g b.wt)	0.93 ±0.03	N/A	0.51±0.01	0.94± 0.01	0.46±0.02	0.47± 0.04

[@]$p<0.05$ compared to non-infected control animals(NIC)

*$p<0.05$ compared to non-infected orally treated control animals(NIC)

#$P<0.05$ compared to non-infected patch treated animals (NIP)

3.7. Effect of CHQ treatment on C-reactive protein concentration in plasma

The concentration of C-reactive protein (CRP) of non-infected non-treated animals (NIC) remained stable throughout the 21 day experimental period. However, CRP of the infected non-treated (IC) group increased during the pre-treatment and treatment periods • (NIC vs IC, P<0.05, Figure 10). Oral treatment of infected rats (IO) resulted in a decrease in CRP concentration when compared to the infected non-treated group *(IC vs IO, P<0.05, Figure 10). Similar results were obtained in animals treated with the once-off transdermal application of the CHQ patch #(IC vs IP, P<0.05, Figure 10). However, the once-off transdermal dermal patch reduced the above mentioned parameter when compared to the oral CHQ ◆ (IO vs IP, P<0.05, Figure 10).

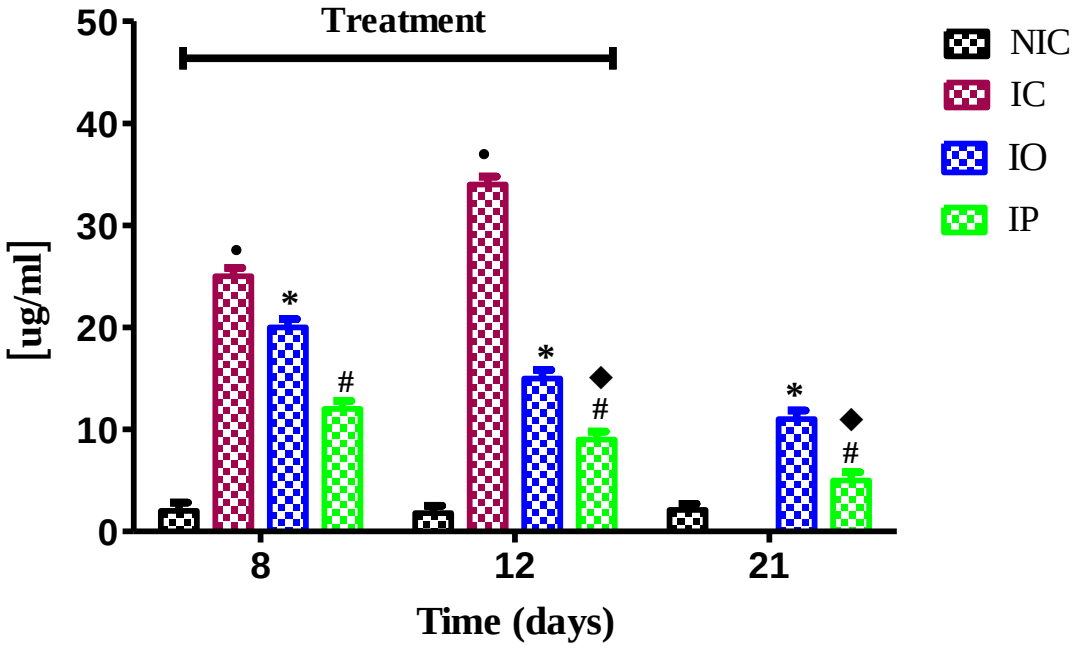

Figure 10: Comparison of the effects of *P. berghei*-infected control and non-infected control (NIC) with *P.berghei*-infected animals treated with oral CHQ (30mg/kg, twice daily) or a once off CHQ matrix patch (56mg/kg) (IO or IP) on C-reactive protein, n=6 in each group. • (NIC VS IC, P<0.05), *(IC vs IO, P<0.05), #(IC vs IP, P<0.05) and ◆ (IO vs IP, P<0.05). Values are presented as means and vertical bars indicate SEM.

4.0 Discussion

The current study investigated whether the novel CHQ-formulation that delivers sustained slow CHQ release into the systemic circulation can minimise drug deposition and organ damage in tissues and therefore alleviate the complication associated with oral CHQ administration. The current study has shown for the first time that our transdermal CHQ-formulation has no morphological effects on various visceral organs. Our findings therefore suggest that transdermal CHQ delivery has the potential to ameliorate the pathophysiological effects that are associated with oral CHQ treatment and could provide an alternative method for the management of malaria.

Oral administration of CHQ resulted in morphological changes in visceral organs. Our results correlate with previous studies which showed the deleterious effects on some vital organs following oral CHQ administration (Cooper and Magwere, 2008). CHQ is a synthetic derivative of 4-aminoquinoline that possesses both blood schizonticidal and gametocytocidal activities against *Plasmodium* parasites (Sullivan *et al.*, 1996). Due to its wide availability and accessibility, CHQ continues to be a drug of choice for treatment and as a prophylaxis against malaria despite the reported *Plasmodium* resistance (WHO, 2006). The histological findings of the present study have shown hyperplastic kupffer cells with scattered haemozoin deposition and portal inflammation as the most common histological changes in the livers of the *P.berghei*-infected group. This damage to the liver is suggested to be due to the invasion of hepatic cells by the parasites during replication (Dekker *et al.*, 1997). It has been shown that during replication, the *Plasmodium* parasites invade hepatic cells where they undergo proliferation and morphological changes (Dekker *et al.*, 1997). This increased migration and replication is associated with hepatocellular damage (Dekker *et al.*, 1997). The oral CHQ treated group showed moderate hyperplastic kupffer cells, portal tract inflammation and moderate deposition of haemozoin pigment when compared to the infected control rats. In addition, oral CHQ treated resulted in mild morphological changes in non-infected rats. Our results are consistent with the previously reported oral CHQ induced hepatocellular damage (Cooper and Magwere, 2008). We speculate that these changes could be attributed to the increased deposition of CHQ in the liver following oral treatment. This accumulation of CHQ in the liver following oral administration has been reported to cause hepatocellular damage (McChesney *et al.*, 1976). Furthermore, CHQ has been found to decrease the availability of reduced glutathione to pathways involved in detoxification which reacts with ferriprotoporphyrin IX which produces highly reactive radicals that generate oxidative stress in the host (Magwere *et al.*, 1997). Indeed, previous studies in our laboratory have reported increased malondialdehyde (MDA) concentrations, diminished superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities following CHQ administration (Thaane, 2014). Hence we speculate that the hepatocellular damage present following oral CHQ administration may have been mediated partly by the production of reactive oxygen species. Transdermal delivery of CHQ showed mild reduction in morphological changes in the organs

of *P.berghei*-infected rats. We speculate that the morphological changes observed in the histology of *P.berghei*-infected animals treated transdermally might be attributed to the malaria parasite and not the treatment. This is supported by the fact that no morphological changes in the histology of the liver were observed in non-infected animals administrated CHQ transdermally. We speculate that the absence of effects on the morphology of the liver is due to the fact that in transdermally treated animals, the drug enters the circulation first before being metabolised in the liver while with oral treatment undergoes first pass metabolism by the liver before drug delivery into the systemic circulation. Since there is no first pass metabolism by the liver, low drug concentrations are required resulting in minimal tissue deposition and hence minimal damage. The liver masses of the infected control animals and the oral CHQ treated infected animals were increased by comparison to non-infected control animals and the pectin CHQ matrix patch treated animals. This observation was consistent with previously reported CHQ induced hepatocellular damage (Cooper and Magwere, 2008). This observation suggests that the toxic effects of oral CHQ may be circumvented by the slow release in a controlled manner of CHQ from our transdermal formulation.

Morphological changes in the kidney were assessed in non-infected and infected animals treated with either oral or transdermally administered CHQ. Results from the present study show that oral CHQ causes defects in the microscopic structure of the kidney in *P.berghei*-infected and non-infected rats. This is substantiated by Musabayane *et al*., 2003, who reported that the malaria parasite and/oral CHQ treatment impaired kidney function resulting in inappropriate renal fluid and electrolyte handling. These changes are believed to be attributed to the increased deposition of CHQ in the heart and kidney following treatment (Dondo and Mubagwa, 1990). Furthermore, Musabayane *et al.*, 1993 suggested that CHQ may influence kidney function resulting in inappropriate renal fluid and electrolytes handling. These changes are believed to be attributed to hepatocellular damage by both the malaria parasite and/ CHQ due to increased deposition of CHQ in tissues such as the heart and kidney following treatment with CHQ administration (Dondo and Mubagwa, 1990; Baguet and Fabre, 1999). It has been shown that about 51% of CHQ is cleared by the kidney unchanged (McChesney *et al.*, 1976). However, a small fraction of CHQ accumulates in the epithelial cells of the kidneys (Gustafsson *et al.*, 1983). This deposition of CHQ in the kidneys is reported to alter kidney function (McChesney *et al.*, 1976). In addition, some of the administered CHQ is deposited in the heart (Baguet and Fabre, 1999) where CHQ exerts antiarrythmic actions to increase heart rate (Dondo and Mubagwa, 1990). The increase in heart rate is thought to alter the perfusion pressure of the kidney, amend renal haemodynamics and electrolyte handling (Dondo and Mubagwa, 1990). Transdermal delivered of CHQ attenuated the features observed in *P.berghei*-infected animals. In addition, no morphological effects were observed in non-infected animals treated with the CHQ patch. These results are suggestive of the ability of the pectin-CHQ matrix patch to provide slow,

sustained CHQ release into the circulation, thereby avoiding drug dumping in the kidneys, which might alter kidney morphology. Our results indicate that transdermal delivery of CHQ via the pectin-CHQ matrix patch has the potential to avert the reported oral CHQ-induced adverse effects on renal fluid and electrolyte handling.

Histological results showed moderate hypertrophy of cardiomyocytes upon oral administration in *P.berghei*-infected rats. These morphological effects observed following oral CHQ were also observed in non-infected rats treated with CHQ, confirming that the drug's cability to affect cardiomyocytes is independent of the malaria infection. As an antimalarial, CHQ acts by inhibiting hemozoin biocrystallization which gives rise to toxic free haem accumulation that is responsible for the death of the parasites (Ginsburg *et al.*, 1998). Haem (iron protoporphyrin IX) is essential in a number of biological processes (Beri and Chandra, 1993). It has been previously shown that haem can directly attack and may impair intracellular targets including the lipid bilayer, the cytoskeleton, intermediary metabolic enzymes, and DNA (Wagener *et al.*, 2003). High concentrations of free haem have also been shown to cause severe toxic effects to the kidney, liver, and cardiac tissues (Kumar and Bandyopadhyay, 2005). Furthermore, free haem undergoes oxygenation resulting in the formation of oxygen free radicals (Francis and Goldberg, 1997). The role of oxidative stress has been suggested in the pathogenesis of heart dysfunction (Piano, 2002). Based on these findings, the current study complements the notion that CHQ may have acted through the generation of excess free haem or reactive oxygen species to induce the cardiomyocyte hypertrophy observed in the treated group. The following results were validated by the increase in CRP concentration. C-reactive protein which is an acute phase protein, is a component of innate immune response and is useful in early detection of inflammation (Kolb-Bachofen, 1991). Measurement of serum CRP concentration is most frequently used for the evaluation of injury in tissues or for the detection of an inflammatory event somewhere in the body (Kushner and Kaplan, 1961). In this study we have shown that CRP concentration is increased in *P.berghei*-infected rats and in rats receiving oral CHQ administration. We speculate that this may be attributed to the lysosomotropic properties of this anti-malarial drug (Allison and Young, 1964). CHQ has been reported to bind and accumulate in various organs including the heart, liver kidneys and spleen (Gustafsson *et al.*, 1987). We speculate that the deposition of CHQ in the organs following oral CHQ administration elicit inflammation or tissue destruction therefore the secretion of CRP observed in this study. The absence of CRP effect in CHQ patch treated animals validates the sustained controlled release of CHQ into the systemic circulation therefore minimising CHQ dumping and tissue damage in these organs.

The spleen is a secondary lymphoid organ, important for immunity and blood filtration and has been reported to decrease parasitaemia (Alves *et al.*, 1996). It is the major site of elimination of parasite-infected erythrocytes (Angus *et al.*, 1997). The spleen is greatly enlarged during malaria in experimental animals and splenomegaly is used as a measure of malaria endemicity (Neva *et al.*,

1970). The current study observed an increase in the size of the spleen in the *P-berghei*-infected groups when compared to the control group. Hyperplasia of the red pulp zone (RP) and white pulp zone (WP) explains the increase in infected spleen size. The delivery of CHQ (orally and transdermal) reduced the size of the spleen. We speculate that this reduction in size of the spleen may be due to the elimination of the malaria parasites from systemic circulation by CHQ. Red pulp disorganization was observed following oral CHQ administration in non-infected rats when compared to CHQ patch treated animals. These results are supported by Muheert, 2013 who showed red pulp disorganization following oral CHQ treatment (Muheet, 2013). The morphological changes of the kidney, liver, heart and spleen in rats treated with oral CHQ are more severe than those seen in rats treated with the transdermal CHQ-formulation. The controlled and sustained release of CHQ from our transdermal formulation, minimised the challenges of dose dumping associated with oral CHQ delivery, suggesting the transdermal treatment could avert the complication associated with oral CHQ on visceral organs.

Conclusion

The results of our current study have shown that the use of the pectin-CHQ matrix patch may alleviate the side-effects that are associated with CHQ dumping in vital tissues following oral administration of CHQ. The current study has demonstrated the feasibility of the use of the pectin CHQ matrix patch as adjunct treatment or as alternative to oral treatment in the management of malaria

References

1 Allison A and Young M. Uptake of dyes and drugs by living cells in culture. *Life Sci;* 1964; **3**: 1407.

2 Alves H, Weidanz W and Weiss L. The spleen in murine *Plasmodium* chabaudi adami malaria: stroma cells, T lymphocytes, and hematopoiesis. *Am J Trop Hyg* 1996; **55**: 370-378.

3 Angus B, Chotivanich K, Udomsangpetch R and White N. In vivo removal of malaria parasites from red blood cells without their destruction in acute *falciparum* malaria. *Blood;* 1997; **90**: 2037–2040.

4 Baguet J and Fabre F. Chloroquine cardiomyopathy with conduction disorders *Heart;* 1999; **81**: 221-223.

5 Beri R and Chandra R. Chemistry and biology of heme: effect of metal salts, organometals, and metalloporphyrins on heme synthesis and catabolism, with special reference to clinical implications and interactions with cytochrome *Drug Metabolism Reviews;* 1993; **25**: 49-152.

6 Cooper R and Magwere T. Chloroquine Novel uses & manifestations. *Indian J Med Res;* 2008; **127**: 305-316.

7 Dekker E, Hellersstein M, Romijn J, Neese R, Peshu N and Endert E. Glucose homeostasis in children with falciparum malaria: precursor supply limits gluconeogenesis and glucose production. *Journal of Clinical Endocrinology and Metabolism;* 1997; **82**: 2514-2521.

8 Dondo F and Mubagwa K. Chloroquine interacts with muscarinic receptors and inhibits cholinergic effects in the heart. *African Journal of Medical Science;* 1990; **19**: 237-243.

9 Francis S and Goldberg D. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annual Review of Microbiology;* 1997; **51**: 97-123.

10 Ginsburg H, Famin O, Zhang J and Krugliak M. Inhibition of glutathione dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochemistry and Pharmacology;* 1998; **56**.

11 Gumede B, Folbb P and Ryffela B. Oral artesunate prevents *Plasmodium berghei* ANKA infection in mice. *Parasitology International;* 2003; **52**: 53-59.

12 Gustafsson L, Walker O, Alvan G, Beermann B, Estevez F, Gleisner L *et al*. Disposition of chloroquine in man after single intravenous and oral doses. *British Journal of Clinical Pharmacology* 1983; **15**: 471-479.

13 Gustafsson L, Lindstrom B, Grahnen A and Alvan G. Chloroquine excretion following malaria prophylaxis. *British Journal of Clinical Pharmacology;* 1987; **24**: 221-224.

14 Kolb-Bachofen V. A review on the biological properties of C-reactive protein. *Immunobiology* 1991; **183**: 133–145.

15 Kumar S and Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett;* 2005; **157**: 175-188.

16 Kushner I and Kaplan M. Studies of acute phase protein. I. An immunohistochemical method for the localization of Cx-reactive protein in rabbits. Association with necrosis in local inflammatory lesions. *J Exp Med* 1961; **114**: 961 –974.

17 Magwere T, Naik Y and Hasler J. Effects of chloroquine treatment on antioxidant enzymes in rat liver and kidney. *Free Radical Biology and Medicine;* 1997; **22**: 321-327.

18 McChesney E, Banks W and Fabian R. Tissue distribution of chloroquine, hydroxychloroquine, and desethylchloroquine in the rat. *Toxicology and Applied Pharmacology* 1976; **10**: 501-513.

19 Muheet A. Studies on chronic administration of chloroquine on gastrocnemius muscle and spleen of Swiss albino mice. *African Journal of Pharmacy and Pharmacology;* 2013; **7**: 524-527.

20 Munjeri O, P H, Osim E and Musabayane C. An investigation into the suitability of amidated pectin hydrogel beads as a delivery matrix for chloroquine. *Journal of Pharmaceutical Sciences;* 1998; **87**: 905-908.

21 Musabayane C, Ndhlovu C, Mamutse G, Bwititi P and Balment R. Acute chloroquine administration increases renal sodium excretion. *Journal of Tropical Medicine and Hygiene;* 1993; **96**: 305-310.

22 Musabayane C, Munjeri O and Matavire B. Transdermal delivery of chloroquine by amidated pectin hydrogel matrix patch in the rat. *Renal Failure;* 2003; **25**: 525-534.

23 Neva F, Sheagren J, Shulman N and Canfield C. Malaria: host-defense mechanisms and complication. *Ann Intern Med;* 1970; **73**: 295–306.

24 Pepys M and Baltz M. Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. *Adv Immunol;* 1983; **34**: 141–212.

25 Piano R. Alcohol and heart failure. *Journal of Cardiac Failure;* 2002; **8**: 239–246.

26 Sullivan D, Gluzman I, Russell D and Goldberg D. On the molecular mechanism of chloroquine's antimalarial action. *Proceedings of the National Academy of Sciences of the United States of America;* 1996; **93**: 11865–11870.

27 Thaane T. Evaluation of the efficacy of Masilinic acid on malaria parasites in *plasmodium berghei*-infected rats: Effects on blood glucose and renal fluid electrolyte handling. Masters thesis, University of Kwa-Zulu Natal, 2014.

28 Wagener F, Volk H, Willis D, Abraham N, Soares M, Adema G *et al*. Different faces of hemeoxygenase system in inflammation. *Pharmacology Review;* 2003; **55**: 551-571.

29 White N. Antimalarial drug resistance. *The Journal of Clinical Investigation* 2004; **113**: 1084 - 1092.

30 WHO. Guidelines for the treatment of malaria (1st ed.). *World Health Organization, Geneva;* 2006; **Report no WHO/HTM/MAL/2006.1108**.

Chapter 4

4.0 Synopsis

The main purpose of the current study was to investigate and compare the effects of using oral chloroquine (CHQ) treatment or a transdermal CHQ patch in the management of malarial infection. To this effect, we looked at changes in haematological parameters as well as plasma cytokine concentrations in male Sprague-Dawley rats. We also looked at the morphological effects on the histology of various visceral organs following malarial infection and subsequent treatment with CHQ. In this study we used a *Plasmodium berghei* model of malaria infection. This rodent model for malaria is considered a good experimental model as it has pathophysiological similarities to *plasmodium* species that infect human beings. CHQ phosphate is a 4-aminoquinoline antimalarial drug that has shizonticidal properties. This antimalarial drug continues to be the major chemotherapeutic agent used in the treatment and prophylaxis of malaria in most malaria endemic tropical countries. The success of this drug is believed to be attributed to the mechanism of action. However, when administered orally, CHQ undergoes the first-pass metabolism by the liver, where the drug undergoes degradation by the liver, decreasing its concentration. As a result of this first-pass metabolism, higher initial drug concentrations are required in order to bring about therapeutic effects. When high concentrations of CHQ are administered this drug has been shown to accumulate in epithelia cells of vital organs disrupting a number of physiological systems and can results onto a number of adverse effects.

To evaluate the effect of CHQ treatment on malarial anaemia, haematological parameters as well as plasma cytokines were monitored in separate groups of non-infected and *P.berghei*-infected male Sprague-Dawley rats treated with orally administrated or topical application of CHQ via pectin matrix patch. The results of the present study showed that both malaria infection and oral CHQ delivery resulted in a reduction in the concentration of red blood cell (RBC) count, haematocrit (HCT), haemoglobin (HGB), red cell distribution width (RDW) and mean corpuscular haemoglobin (MCH). Studies have reported various mechanisms that contribute to anaemia during malarial infection. The decrease in RBC, HCT and HGB in malaria may be attributed to the uncontrolled destruction of RBC's by the plasmodium parasite. Normally RBCs are replaced by erythropoiesis however this is also affected in malaria therefore this imbalance between destruction and replacement of RBC's is believed to be one of the mechanisms resulting into persistent anaemia. Oral CHQ treatment reduced RBC indices of both malaria infected and non-infected animals. The observation is consistent with the previous reports suggesting quinoline drugs to act as haptens and binds with specific RBC proteins to elicit an immune response leading to the destruction of red blood cells, thus aggravating anaemia. Furthermore, the increase in CRP following oral treatment validates the above results on the effects of oral CHQ on anaemia. These results correlate with a recent study which showed that binding of CRP

to infected red blood cells (RBCs) increased the removal of damaged RBCs from the circulation which could lead to a more pronounced anaemia. Interestingly, topical application of pectin CHQ matrix patch did not alter RBC indices of non-infected animals, suggestive of the ability of the patch to circumvent the toxic effects of oral CHQ delivery. The current study further elucidated the mechanisms through which RBC indices were reduced in the infected and oral CHQ treated animals. The current study measured plasma cytokines to give clear mechanism of the observed changes in RBC indices.

Studies have reported various mechanisms that contribute to malarial aneamia including the increase in the concentration of pro-inflammatory cytokines such as TNF-α and IL-10. Studies have shown that elevated TNF-α cytokine concentration contributes to bone marrow suppression and red cell destruction, whereas elevated IL-10 is thought to stimulate hematopoiesis. Our results showed an increase in the concentration of the concentration of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in *P.berghei*-infected rats. Following treatment with CHQ a decrease in the concentration of these cytokines was observed. During day 12 of treatment and during post-treatment period there was an increase in pro-inflammatory cytokine concentration in oral CHQ treated animals, thus aggravating anaemia. The reported increase in pro-inflammatory cytokine concentration in the current study may be attributed to the increased deposition of CHQ in tissues such as the heart, liver, spleen and kidney following treatment with oral CHQ discussed below. Treatment with the pectin-CHQ matrix patch resulted in a decrease in pro-inflammatory cytokine concentration and therefore no anaemia in these animals. This suggests better management of the drug by the body in this mode of treatment.

Findings from the current study have shown for the first time that our transdermal CHQ delivery system does not result in morphological changes to the liver, kidney, heart and spleen in non-infected animals while in orally treated animals, slight morphological changes were observed in the organs. Also, both the oral and the transdermal delivery methods were able to attenuate the morphological changes associated with a malarial infection in these organs. This suggests that our CHQ concentration in the patch was able to decrease the effects of parasiteamia while preserving organ function, a novel finding. These findings therefore suggest that transdermal CHQ delivery has the potential to ameliorate the pathophysiological effects that are associated with oral CHQ treatment and could therefore be used in conjunction with or as an alternative treatment in the management of malaria.

4.1 Conclusion

The results of the current study have demonstrated that the once off application of the CHQ-formulation has no morphological effects when compared to oral administration of CHQ on various organs. The ability of the pectin-CHQ matrix patch to provide slow, sustained CHQ releases into the circulation, avoids drug dumping in various tissue organs therefore circumventing the adverse effects associated with oral administration of CHQ. In addition, our results show that both CHQ and malaria parasite result in the development of anaemia by affecting RBCs and plasma cytokine concentration. Topical application of pectin CHQ matrix patch did not alter RBC indices of non-infected animals, suggestive of the ability of the patch to circumvent the toxic effects of oral CHQ delivery. The results presented in this thesis suggest that topical application of pectin CHQ matrix does not only avoid drug dumping in various tissue organs, but also may circumvent the malaria associated anaemia.

4.2 Recommendations

We have demonstrated for the first time that our transdermal CHQ delivery system does not result in morphological changes to the liver, kidney, heart and spleen, while in orally treated animals, slight morphological changes were observed on the organs. Future studies should, therefore, assess inflammatory biomarkers in these tissue organs. In the present study we have reported increased pro-inflammatory cytokines following oral CHQ administration. Oxidative stress could be an important mediator of anaemia. Further studies should therefore measure such markers. Studies that will elucidate the possible mechanisms responsible for the reduction in pro-inflammatory cytokines are required. For instance, measurements of hepcidin, a recently discovered peptide hormone, that plays a major role in iron regulation and is thought to play a central role in the anaemia of chronic inflammation.

5.0 References

1 Abraham R, Hendy R and Grasso P. Formation of myeloid bodies in rat liver lysosomes after chloroquine administration. *Exp Mol Path;* 1968; **9**: 212-229.

2 Alexander R. Inflammation and coronary artery disease. *New Engl J Med;* 1994; **331**: 468 – 469.

3 Allison A and Young M. Uptake of dyes and drugs by living cells in culture. *Life Sci;* 1964; **3**: 1407.

4 Alves H, Weidanz W and Weiss L. The spleen in murine *Plasmodium* chabaudi adami malaria: stroma cells, T lymphocytes, and hematopoiesis. *Am J Trop Hyg* 1996; **55**: 370-378.

5 Angus B, Chotivanich K, Udomsangpetch R and White N. In vivo removal of malaria parasites from red blood cells without their destruction in acute *falciparum* malaria. *Blood;* 1997; **90**: 2037–2040.

6 Aringer M and Smolen J. SLE – complex cytokine effects in a complex autoimmune disease: tumor necrosis factor in systemic lupus erythematosus. *Arthritis Res Ther;* 2003; **5**: 172–177.

7 Armitage A, Pinches R, Eddowes L, Newbold C and Drakesmith H. *Plasmodium falciparum* infected erythrocytes induce hepcidin (HAMP) mRNA synthesis by peripheral blood mononuclear cells. *British Journal of Haematology;* 2009; **147**: 769–771.

8 Baguet J and Fabre F. Chloroquine cardiomyopathy with conduction disorders *Heart;* 1999; **81**: 221-223.

9 Ballou S and Kushner I. C-reactive protein and the acute phase response. *Adv Intern Med;* 1992; **37**: 313–336.

10 Baughman R, Lower E and Du Bois R. Sarcoidosis. *Lancet* 2003; **361**: 1111–1118.

11 Beri R and Chandra R. Chemistry and biology of heme: effect of metal salts, organometals, and metalloporphyrins on heme synthesis and catabolism, with special reference to clinical implications and interactions with cytochrome *Drug Metabolism Reviews;* 1993; **25**: 49-152.

12 Bjo¨rkman A. Malaria associated anaemia, drug resistance and antimalarial combination therapy. *International Journal for Parasitology;* 2002; **32**: 1637–1643.

13 Braczkowski R, Romanawsky W, Danikiewicz A, Muc-Wierzgon M, Blazelonis A and Zubelewicz B. Decrease of erythropoietin level by human recombinant tumour necrosis factor alpha in patients with advanced cancer. *J Biol Regul Homeost;* 2001; **15**: 366–369.

14 Breman JG, Alilio MS and Mills A. Conquering the Intolerable Burden of Malaria: What's New, What's Needed: A Summary. *American Journal of Tropical Medicine and Hygiene;* 2004; **71**: 23-79.

15 Cerletti C, Tamburrelli C, Izzi B, Gianfagna F and de Gaetano G. Platelet-leukocyte interactions in thrombosis. *Thrombosis Research;* 2012; **129**: 263–266.

16 Chang K and Stevenson M. Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. *International Journal for Parasitology* 2004; **34**: 1501–1516.

17 Chaturvedi P and Chaturvedi M. Effect of African potato (Hypoxis hemerocallidea) extract on oxidative stress induced by chloroquine in Albino rats. *African Journal of Food, Agriculture, Nutrition and Development;* 2011; **11**: 76-89.

18 Chen G and Goeddel D. TNF-R1 Signaling: A Beautiful Pathway. *Science;* 2002; **296**: 1634-1635.

19 Chirico E and Pialoux V. Role of oxidative stress in the pathogenesis of sickle cell disease. *International Union of Biochemistry and Molecular Biology Life* 2012; **64**: 72-80.

20 Clark I, Virelizier J, Carswell E and Wood P. Possible importance of macrophage-derived mediators in acute malaria. *Infect Immun;* 1981; **32**: 1058-1066.

21 Clark I and Cowden W. The pathophysiology of *P. falciparum* malaria. *Pharmacology and Therapeutics;* 2003; **99**: 221-260.

22 Clark I, Budd A, Alleva L and Cowden W. Human malarial disease: A consequence of inflammatory cytokine release. *Malaria Journal (Biomed Central);* 2006; **10**: 1186/1475-2875-1185-1185.

23 Cooper R and Magwere T. Chloroquine Novel uses & manifestations. *Indian J Med Res;* 2008; **127**: 305-316.

24 Dekker E, Hellersstein M, Romijn J, Neese R, Peshu N and Endert E. Glucose homeostasis in children with falciparum malaria: precursor supply limits gluconeogenesis and glucose production. *Journal of Clinical Endocrinology and Metabolism;* 1997; **82**: 2514-2521.

25 Dondo F and Mubagwa K. Chloroquine interacts with muscarinic receptors and inhibits cholinergic effects in the heart. *African Journal of Medical Science;* 1990; **19**: 237-243.

26 Drakesmith H and Prentice A. Hepcidin and the iron-infection axis. *338;* 2012; **Science**: 768-772.

27 Dreyfuss M, Stoltzfus R, Shrestha J, Pradhan E, LeClerq S, Khatry S *et al*. Hookworms, Malaria and Vitamin A deficiency contribute to anemia and Iron deficiency among pregnant women in the plains of Nepal. *J Nutr;* 2000; **130**: 2527-2536.

28 Ekvall H. Malaria and anemia. *Curr Opin Hematol;* 2003; **10**: 108-114.

29 Eric M, Kevin M and Laird J. Determinants of Anemia among Preschool Children in Rural, Western Kenya. *Am J Trop Med Hyg;* 2013; **88**: 757–764.

30 Faquin W, Schneider T and Goldberg M. Effect of inflammatory cytokines on hypoxia-induced erythropoietin production. *Blood;* 1992; **79**: 1987–1994.

31 Francis S and Goldberg D. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annual Review of Microbiology;* 1997; **51**: 97-123.

32 Francis S, Sullivan DJ and Goldberg D. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu Rev Microbiol* 1997; **51**: 97–123.

33 Fujiwara N and Kobayashi K. Macrophages in Inflammation. *Current Drug Targets - Inflammation & Allergy;* 2005; **4**: 281-286.

34 Gabay C and Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 1999; **340**: 448–454.

35 Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood;* 2003; **102**: 783–788.

36 Ganz T. Hepcidin—a regulator of intestinal iron absorption and iron recycling by macrophages. *Best Pract Res Clin Haematol;* 2005; **18**: 171–182.

37 Ganz T. Hepcidin and the Global Burden of Iron Deficiency. *Clinical Chemistry;* 2015; **61**: 577-578

38 Ginsburg H, Famin O, Zhang J and Krugliak M. Inhibition of glutathione dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochemistry and Pharmacology;* 1998; **56**: 1305-1313.

39 Ginsburg H, Famin O, Zhang J and Krugliak M. Inhibition of glutathione dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochemistry and Pharmacology;* 1998; **56**.

40 Gumede B, Folbb P and Ryffela B. Oral artesunate prevents *Plasmodium berghei* ANKA infection in mice. *Parasitology International;* 2003; **52**: 53-59.

41 Gustafsson L, Walker O, Alvan G, Beermann B, Estevez F, Gleisner L *et al*. Disposition of chloroquine in man after single intravenous and oral doses. *British Journal of Clinical Pharmacology* 1983; **15**: 471-479.

42 Gustafsson L, Lindstrom B, Grahnen A and Alvan G. Chloroquine excretion following malaria prophylaxis. *British Journal of Clinical Pharmacology;* 1987; **24**: 221-224.

43 Haddad J. Cytokines and related receptor-mediated signaling pathways *Biochemical and Biophysical connunications;* 2002; **297**: 700-713.

44 Hadebe S, Ngubane P, Serumula M and Musabayane C. Transdermal Delivery of Insulin by Amidated Pectin Hydrogel Matrix Patch in Streptozotocin-Induced Diabetic Rats: Effects on Some Selected Metabolic Parameters. *Plos one;* 2014; **9**: 1-10.

45 Haldar K and Mohandas N. Malaria, erythrocytic infection and anemia. *American Society of Hematology;* 2009: 89-93.

46 Hedayat M, Mahmoudi M, Rose N and Rezaei N. Proinflammatory cytokines in heart failure: double-edged swords. *Heart Fail Rev;* 2010; **15**: 543–562.

47 Howard C, McKakpo U, Quakyi I, Bosompem K, Addison E and Sun K. Relationship of hepcidin with parasitemia and anaemia among patients with uncomplicated *Plasmodium falciparum* malaria in Ghana. *Blood;* 2007; **121**: 3016-3022.

48 Jacobs P, Radziach P and Stevenson M. A Th1-associated increase in tumor necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice. *Infect Immun* 1996; **64**: 535-541.

49 Jakubowski JA, Thompson C, Vaillancourt R, Valeri C and Deykin D. Arachidonic acid metabolism by platelets of differing size. *British Journal of Haematology;* 1983; **53**: 503-551.

50 Jang C, Choi J, Byun M and Jue D. Chloroquine inhibits production of TNF-alpha, IL-1beta and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes. *Rheumatology;* 2006; **45**: 703-710.

51 Jaye D and Waites K. Clinical applications of C-reactive protein in pediatrics. *Pediatr Infect Dis J* 1997; **16**: 735– 747.

52 Jelkmann W, Fandrey J, Frede S and Pagel H. Inhibition of erythropoietin production by cytokines. Implications for the anemia involved in inflammatory states. *Ann NY Acad Sci;* 1994; **718**: 300–309.

53 Jürgen S, Athena C, Dirk S and Stefan R. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochima et Biophysica Acta;* 2011; **1813**: 878-888.

54 Kogstad D and Schlesinger P. A perspective on antimalarial action: effects of weak bases on *Plasmodium Falciparum*. *Biochem Pharmacol;* 1986; **5**: 547-552.

55 Kolb-Bachofen V. A review on the biological properties of C-reactive protein. *Immunobiology* 1991; **183**: 133–145.

56 Kolb-Bachofen V. A review on the biological properties of C-reactive protein. *Immunobiology* 1991; **183**: 133– 145.

57 Kumar A. Thrombocytopenia- an indicator of acute vivax malaria. *Indian J Pathol Microbiol;* 2006; **49**: 505-508.

58 Kumar S and Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett;* 2005; **157**: 175-188.

59 Kurtzhals J, Adabayeri V, Goka B, Akanmori B, OliverCommey J, Nkrumah F *et al.* Low plasma concentrations of interleukin 10 in severe malarial anemia compared with cerebral and uncomplicated malaria. *Lancet;* 1998; **351**: 1768–1772.

60 Kushner I and Kaplan M. Studies of acute phase protein. I. An immunohistochemical method for the localization of Cx-reactive protein in rabbits. Association with necrosis in local inflammatory lesions. *J Exp Med* 1961; **114**: 961 –974.

61 Lyke K, Burges R, Cissoko Y, Sangare L, Dao M, Diarra I *et al.* Serum Levels of the Proinflammatory Cytokines Interleukin-1 Beta (IL-1), IL-6, IL-8, IL-10, Tumor Necrosis Factor Alpha, and IL-12(p70) in Malian Children with Severe *Plasmodium falciparum* Malaria and Matched Uncomplicated Malaria or Healthy Controls. *Infection and Immunity;* 2004; **72**: 5630–5637.

62 Magwere T, Naik Y and Hasler J. Effects of chloroquine treatment on antioxidant enzymes in rat liver and kidney. *Free Radical Biology and Medicine;* 1997; **22**: 321-327.

63 Mbajiorgu E, Aire T, Volk W, Albert M and Debusho L. Haematological profile of male rats treated with ethanol and/ or Chloroquine and fed normal or low Protein diet. *The Internet Journal of Hematology;* 2006; **3**: 1-11.

64 McChesney E, Banks W and Fabian R. Tissue distribution of chloroquine, hydroxychloroquine, and desethylchloroquine in the rat. *Toxicology and Applied Pharmacology* 1976; **10**: 501-513.

65 McDevitt M, Xie J and Gordeuk V. The anemia of malaria infection: role of inflammatory cytokines. *Current Hematology;* 2004; **3**: 97–106.

66 Means R. The anaemia of infection. *Baillie ´re's Best Pract Res Clin Haematol;* 2000; **13**: 151–162.

67 Mohamed-Ali V, Pinkney J and Coppack S. Adipose tissue as an endocrine and paracrine organ. *Int J Obesity* 1998; **22**: 1145-1158.

68 Moldawer L, Marano M, Wei H, Fong Y, Silen M, Kuo G *et al.* Cachectin/tumor necrosis factor-alpha alters red blood cell kinetics and induces anemia in vivo. *Fed Am Soc Exp Biol J* 1989; **3**: 1637–1643.

69 Moss D, Fargion S, Fracanzani A, Levi S, Cappellini M, Arosio P *et al.* Functional role of the ferritin receptors of human liver, hepatoma, lymphoid and erythroid cells. *J Inorg Biochem;* 1992; **47**: 219–227.

70 Muheet A. Studies on chronic administration of chloroquine on gastrocnemius muscle and spleen of Swiss albino mice. *African Journal of Pharmacy and Pharmacology;* 2013; **7**: 524-527.

71 Munjeri O, P H, Osim E and Musabayane C. An investigation into the suitability of amidated pectin hydrogel beads as a delivery matrix for chloroquine. *Journal of Pharmaceutical Sciences;* 1998; **87**: 905-908.

72 Musabayane C, Ndhlovu C, Mamutse G, Bwititi P and Balment R. Acute chloroquine administration increases renal sodium excretion. *Journal of Tropical Medicine and Hygiene;* 1993; **96**: 305-310.

73 Musabayane C, Munjeri O and Matavire B. Transdermal delivery of chloroquine by amidated pectin hydrogel matrix patch in the rat. *Renal Failure;* 2003; **25**: 525-534.

74 Nanri A, Moore M and Kono S. Impact of C-Reactive Protein on Disease Risk and Its Relation to Dietary Factors: Literature Review. *Asian Pacific J Cancer Prev;* 2007; **8**: 167-177.

75 Naumann B, PA W, Sarangapani R, Hu S, Dixit R and Sargent E. Investigations of the use of bioavailability data to adjust occupational exposure limits for active pharmaceutical ingredients. *Toxicological Sciences;* 2009; **112**: 196-210.

76 Nemeth E, Tuttle M, Powelson J, Vaughn M, Donovan A, Ward D *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004; **306**: 2090–2093.

77 Neva F, Sheagren J, Shulman N and Canfield C. Malaria: host-defense mechanisms and complication. *Ann Intern Med;* 1970; **73**: 295–306.

78 Ogbonna A and Unekeb C. Artemisinin-based combination therapy for uncomplicated malaria in sub-Saharan Africa: the efficacy, safety, resistance and policy implementation since Abuja 2000. *Transactions of The Royal Society of Tropical Medicine and Hygiene;* 2008; **102**: 621-627.

79 Omotosho O, Adebiyi M and Oyeyemi M. Comparative study of the haematology and serum biochemistry of male Wistar Rats treated with Chloroquine and Artesunate. *J Phys Pharm* 2014; **4**: 413-419.

80 Pang G, Couch L, Batey R, Clancy R and Cripps A. GM-CSF, IL-1a, IL-1b, IL-6, IL-8, IL-10, ICAM-1 and VCAM-1 gene expression and cytokine production in human duodenal fibroblasts stimulated with lipopolysaccharide, IL-1aand TNF-α *Clin Exp Immunol* 1994; **96**: 437 – 443.

81 Pepys M and Baltz M. Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. *Adv Immunol;* 1983; **34**: 141–212.

82 Phillips R and Pasvol G. Anaemia of *Plasmodium falciparum* malaria *Baillière's Clinical Haematology* 1992; **5**: 315–330.

83 Piano R. Alcohol and heart failure. *Journal of Cardiac Failure;* 2002; **8**: 239–246.

84 Picot S, Peyron F, Donadille A, Vuillez J, Barbe G and Ambroise-Thomas P. Chloroquine-induced inhibition of the production of TNF,but of IL-6, is affected by disruption of iron metabolism. *Immunology;* 1993: 127-133.

85 Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P *et al.* A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J BiolChem;* 2001; **276**: 7811–7819.

86 Richards A. Tumour Necrosis Factor and Associated Cytokines in the Host's Response to Malaria *International Journal for Parasitology;* 1997; **27**: 1251-1263. .

87 Rowe A, Rowe S, Snow R, Korenromp E, Schellenberg J, Stein C *et al.* The burden of malaria mortality among African children in the year. *Int J Epidemiol;* 2006; **35**: 691-704.

88 Schneider P, Korolenko T and Busch U. A review of drug induced lysosomal disorders of the liver in man and laboratory animals. *Microsc Res Tech;* 1997; **36**: 253-275.

89 Seta Y, Shan K, Bozkurt B, Oral H and Mann D. Basic Mechanisms in Heart Failure: The Cytokine Hypothesis. *Cardiac failure;* 1996; **2**.

90 Sibiya H. Evaluation of the efficacy of transdermal delivery of chloroquine on *Plasmodium berghei*-infected male sprague-dawley rats: Effects on blood glucose and renal electrolyte handling.MMeD thesis thesis, University of KwaZulu Natal. 2013.

91 Sidjanski S and Vanderberg J. Delayed migration of *Plasmodium* sporozoites from the mosquito bite site to the blood. *The American Journal of Tropical Medicine and Hygiene* 1997; **57**: 426-429.

92 Stenvinkel P and Alvesrand A. Inflammation in End-stage Renal Disease: Sources, consequences, and therapy. *Seminar in Dialysis;* 2002; **15**: 329-337.

93 Stevenson M and Riley E. Innate immunity to malaria. *Nature Rev Immunol;* 2004; **4**: 169–180.

94 Sullivan D, Gluzman I, Russell D and Goldberg D. On the molecular mechanism of chloroquine's antimalarial action. *Proceedings of the National Academy of Sciences of the United States of America;* 1996; **93**: 11865–11870.

95 Taverne J, Sheikh N, De Souza J, Playfair J, Probert L and Kollias G. Anaemia and resistance to malaria in transgenic mice expressing human tumor necrosis factor. *Immunology* 1994; **82**: 397–403.

96 Thaane T. Evaluation of the efficacy of Masilinic acid on malaria parasites in *plasmodium berghei*-infected rats: Effects on blood glucose and renal fluid electrolyte handling. Masters thesis, University of Kwa-Zulu Natal, 2014.

97 Ulich T, del Castillo J and Yin S. Tumor necrosis factor exerts dosedependent effects on erythropoiesis and myelopoiesis in vivo. *Exp Hematol;* 1990; **18**: 311-315.

98 Van den Born B, Dijikmans B, Rooij H, Lecessi S and Verweij C. Chloroquine and Hyroxy-chloroquine equally effect Tumour Necrosis Factor Alpha, Interleukin 6 and Interferon Gamma by peripheral nuclear cells. *Journal of Rheumatology;* 1997; **24**: 55-60.

99 Vinik A, Erbas T, Sun Park T, Nolan R and Pittenger G. Platelet dysfunction in type 2 diabetes. *Diabetes Care;* 2001; **24**: 1476–1485.

100 Wagener F, Volk H, Willis D, Abraham N, Soares M, Adema G *et al*. Different faces of hemeoxygenase system in inflammation. *Pharmacology Review;* 2003; **55**: 551-571.

101 Weber S and Levitz S. Chloroquine interferes with lipopolysaccharide-induced TNF-alpha gene expression by a non lysosomotropic mechanism. *J Immunol;* 2000; **165**: 1534-1540.

102 Weinstein D, Roy C, Fleming M, Loda M, Wolfsdorf J and Andrews N. Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. *Blood;* 2002; **100**: 3776–3781.

103 Wenisch C, Wenisch H, Parschalk B, Vanijanonta S, Burgmann H and Exner M. Elevated levels of soluble CD14 in serum of patients with acute *Plasmodium falciparum* malaria. *Clinical Experimental Immunology;* 1996; **105**: 74-78.

104 White N. Antimalarial drug resistance. *The Journal of Clinical Investigation* 2004; **113**: 1084 - 1092.

105 WHO. Assessment of the safety of artemisinin compounds in pregnancy, report of two informal consultations by WHO in 2002 2003; **Report no. WHO/CDS/MAL/2003.1094**.

106 WHO. Guidelines for the treatment of malaria (1st ed.). *World Health Organization, Geneva;* 2006; **Report no WHO/HTM/MAL/2006.1108**.

107 WHO. Malaria guidlines. Report no.WHO/CDS/RBM/2000.17. *Media centre;* 2008.

108 WHO. Malaria guidlines. *Media centre;* 2008; **Report no.WHO/CDS/RBM/2000.17.** .

109 WHO. World malaria report 2015 2015.

110 Wickramasinghe S and Abdalla S. Blood and bone marrow changes in malaria. *Baillie´re's Best Pract Res Clin Haematol;* 2000; **13**: 277–299.

111 Williams N, Bertoncello I, Jackson H, Arnold J and Kavnoudias H. The role of interleukin 6 in megakaryocyte formation, megakaryocyte development and platelet production. *Ciba Found Symp;* 1992; **167**: 160-170.

112 Young B, Gleeson M and Cripps A. C-reactive protein: a critical review. *Pathology;* 1991; **23**: 118–124.

113 Yudkin J, Kumari M, Humphries S and Mohamed-Ali A. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* 2000; **148**: 209 – 214.

114 Zhang J, Krugliak M and Ginsburg H. The fate of ferriprotorphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. *Molecular and Biochemical Parasitology;* 1999; **99**: 129–141.

APPENDICES

APPENDIX I

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

14 July 2014

Reference: 105/14/Animal

Miss N Gumede
Dept of Physiology
School of Laboratory Medicine &
Medical Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Miss Gumede

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2014** on the following project:

"Quantification of chloroquine in various organs of *P. berghei* infected rats."

Yours sincerely

[redacted]

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar – Mr C Baloyi
Research Office – Dr N Singh
Supervisor – Prof. C Musabayane
HOS – Prof. W Daniels
BRU – Dr S Singh

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
Telephone: +27 (0)33 260 5463/35 **Facsimile:** +27 (0)33 260 5105 **Email:** animalethics@ukzn.ac.za **Website:** www.ukzn.ac.za
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

APPENDIX II

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

17 December 2014

Reference: 034/15/Animal

Miss N Gumede
Dept of Physiology
School of Laboratory Medicine &
Medical Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Miss Gumede

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2015** on the following project:

"Quantification of chloroquine in various organs of *P. berghei* infected rats."

Yours sincerely

[redacted]

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar
Research Office – Dr N Singh
Supervisor – Prof. C Musabayane
HOS – Prof. W Daniels
BRU – Dr S Singh

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
elephone: +27 (0)33 260 5463/35 **Facsimile:** +27 (0)33 260 5105 **Email:** animalethics@ukzn.ac.za **Website:** www.ukzn.ac.za
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

APPENDIX III

COLLEGE OF HEALTH SCIENCES RESEARCH SYMPOSIUM

10-11 September 2015

K-RITH TOWER BUILDING

*Mbatha, B., ***Gumede, N.M.,** *Sibiya, H., *Musabayane C.T.

Evaluation of the efficacy of Syzygium aromaticum-derived oleanolic acid on malaria parasite in *Plasmodium berghei*-infected male Sprague-Dawley rats: effects on blood glucose and

renal electrolytes handling.

EVALUATION OF THE EFFICACY OF *SYZYGIUM AROMATICUM*-DERIVED OLEANOLIC ACID ON MALARIA PARASITE IN *PLASMODIUM BERGHEI*-INFECTED MALE SPRAGUE-DAWLEY RATS: EFFECTS ON BLOOD GLUCOSE AND RENAL ELECTROLYTES HANDLING.

*Mbatha, B., ***Gumede, N.M.,** *Sibiya, H., * Musabayane C.T.

**School of Laboratory Medicine and Medical Sciences & *Life sciences. University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa*

Aim
Previous studies have shown that Syzygium aromaticum-derived oleanolic acid (OA) possesses anti-inflammatory, antibacterial and anti-parasitic properties. Against this background the current study was designed to investigate the effects of OA on Plasmodium berghei parasites. We also evaluated the effects of this triterpene on glucose homeostasis and renal electrolytes handling in infected experimental animals.

Methods
Parasitaemia, blood glucose and renal function were monitored in separate groups of control and Plasmodium berghei-infected male Sprague-Dawley rats following oral administration of OA (40, 80, 160 mg/kg) over a period of 21days. The study was divided into pre-treatment (days 0-7), treatment (days 8-12) and post-treatment (days13-21) periods.

Results and discussion
OA was able to reduce and clear the malaria parasites from 19.9 ± 0.4 % to 0.0 ± 0.0 % within a period of 6 days. Oral administration of OA, significantly increased blood glucose levels of infected animals and euglycaemic levels were maintained throughout the post-treatment period (6.31 ± 0.3 mmol/L). Following treatment, there was an increase in urinary sodium (Na+) output with a concomitant increase in AVP concentrations. Furthermore, OA significantly decreased urinary potassium (K+) output of infected animals. The current data demonstrates the potential use of OA for chemoprophylaxis and treatment of malaria.