A New Adjuvant Therapy for Preeclampsia

Rahana Abdul Rahman

MD (UKM)

The Ritchie Centre, Department of Obstetrics and Gynaecology

School of Clinical Sciences, Monash University

Clayton, Victoria, Australia

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TABLE OF CONTENTS

Abstract

Preeclampsia (PE) is one of the leading causes of maternal and perinatal mortality and morbidity. The incidence is higher in developing as compared to developed countries. One of the main complications of the disease is induced preterm delivery, which has considerable impact on both health and economic burden of a country. Despite the advancement in the health sector, there is still no effective treatment for PE to improve both maternal and fetal outcomes. Therefore, there is an urgent need to discover new therapies that can be used safely during pregnancy.

The pathophysiology of PE is still partially understood. However, accumulating body of evidence have shown that the major players involved are placental hypoxia-reperfusion injury, excessive oxidative stress and widespread maternal endothelial dysfunction. Placental hypoxia-ischaemic reperfusion injury originated from the failure of maternal spiral artery remodelling which leads to release of various cytokines and toxic factors into the maternal circulation. These factors consist of an imbalanced pro- and anti-angiogenic factor, pro- and anti-inflammatory cytokines of which triggered the exaggerated oxidative stress and target the maternal vasculature system. The net result is endothelial dysfunction in almost all of the vital organ systems including the brain, liver and the kidneys.

Theoretically, targeting the factors associated with the pathophysiology of PE either individually or collectively will produce an improved clinical outcome for preterm PE. Improvement of the maternal spiral artery remodelling will prevent the disease but this will require an effective method of identifying women at high risk.

Reduction of oxidative stress and endothelial dysfunction will slow down the disease process and hence prolonging the pregnancy to improve the survival rate of the fetus without compromising the maternal outcome. There are many drugs or biological agents that have been researched to target these underlying pathologies of PE. Most of them have shown promising results in the *in vitro* and animal study. Only a few drugs that have been used to treat other diseases had been demonstrated to be safe in pregnancy and beneficial for the treatment of PE. One such drug is hydroxychloroquine (HCQ).

HCQ is an antimalarial drug that is widely used for autoimmune diseases such as SLE, rheumatoid arthritis and Sjogren's syndrome. Its safety in pregnancy had been established by numerous clinical studies. The impact of this drug on PE is not well known but the mechanism of action targets most of the pathologies in PE. As it has been shown to improve the clinical course of SLE, which has striking similarity with PE, I hypothesised that treatment with HCQ may improve the clinical outcome of PE. Therefore, in this section, I have reviewed the use of HCQ in pregnancies amongst women with autoimmune disorders such as SLE and rheumatoid arthritis and subsequently focused on the outcome of pregnant SLE women who were treated with or without HCQ. Following this, I examined the effect of HCQ on the human placental function in PE by measuring the placental explant release of anti-angiogenic factors such as sFlt-1 and sEng including pro-inflammatory cytokine namely TNF-α. The levels of 8-isoprostane productions and activin A, markers of oxidative stress was also performed. The assessment of the effects of HCQ on the maternal oxidative stress and endothelial dysfunction was conducted on primary HUVECs by measuring the levels of produced 8-

isoprostane, NOX2 mRNA expression, levels of ET-1, endothelial cell permeability assay and ZO-1 staining. In order to obtain a better idea on the impact of HCQ on patients, a retrospective clinical study was conducted to compare the adverse pregnancy outcomes between SLE women treated with HCQ and those who were not.

In conclusion, my studies have demonstrated that *in vitro* HCQ does not significantly impair endothelial cell viability, significantly decreases TNF-α induced oxidative stress and endothelial dysfunction but does not improve the placental hypoxia-ischaemic reperfusion injury. The retrospective cohort study of pregnancy outcomes in women with SLE showed that HCQ, when taken in conjunction with corticosteroids and azathioprine was associated with a higher rate of preterm birth, most likely due to a higher rate of concurrent medical illness in those women taking HCQ.

Publications during enrolment

Published journal articles:

1. **Rahman R**, Murthi P, Singh H, Gurusinghe S, Mockler JC, Lim R, Wallace EM.The effects of hydroxychloroquine on endothelial dysfunction. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health. 2016; 6(4): 259-262.

2. **Abd Rahman R**, DeKoninck P, Murthi P, Wallace EM. Treatment of preeclampsia with hydroxychloroquine: a review. The Journal of Maternal-Fetal & Neonatal Medicine. 2017; 21(1): 1-5.

Thesis including published works declaration

I hereby declare that this thesis contains no materials which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer-reviewed journal and three unpublished publications. The core theme of the thesis is preeclampsia. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within The Ritchie Centre under the supervision of Professor Euan Wallace.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Thesis including published works declaration

In the case of chapters 2 to 5 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication Status	Nature and extent (%) of student contribution
2	Treatment of preeclampsia with hydroxychloroquine: a review	Published	80
3	The effects of hydroxychloroquine on placental and endothelial function in preeclampsia. Submitted to PLoS one Journal on 24/11/2016.	Accepted	75
4	Effects of hydroxychloroquine on endothelial dysfunction.	Published	90
5	Hydroxychloroquine and pregnancy outcomes in women with systemic lupus erythematosus	Submitted	95

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] Date: 15/3/2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main Supervisor signature: [redacted] Date: 15/3/2017

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List of abbreviations

APS	antiphospholipid syndrome
BBB	blood brain barrier
DMARD	disease-modifying antirheumatic drug
ELISA	Enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
EVT	extravillous cytotrophoblast
HBSS	Hank’s Balanced Salt Solution
HCQ	hydroxychloroquine
HELLP	haemolysis, elevated liver enzymes, low platelet
HUVECs	Human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
ICU	intensive care unit
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-10	interleukin-10
IUGR	intrauterine growth restriction
LPS	lipopolysaccharide

M199	Medium 199
MAP	mean arterial pressure
MDA	malondialdehyde
MLT	melatonin
NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase enzymes
NF-κB	Nuclear factor- κB
NO	nitric oxide
NOX	nicotinamide adenine dinucleotide phosphate-oxidase enzymes
PAPP-A	pregnancy-associated plasma protein-A
PBS	phosphate-buffered saline
PE	preeclampsia
PI	pulsatility index
PlGF	placental growth factor
PMNs	polymorphonuclear leucocytes
RA	rheumatoid arthritis
RCT	randomised clinical trials
ROS	reactive oxygen species
RUPP	reduced uterine perfusion pressure
sEng	soluble endoglin
sFlt1	soluble fms-like tyrosine kinase 1
SGA	small for gestational age

SLE	systemic lupus erythematosus
SOD	superoxide dismutase
TGF	transforming growth factor
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2
X	xanthine
XDH	xanthine dehydrogenase
XO	xanthine oxidase
ZO-1	zona occludens-1

CHAPTER ONE

Literature review

1.1 Overview of preeclampsia

Hypertensive disease in pregnancy is the second most common cause of maternal mortality after haemorrhage, complicating 5% of pregnancies(1).The incidence of maternal death was high in Latin America and the Caribbean (25%) when compared to the Asian and African countries with average incidence of9% (2).Even in developed countries such as the USA, the incidence of hypertensive disease in pregnancy seems to be rising (3, 4).It is associated with considerable maternal and perinatal morbidity such as renal failure, stroke, cardiac arrest, HELLP syndrome, abruptio placentae, intrauterine death, fetal growth restriction and iatrogenic preterm delivery(5). Furthermore, the need for preterm delivery imposes significant health and economic burden to the affected countries (6).

Hypertensive disease in pregnancy can be classified as preeclampsia-eclampsia, gestational hypertension, chronic hypertension and preeclampsia superimposed on chronic hypertension(7). PE is defined by elevated systolic blood pressure of 140/90 mmHg or more after 20weeks of gestation and it may be associated with renal, haematological, liver, neurological, pulmonary oedema, proteinuria and fetal growth restriction (7). Women with severe PE may experience symptoms such as

headache, blurring of vision, abdominal pain or vomiting. More often than not, proteinuria is present, although it is not a pre-requisite before making the diagnosis.

The outcome of PE is dependent on the severity of the disease, gestational age at diagnosis, quality of treatment and presence of co-morbidities. In particular, pregnancies complicated by early-onset PE at less than 34 weeks, are associated with 20-fold increase in maternal mortality and increased rates of maternal and perinatal morbidities (8-10). The management of early onset PE continues to pose significant challenges to the obstetrician who tries to balance the maternal risks with the fetal benefits of prolonging the pregnancy.

Some of the serious maternal complications are maternal death, acute pulmonary oedema, acute renal failure, liver haemorrhage or failure, disseminated intravascular coagulopathy, eclampsia, HELLP syndrome and cerebrovascular accidents(11). Following pre-eclamptic pregnancies, there is an increased risk of developing metabolic syndrome in later life which consists of excess abdominal weight, lipid abnormalities, hypertension and hyperglycaemia. Additionally, there is a greater risk of developing cardiovascular diseases, including coronary artery disease and stroke as well as chronic hypertension(12). These risks were observed to be highest with early onset severe disease (13, 14). Similarly, fetal complications are high including IUGR, prematurity and intrauterine death arising from placental abruption or placental insufficiency. There is an increased rate of neonatal ICU admissions, requirement of mechanical ventilation, respiratory distress syndrome, intracerebral haemorrhage and lower birth weight (15). Severe

prematurity poses greater risk for the newborns such as necrotising enterocolitis, hypoxic brain injury, chronic lung disease, retinopathy of prematurity and even death (16). In later life, growth restricted fetuses remain at increased risks of diseases such as diabetes mellitus, coronary heart disease, hypertension and hyperlipidemia (17).

Ideally, women at high risk of PE should be identified in the early pregnancy to determine whether they will benefit from preventative treatment such as aspirin. Amongst those considered to be in the high risk group are women with antiphospholipid syndrome, PE in the previous pregnancy, chronic hypertension, pregestational diabetes, prepregnancy BMI > 30 and those conceiving via assisted reproductive techniques(18).Apart from this, the mean arterial blood pressure at the first antenatal visit is also considered as one of the important determinants of the risk of PE (19). In mild hypertension as characterised by diastolic blood pressure not exceeding 110 mmHg without organ involvement, the risk ranges from 10% -25%(20, 21). However, in severe chronic hypertension, the risk will doubleto46% -52%(22, 23). Other predisposing factors are positive family history and pregnancy related factors such as multiple pregnancy and nulliparity(24-27).

Upon identification of the high risk group for PE, it is beneficial if the incidence of PE can be predicted in each of these patients. Prediction of PE based on maternal characteristics alone, only identified about 30% of cases. However, the detection rate for early onset PE (before 34 weeks) by a combination of maternal characteristics and uterine artery doppler at 22-24 weeks of gestation increased to 95.7%. Moreover, when the mean arterial blood pressure in the first trimester was

added the detection rate further increased to 100%(28). Likewise, the combination of mean arterial blood pressure, uterine artery pulsatility index, serum PAPP-A and PlGF resulted in93% detection rate of early onset PE (29).

To date, apart from low dose aspirin and calcium supplementation, available therapies that are effective in preventing the occurrence or recurrence of PE in women at high risk are limited. Low dose aspirin is one of the earliest and widely used drugs as preventative therapy for PE. The outcomes of the clinical trials differed and this may be attributed to the difference in the criteria of patients who were recruited, dosage of aspirin as well as the gestational age of patients recruited(30-33). Likewise, there were conflicting results in regard to the time to initiate low dose aspirin in obtaining the maximum beneficial effect(34). However, most recently, it has been shown that commencement of low dose aspirin in early in pregnancy in women at high risk of preeclampsia can reduce the risk by 60% (35). The evidence regarding calcium supplementation is a little more conflicting. In a study by Villar *et al,* other supplements like calcium was shown to be beneficial in nulliparous women to prevent PE (36).However, Levine *et al*. did not obtain similar results(36, 37). The consensus position is that low dose calcium supplement, of 1g per day, in those women with calcium deficiency is beneficial in reducing the risk of PE and its complications (38) whereas supplementation in women replete for calcium is not beneficial. The role of vitamin D supplementation in preventing preeclampsia is even more controversial. A series of reports suggested that maternal vitamin D deficiency is associated with increased risks of several adverse pregnancy outcomes, including preeclampsia(39). However, the evidence that vitamin D supplementation reduces the risk of preeclampsia is not

strong. While supplementation is safe(40) , there is no direct evidence that supplementation reduces risks(41).

Due to the unavailability of effective preventative therapy, obstetricians have been relying on the antihypertensive agents to avoid further maternal complications from acute severe hypertension such as cerebrovascular accidents and left ventricular failure (42). The challenge in managing early onset PE is to prolong the pregnancy to increase the chance of survival of the foetus and at the same time to minimize both maternal and fetal morbidity and mortality. The ultimate treatment is delivery regardless of the gestational age. Among the commonly used drugs are methyldopa, β-adrenergic blocking agent, hydralazine and calcium channel blockers. Methyldopa is a suitable treatment option which is effective and safe, especially when the onset of PE is less than 28 weeks (42). Another option is Labetalol but it was reported to be of concern as it was said to cause IUGR when used in the first trimester(43). This, however, was disputed by a recent study which used labetalol to control the blood pressure, whereby no differences were observed in regard to SGA (44). On the other hand, Nifedipine is another option to treat PE but without any effects to the growth of the fetus (45). In acute hypertensive crisis, parenteral antihypertensive agents are indicated to protect against hypertensive encephalopathy, intracranial bleeding and congestive cardiac failure. However, these agents such as labetalol or hydralazine must be used with caution in view of their side effects to both mother and fetus. Usage of intravenous hydralazine is associated with higher incidence of maternal palpitation and tachycardia, whilst intravenous labetalol causes more neonatal hypotension and bradycardia(46).

One of the severe complications of PE is eclampsia which is defined as generalised seizure in the presence of elevated blood pressure (47).It usually affects women with established PE and rarely occurs in those without prior symptoms. Amongst the symptoms experienced are persistent occipital or frontal headache, blurred vision, epigastric or right upper quadrant pain and nausea or vomiting. Magnesium sulphate has been the drug of choice for prevention or treatment as its use is associated with significant reduction in the rate of eclampsia(48).

1.2 Pathophysiology of preeclampsia

The complete pathophysiology of PE is still not completely understood making it difficult to prevent the disease and to find an effective treatment to improve the maternal and perinatal outcomes. It has been proposed to be a 'two-staged' disease (Figure 1). The first stage originates from the placenta whereby there is defective placentation without any overt clinical manifestation. This is followed by the second stage, which is a consequent of the defective placentation, thus causing the clinical syndrome (49). Based on the accumulating body of evidence, each stage is characterised by specific pathology. Therefore, theoretically any intervention in the first stage will be able to prevent the disease and in the second stage, targeted treatment will reduce the severity of the disease.

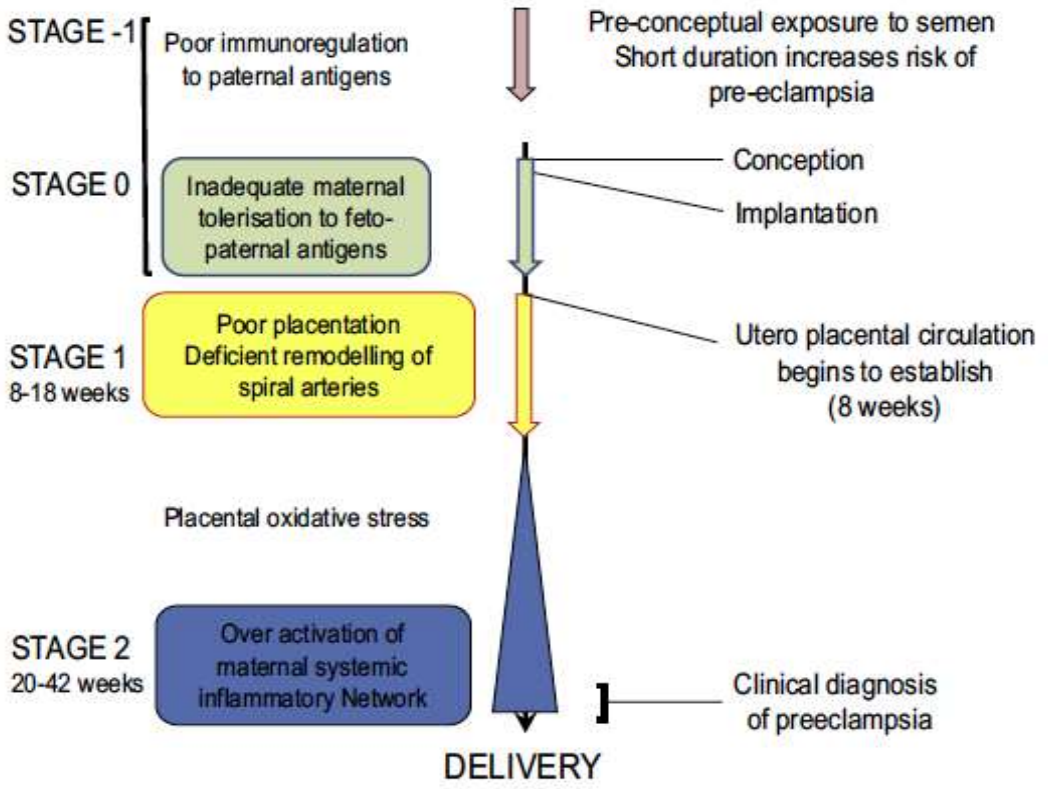

Figure 1: *PE is a two-staged disease whereby in stage 1 there is poor placentation without any clinical manifestation. In stage 2 there is widespread maternal endothelial dysfunction accompanied by the clinical syndrome of PE. Image adapted from Redman CWG, Preeclampsia: a multi-stress disorder. Rev Med Interne. 2011;32 Suppl 1:S41-44.*

1.2.1 Uterine spiral artery remodelling

In a normal pregnancy, maternal spiral artery remodelling occurs early in two phases to prepare the uteroplacental vascular system for the growing fetus. The uterine arteries, which mainly supply the uterus, branch into arcuate system that gives rise to the radial arteries (Figure 2). The spiral arteries arise from the termination of the radial arteries in the myometrium and traverse into the endometrium (50). Invasion of the spiral arteries in the endometrium by the endovascular trophoblast replaces the elastic lamina with fibrinoid materials. This

occurs at 10 to 12 weeks of gestation and deeper invasion into the myometrium segment occurs at 12 to 16 weeks(51). The spiral arteries are transformed into distended funnel-shaped tortuous vessels characterised by loss of vasomotor response and low resistance to allow more blood flow into the placenta(50). Prior to the spiral artery remodelling, there is a relative hypoxic environment(52). Upon establishment of the uterovascular system by the end of the first trimester, the oxygen tension rapidly increases(53).

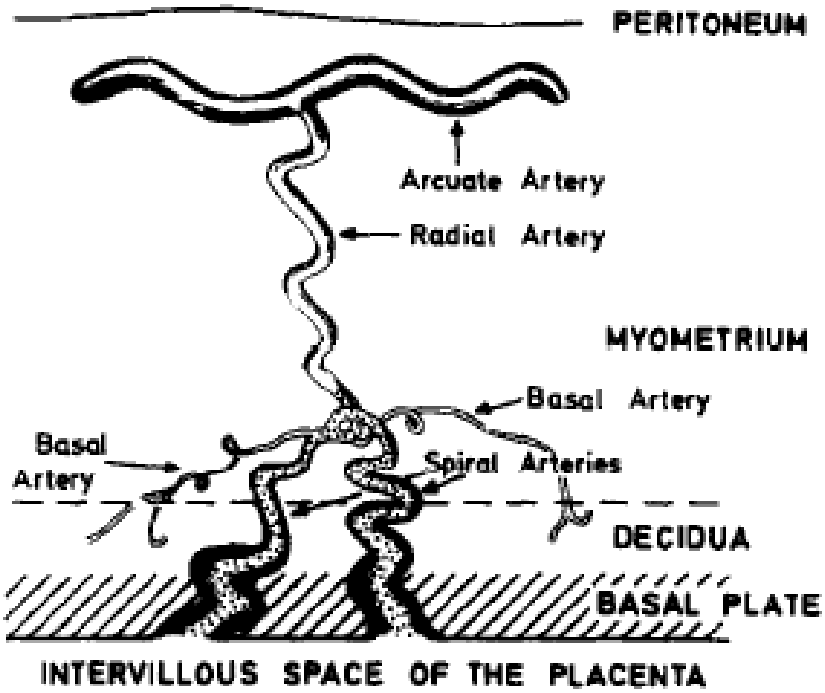

Figure 2*: The anatomy of maternal uteroplacental vascular system. Uterine arteries give rise to the arcuate system which branch into radial arteries. Spiral arteries are the termination of the radial arteries that supply the endometrium. Image adapted from Brosens I, The physiological response of the vessels of the placental bed to normal pregnancy, J. Path. Bact. 1967; 93(2): 569-579(54).*

In PE, the maternal spiral arteries in the myometrial segment failed to undergo the physiological changes and presence of atheromatous lesions (55, 56). As a result,

the diameter of the uterovascular system will remain small with high resistance, causing inadequate blood flow to the placenta and the growing fetus. Additionally, the vessels are responsive to vasomotor stimuli causing periodic vasoconstriction. This causes intermittent placental perfusion which leads to fluctuation in the oxygen tension, hence giving rise to ischaemia-reperfusion injury (57).

1.2.2 Placental ischaemia-reperfusion injury

Ischaemia-reperfusion injury can be detrimental as it generates a large amount of reactive oxygen species (ROS) (58). The source of ROS originates from mitochondria and XDH/XO with major contribution from the latter. XDH converts purine to uric acid while XO metabolises xanthine and hypoxanthine also to uric acid. In the presence of hypoxia, XDH is converted to XO along with the production of free radicals. XO is produced by the placenta and is shown to be increasing in PE as a response to reperfusion injury(57, 59). Consequently, excessive oxidative stress ensues as it overwhelms the antioxidant defences.

Ischaemia-reperfusion injury to the placenta causes the release of various cytokines into the maternal circulation. Hypoxia alone triggers the release of pro-inflammatory cytokines such as TNF-α, IL-1β and anti-angiogenic factors, namely sFlt-1 and sEng (60-62). In response to oxidative stress, more cytokines are produced from the placenta, such as 8-isoprostane and activin A(63, 64).As this exaggerated production of cytokines enter the maternal circulation, the maternal vascular systems are targeted, leading to widespread endothelial dysfunction.

1.2.3 Endothelial dysfunction

In the past, the complications of PE were thought to be caused by the elevated blood pressure. To date, as more evidence had emerged, we believe that the molecular levels of the complications precede the clinical manifestations with the main culprit is thought to be endothelial dysfunction. Earlier, the theory of PE being a “two-staged” disease had been mentioned and the first stage had been discussed. The second stage of the disease is basically the result of the cytokines targeted on the maternal vascular system (Figure 1). As a response to the various elevated cytokines released from the injured placenta, the maternal circulation is in a massive systemic inflammatory state and exaggerated oxidative stress. Numerous published data supported this theory (65-69).

Normal, healthy endothelial cells have various important functions such as regulation of blood vessel tone, oxidative stress, thrombotic and inflammatory pathways and many others. Therefore, the cells have the ability to produce various agonist or antagonist molecules to maintain the normal homeostasis (70). In response to insults or stimuli, the endothelial cells become dysfunctional when there is an imbalance between the agonist and antagonist molecules. In the maternal circulation of preeclamptic women, there are increased levels of pro-inflammatory cytokines, anti-angiogenic molecules and markers of oxidative stress(66, 71, 72). Various *in vitro* experiments had demonstrated that when normal HUVECs are incubated in PE serum, there is evidence of endothelial dysfunction as well as oxidative stress (73-76). Both of these pathologies affect almost all organ systems, causing damages that are manifested as the clinical syndrome of PE.

The central nervous system is affected by PE as evidenced by complications of eclampsia, which is characterised by generalised seizure in the presence of elevated blood pressure. This can be preceded by symptoms of headache and blurring of vision(77). The presence of elevated levels of sFlt-1 in the maternal circulation had caused an increase in vascular permeability of the BBB which leads to leaky vessels. Consequently, the development of cerebral oedema is manifested as the neurological symptoms in severe PE and eclampsia (78).Similarly, kidney injury in PE involves glomerular endotheliosis affecting the glomerular capillaries which is characterised by glomerular endothelial cell enlargement which appears bloodless(79). This leads to loss of endothelial cell integrity and the vessels become leaky causing proteinuria(80).Endothelial dysfunction also affects the maternal liver in the severe spectrum of the disease i.e. HELLP syndrome(81).

1.3 Treatment update forpreeclampsia

The goal of treatment in PE is to minimise maternal morbidities, followed by the delivery of a live born healthy baby. As the pathophysiology of the disease is slowly being solved though it is incomplete, many researchers aim to find a treatment that targets the underlying problem focusing on stabilising or reversing the pathology.

1.3.1 Therapies to correct angiogenic factors

A normal placental and fetal development require both pro- and anti-angiogenic factors to be in a balance. The pro-angiogenic factors, namely VEGF and PlGF are antagonised by anti-angiogenic factors i.e sFlt-1 and sEng. In the maternal

circulation of preeclamptic women, the levels of sFlt-1 are elevated while the levels of PlGF are significantly low when compared to normal pregnancy (82). Administration of recombinant VEGF factor was shown to reduce the blood pressure and improved the kidney function in a rat model of PE(83, 84). Similar results were obtained using recombinant PlGF in a study by Spradley *et.al* using the same animal model of PE, without any teratogenic effect to the fetus (85).

The most recent drugs shown to have similar effects are metformin, pravastatin and esomeprazole, which apart from reducing the levels of the anti-angiogenic factors, they also have positive effects for angiogenesis and improve endothelial dysfunction (86-88). Metformin is a good option as it has been used to treat women with established as well as gestational diabetes during pregnancy. Nevertheless, it has not been used to treat PE(89). On the other hand, pravastatin has recently been used in a pilot randomised controlled trial involving 10 women with high risk of PE. Although statistically not significant, it was associated with lower rates of PE, induced preterm delivery and NICU admission. There was no maternal or fetal and neonatal adverse effects observed (90).Apart from statin, esomeprazole which is a proton pump inhibitor, is currently being used in a double blind, placebo controlled clinical trial in South Africa, involving 120 women with early onset PE within 26 to 31+6 weeks gestation. The primary outcome measure is prolongation of pregnancy along with secondary outcomes which includes maternal, fetal and neonatal mortality and morbidity, maternal serum biomarkers including sFlt, sEng and ET-1 and placental samples (88).

1.3.2 Therapies to correct inflammatory cytokines

Inflammation is a powerful mechanism implicated in most human diseases. It plays an important role in causing endothelial dysfunction and oxidative stress in PE(91, 92). Cyclosporin A, which is an immunosuppressant drug is effective in improving the blood pressure in a rat model of PE (LPS induced) via reduction of serum levels of pro-inflammatory cytokines i.e IL-6 and TNF-α (93). It has been used in human pregnancy mainly for post-allogenic organ transplant patients and also autoimmune diseases such as SLE and, RA (94). It appears to be safe for use during pregnancy, but both maternal and fetal outcomes are difficult to be assessed owing to the coexistent comorbidities in the recruited cohort of patients.

In most diseases that involve exaggerated inflammation, there is an imbalance between pro- and anti-inflammatory cytokines. IL-10 is an anti-inflammatory cytokine which was given in the RUPP rat model intraperitoneally and resulted in a reduction of mean arterial pressure, levels of IL-6, TNF-α and oxidative stress (95). It is a promising therapy, but requires more research to assess the effects on fetus and neonates.

1.3.3 Therapies to reduce oxidative stress

Excessive oxidative stress can be overcome either by reducing the production of the ROS or increasing the activity of antioxidant defence system. Resveratrol, which can be found mainly in grapes was found to reduce oxidative stress both *in vitro* and *in vivo* by increasing the level of SOD an anti-oxidant enzyme and decreasing the level of MDA which is a marker for lipid peroxidation(96). In

addition, it also reduces the level of sFlt-1 released from preeclamptic placental explants (97).

Melatonin is a hormone found in human secreted by the pineal gland to help in maintaining the body's circadian rhythm and taken as a treatment for jet lag. Melatonin was discovered to be a powerful scavenger for free radicals and since then had been thought to have the ability to treat PE based on *in vivo* evidence (98, 99). It is currently being used in a clinical study involving women with established PE (100).

1.3.4 Therapies to improve endothelial dysfunction

NO is one of the most important components produced by the endothelial cells. It is not only a vasodilating substance, but also inhibits inflammation and platelet aggregation (101). Sildenafil citrate or Viagra is used to treat erectile dysfunction and pulmonary hypertension. Its mechanism of action is via inhibition of PDE-5, an enzyme present in the vascular smooth muscle. This will prolong the effects of NO signalling and leads to vasodilatation(102). In animal model of PE, it reduces the blood pressure, fetal growth and endothelial dysfunction without any teratogenic effect to the fetus (103, 104). In 2011, the Canadian group had used sildenafil to treat 10 women with severe early onset IUGR as early as 21 weeks. They observed an improvement in the fetal growth, no maternal side effects, but no assessment has been made on either short or long term side effects on the neonates (105).

Glyceryl trinitrate (GTN) is a vasodilating agent used primarily in pulmonary hypertension and had been used to treat severe PE when the oral

antihypertensive agent failed to control the blood pressure (106). It contains NO within its structure and act as a provider of NO in the tissues (107). Additionally, it also inhibits the production of sFlt-1 and sEng from placental explants exposed to hypoxia (108).However, GTN is only available in infusion form, which is rather inconvenient when used for outpatient treatment.

Hydroxychloroquine (HCQ) is an anti-malarial drug which is known to have both anti-inflammatory and immunomodulatory properties. It is widely used in autoimmune disorders such as SLE, rheumatoid arthritis and Sjogren's syndrome. Treatment of SLE patients with HCQ is associated with decreased serum level of pro-inflammatory cytokines namely IL-6, IL-8 and TNF-α (109). Other effects of HCQ on the immune systems include alteration in the lysosomal pH inhibiting its functions, inhibition of prostaglandins and suppression of T and B cell receptors signalling (110-112). A recent *in vivo* study involving a mouse model of severe SLE had demonstrated a reduction in blood pressure and improvement in the endothelial dysfunction, as well as organ damage (113). It has good and sufficient data on the safety to both mother and fetus when used during pregnancy with minimal data on the reduction in the incidence of PE (114, 115).

1.4 Rationale and aims of studies

The treatment of preterm PE has been limited to the use of antihypertensive drugs, whereby they neither delay the disease progress nor improve the clinical outcomes. This is understandably due to its target on the end point of the disease that cannot be altered. Nevertheless, the "treatment" does help in minimising the impact of the disease imposed by the underlying pathologies. Theoretically, a drug

or substance that targets the placental injury or the widespread maternal endothelial dysfunction in PE may be an answer to the problem. HCQ appears to be a potential drug that exerts its effects via various molecular pathways that are similar to the pathophysiology of PE.

In order to assess the potential use of HCQ in preterm PE, in chapter 2 I reviewed the mechanisms of action of HCQ and similarity of the pathways targeted in both SLE and PE. Chapter 3 summarises the results of *in vitro* and *ex vivo* studies of the effects of HCQ on the pathophysiology of PE, specifically focusing on placental ischaemic injury, placental oxidative stress and endothelial cell dysfunction. In Chapter 4, the results of further studies expanding the effects of HCQ on endothelial dysfunction are reported in more detail. Last, in Chapter 5 I describe the possible benefits of HCQ on maternal and perinatal outcomes in pregnant women with systemic lupus erythematosus who were treated with HCQ throughout pregnancy.

CHAPTER TWO

Treatment of preeclampsia with hydroxychloroquine: a review

2.1 Preamble

Hydroxychloroquine is one of the treatments recommended for all women with systemic lupus erythematosus (SLE). This is due to its beneficial effects in improving survival by reducing the rates of flares and end organ damage(116). The mechanisms of action vary targeting multiple molecular pathways to exert its effects. Some of the pathways targeted in SLE are similar to the pathophysiological pathways of PE.

SLE is an autoimmune disorder which is characterised by systemic inflammation with resulting endothelial dysfunction(117, 118). Pregnancy complications in women with SLE mainly arise as a result of these pathologies. Additionally, there are also genetic susceptibilities upon which SLE can be inherited(119). Amongst the complications of SLE during pregnancy is the increased risk of PE(120). There may be some similarities in the pathophysiology of both of these diseases. To date, there is no definitive treatment for both conditions despite extensive research attempting to use biological agents to target the underlying pathology.

One of the treatments widely used in SLE is HCQ. It exerts anti-inflammatory, antioxidant and immunomodulatory properties(117, 121).Therefore, theoretically the pathways involved in SLE that it targets may be beneficial for PE as well. These various molecular pathways have been recently discovered highlighting the mechanisms of action for this drug. Hence, this review is aimed to explore the possible use of antimalarial drugs in general for the treatment of PE as an adjuvant therapy.

2.2 Treatment of preeclampsia with hydroxychloroquine: a review

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REVIEW ARTICLE

Treatment of preeclampsia with hydroxychloroquine: a review

Rahana Abd Rahman[a,c], Philip DeKoninck[b], Padma Murthi[b] and Euan M. Wallace[b,c]

[a]Department of Obstetrics and Gynecology, Faculty of Medicine, National University of Malaysia, Kuala Lumpur, Malaysia;
[b]Department of Medicine, School of Clinical Sciences, Monash University, Monash Medical Centre, Clayton, Victoria, Australia;
[c]The Ritchie Centre, Hudson Institute of Medical Research, Clayton, Victoria, Australia

ABSTRACT

In this review, we discuss the potential use of antimalarial drugs as an adjuvant therapy for preeclampsia, focusing on the mechanisms of action of this class of drugs in the context of preeclampsia. In particular, hydroxychloroquine has been shown to have various beneficial effects on patients with systemic lupus erythematosus. There are several pathways targeted by the antimalarial drugs that are similar to the pathophysiology of preeclampsia and hence offering opportunities to develop novel therapies to treat the disease. Given the safety profile of hydroxychloroquine in pregnancy, there is merit in exploring the efficacy of this drug as an adjuvant therapy in women with early onset preeclampsia.

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Introduction

While initially introduced to treat malaria, due to their anti-inflammatory actions, antimalarial drugs have become widely used to manage autoimmune disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and Sjogren's syndrome. The first widespread recognition of their potential anti-inflammatory effects was when quinacrine was dispensed as prophylactic antimalarial to American soldiers in Second World War and it was noted to improve rashes and inflammatory arthritis [1]. Subsequently, they were used for many years in the treatment of cutaneous lupus without objective evidence of their efficacy. It was a report in 1951, of the use of mepacrine in the treatment of SLE that triggered more formal interest in the application of these drugs outside of malaria [2].

It is now known that antimalarial drugs exert their therapeutic effects via different molecular pathways, such as antioxidant, anti-inflammatory and antithrombotic mechanisms depending on the target cells and disease process(es). For example, at therapeutic concentrations, they inhibit reactive oxygen species (ROS) production by neutrophils and at higher concentrations, can themselves scavenge ROS [3]. Antimalarials are also potent anti-inflammatory and immunomodulatory agents, acting mainly by inhibition of phagocytosis and antigen presentation, binding and stabilising DNA and inhibition of matrix metalloproteinases (MMP) [4–6]. Most recently, it has been shown that hydroxychloroquine inhibits toll-like receptor (TLR) signalling, thereby reducing the production of pro-inflammatory cytokines [7].

In the context of pregnancy, it is well known that women with SLE have a 3–4 fold higher risk of developing preeclampsia than women without SLE [8]. In this regard, it is interesting that there are some similarities in the underlying pathophysiology between SLE and preeclampsia. For example, in preeclampsia, oxidative stress plays a pivotal role in the placental dysfunction as a result of ischaemia-reperfusion injury as a consequence of inadequate placentation [9]. The syncytiotrophoblast (STB) becomes dysfunctional owing to the excessive oxidative stress resulting in apoptosis or necrosis, known as trophoblast debris, which is released more in preeclampsia [10]. Together with this, there is also excessive placental release of anti-angiogenic factors, such as activin A [11], sFlt-1, soluble endoglin [12] and pro-inflammatory cytokines, such as TNF-α [9] into the maternal circulation. In turn, these factors target the maternal vessels causing endothelial activation and stimulation of endothelin-1 (ET-1) production [13–15]. Similarly in SLE, altered endothelial function is the main feature of the disease, which precedes the development of hypertension [16]. Therefore, there is a possibility that the benefits of using hydroxychloroquine in SLE patients are also

CONTACT Euan M. Wallace euan.wallace@monash.edu Department of Obstetrics and Gynecology, Monash University, Monash Medical Centre, 246 Clayton Road, Clayton, Victoria 3168, Australia

applicable to pregnancies complicated with preeclampsia.

In this review, we highlight the recent insights into the mechanisms of action of hydroxychloroquine and consider whether these mechanisms may have application to the treatment of women with established preeclampsia.

Mechanisms of action with a view to treat preeclampsia

While antimalarial agents have been used to treat lupus and other inflammatory conditions for nearly 70 years, it is only relatively recently that their actual mechanisms of action have become apparent. Promisingly, many of these may have direct relevance to placentation and preeclampsia which involves multiple molecular pathways.

Anti-inflammatory effects

Systemic inflammatory disorders involving extensive tissue damage such as SLE are characterised by important changes in the innate immune system. Toll-like receptors play an important role in the underlying pathophysiological mechanisms of this condition [7]. DNA methylation is an essential process for gene regulation involved in development and disease which typically occurs in a CpG dinucleotide. Hypomethylated CpG is believed to activate B and dendritic cells via receptor such as TLR9 [17]. There are elevated levels of hypomethylated cytosine guanine dinucleotide (CpG) in the plasma of SLE patients, which induces the production of interferon-α (IFN-α). This in turn, promotes the production of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α). The main source of IFN-α is believed to be from the plasmacytoid dendritic cells that express both TLR 7 and 9 [17]. The role of antimalarial drugs in the treatment of SLE, and hydroxychloroquine in particular is to reduce the production of TNF-α induced by TLR7 and TLR9 in plasmacytoid dendritic cells [18].

In preeclampsia, there is increased release of syncytiotrophoblast microparticles due to excessive placental apoptosis secondary to the ischaemia-reperfusion injury [19]. The release of these placental microparticles is accompanied by an increased release of cell-free foetal DNA which are ligands for TLR 3, 7 and 9. Via TLR binding and activation, the cell-free DNA triggers the production of pro-inflammatory cytokines such as interleukin-6 and TNF-α leading to widespread inflammation and subsequent organ injury [20]. The inhibition of TLR signalling by antimalarials may offer a novel approach to interrupt this aspect of the pathogenesis of preeclampsia. It would certainly be worth assessing whether hydroxychloroquine could block cell-free DNA-mediated TLR activation in endothelial cells.

Antioxidant effects

Oxidative stress is thought to play a central role in the development of preeclampsia [9]. In particular, oxidative stress within the placenta leads to the excess release of activin A and 8-isoprostane [11] while oxidative stress in the maternal endothelium leads to endothelial disruption and dysfunction [21]. The sources of intracellular reactive oxygen species (ROS) in the endothelial cells are mainly from mitochondria and NADPH oxidases (NOX), which activates NFKB signalling and triggers apoptosis and necrotic cell death depending on the severity [21]. While it has been recently suggested that NOX inhibitors, such as apocynin may be effective therapies for endothelial dysfunction in preeclampsia, these have not been tested clinically [22]. Other approaches to antioxidant therapies are needed.

In this regard, SLE is also associated with excessive oxidative stress, characterised by elevated serum levels of malondialdehyde (MDA), a marker for oxidative stress [23]. Intriguingly, hydroxychloroquine inhibits the production of ROS by affecting the function of polymorphonuclear cells at therapeutic concentration, but has the ability to scavenge at higher concentration [3]. More recently, it has been shown in a mouse model of SLE that hydroxychloroquine can protect against oxidative stress-induced endothelial dysfunction and thereby improve renal function by the inhibition of NOX activity [24,25]. Whether hydroxychloroquine can mitigate NOX activity in the placenta [26] or in endothelium [21] has not been explored and is certainly worthy of study.

Vascular protective effects

The major source of ROS in the vessels is NADPH oxidase which is activated by factors such as TNF-α, angiotensin II, thrombin, activin and platelet-derived growth factor (PDGF) resulting in oxidative stress and hence endothelial dysfunction [21,27–30]. This is characterised by excessive endothelial release of pro-inflammatory cytokines and chemokines, and cell adhesion molecules such as VCAM, ICAM and E-selectin [31]. In SLE, chronic endothelial dysfunction secondary to chronic inflammation and oxidative stress underlies the increased risks of hypertension, stroke and renal disease [16,32]. However, hydroxychloroquine mitigates the

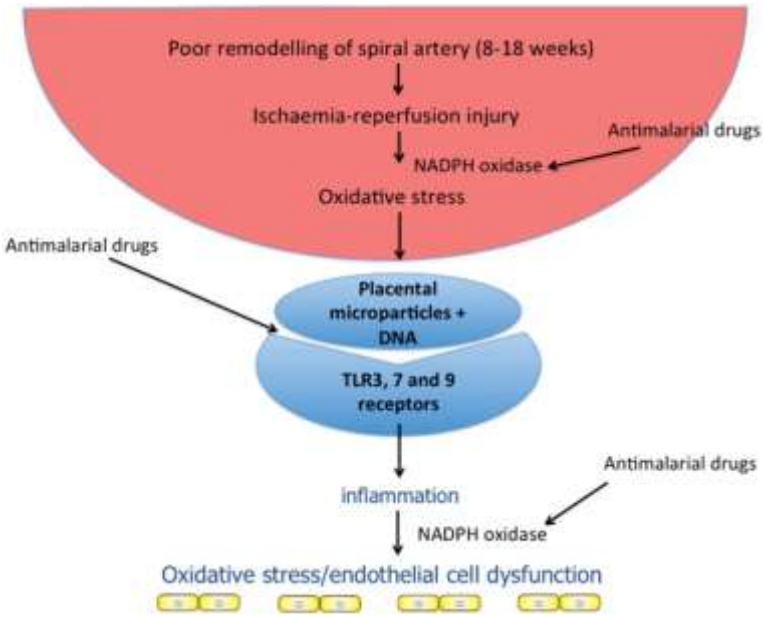

Figure 1. Role of antimalarial drugs in targeting the pathways involved in preeclampsia based on their mechanisms of action. Antimalarial drugs can be used to mitigate oxidative stress in the placentae as well as in the endothelium via inhibition of NADPH oxidase activity. Additionally, they can target the TLRs to prevent the production of pro-inflammatory cytokines.

inflammation and oxidative stress, and with prolonged treatment, improves the endothelial function.

There is considerable evidence that endothelial cell dysfunction also plays a major role in the pathophysiology of preeclampsia. For instance, the placenta releases toxic factors that target the maternal vasculature by upregulating NADPH oxidase expression to induce oxidative stress and affect the endothelial cell integrity [21]. For this reason, the use of hydroxychloroquine could be beneficial in improving the endothelial functions.

Use of hydroxychloroquine in pregnancy

Hydroxychloroquine has a good safety track-record as a treatment of SLE and rheumatoid arthritis during pregnancy. While there have been reported cases of teratogenic effects associated with the use of chloroquine during pregnancy [33], this is not the case with hydroxychloroquine. One of the earliest reported anecdotal experiences of using hydroxychloroquine during pregnancy was in 1983 [34]. A patient was treated with 200 mg per day throughout the pregnancy starting from 16 weeks gestational age. There were no complications or unwanted side effects to both mother and foetus. Subsequently, there have been multiple case reports and case series published, with no evidence of an increase in the incidence of foetal abnormalities [35] or other sequelae in the offspring into early childhood [36]. Due to the immunosuppressive effect of hydroxychloroquine, studies had also been undertaken to assess immune system development in the offspring of women who took it across pregnancy. No immune effects in the children have been observed [37].

These follow-up studies are reassuring because hydroxychloroquine crosses the placenta and the concentrations in both maternal and cord blood are comparable [38]. It is also excreted into the breast milk, albeit in a very limited amount [39]. The American Academy of Pediatrics (AAP) considers hydroxychloroquine acceptable for use during breastfeeding [40]. Furthermore, the use of antimalarial drugs, hydroxychloroquine in particular is associated with only mild side effects such as gastrointestinal discomfort, headache and pruritus.

Conclusions

There are several interesting pathways that antimalarial drugs can target to improve the pathophysiological changes resulting in preeclampsia (summarised in Figure 1). Hydroxychloroquine can exert its antioxidant effect in both the placenta and in the endothelium by reducing the production of free radicals via inhibition of NADPH oxidase activity. Additionally, suppression of TLR receptor activation could prevent inflammation and hence improve endothelial cell function.

Clearly, the antimalarial drugs possess various beneficial effects provoking strong interest amongst researchers. The excellent safety profile with minimal side effects and together with the evidence of targeting similar pathophysiology pathway as in preeclampsia, makes it an interesting option to be explored further as an adjuvant therapy to treat established preeclampsia.

Disclosure statement

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ORCID

Padma Murthi http://orcid.org/0000-0003-2535-5134
EuanM. Wallace http://orcid.org/0000-0002-4506-5233

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CHAPTER THREE

Hydroxychloroquine: an adjuvant therapy for preeclampsia?

3.1 Preamble

In view of the similarity in the pathways targeted by HCQ in both SLE and PE, I have further performed *in vitro* experiments to assess the effect of the drug on placental function and endothelial cells. The aim of this research is to explore the possibility of using HCQ in women with established diagnosis of PE. Hence, I have decided to investigate whether the drug is able to improve the function of an injured placenta and endothelial dysfunction.

PE is postulated to be a “two-staged” disease whereby the first stage comprises mainly of the ischaemia-reperfusion injury that leads to excessive oxidative stress and release of various toxic factors but the patient remains asymptomatic. This is followed by the second stage that involves targeted injury to the maternal vasculature system by the toxic factors giving rise to the clinical syndrome of PE(49, 122).Improvement in the placental injury and endothelial dysfunction theoretically delay the progress of the disease resulting in improvement in both maternal and perinatal outcomes.

This is the first *in vitro* study that assessed the effect of HCQ on the pathophysiology of PE. The association of HCQ with anti-inflammatory and

antioxidant effects have been published previously based on *in vitro* data (109, 121). Recently, newly added data on *in vivo* studies were published involving a mouse model of lupus. Gomez-Guzman *et al.* had demonstrated that HCQ treatment had prevented hypertension, proteinuria, renal injury and endothelial dysfunction (113). This was supported by Virdis *et al.* who showed that early treatment with HCQ had prevented endothelial dysfunction in a mouse model of lupus (123). All these developments support further assessment of HCQ as an adjuvant therapy in PE.

3.2 Hydroxychloroquine: an adjuvant therapy for preeclampsia?

PLOS ONE

Hydroxychloroquine: an adjuvant therapy for preeclampsia?

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Corresponding Author:	Padma Murthi Monash University Faculty of Medicine Nursing and Health Sciences Clayton, AUSTRALIA
Keywords:	hydroxychloroquine; Preeclampsia; 8-isoprostane; activin A; fms-like tyrosine kinase 1; soluble endoglin; tumour necrosis factor-α; NOX2; ZO-1; endothelial dysfunction
Abstract:	Background It is generally accepted that the widespread maternal endothelial dysfunction in women with preeclampsia is secondary, at least in part, to excessive placental release of anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng), tumour necrosis factor-α (TNF-α) and activin A. This offers opportunities for the development of novel therapies for preeclampsia that target the inflammation and oxidative stress induced by these factors. The antimalarial hydroxychloroquine is an anti-inflammatory that has been shown to improve endothelial health in lupus. Whether it can improve placental and endothelial health in preeclampsia has not been previously explored. Objective To assess whether hydroxychloroquine can alter ex-vivo placental production of sFlt-1, sEng, TNF-α, activin A and 8-isoprostane and/or improve endothelial dysfunction in vitro. Study Design We used in vitro term placental explants to assess the effects of hydroxychloroquine on the ex-vivo placental production of sFlt-1, sEng, TNF-α, activin A and 8-isoprostane and human umbilical vein endothelial cells (HUVECs) to assess the effects of hydroxychloroquine on in vitro markers of endothelial dysfunction. Results Hydroxychloroquine had no effect on the release of sFlt-1, sEng, TNF-α, activin A or 8-isoprostane from placental explants exposed to hypoxic injury or oxidative stress. Hydroxychloroquine significantly mitigated TNF-α and preeclamptic serum induced HUVEC monolayer permeability and rescued loss of zona occludin-1 (ZO-1). Hydroxychloroquine also mitigated TNF-α induced HUVEC production of 8-isoprostane and NOX2 expression but not that induced by preeclamptic serum. Discussion Hydroxychloroquine has no apparent effects on placental explants but may be useful as an endothelial protectant in women with established preeclampsia.
Order of Authors:	Rahana Abd Rahman
	Padma Murthi
	Harmeet Singh
	Seshini Gurusinghe
	Bryan Leaw
	Joanne C Mockler
	Rebecca Lim
	Euan M Wallace

Hydroxychloroquine: an adjuvant therapy for preeclampsia?

Authors: Raihana A. Rahman[1,3,4], Padma Murthi[2], Harmeet Singh[5], Seshini Gunasinghe[1], Bryan Leaw[5], Joanne C.Mockler[1], Rebecca Lim[1,5], Euan M.Wallace[1,5]*.

Affiliations:

[1]Departments of Obstetrics and Gynaecology, School of Clinical Sciences, Monash University, Monash Medical Centre, Clayton, Victoria, Australia.

[2]Departments of Medicine, School of Clinical Sciences, Monash University, Monash Medical Centre, Clayton, Victoria, Australia.

[5]The Ritchie Centre, Hudson Institute of Medical Research, Clayton, Victoria, Australia.

[4]Department of Obstetrics and Gynaecology, Faculty of Medicine, National University of Malaysia, Kuala Lumpur, Malaysia.

***Corresponding author:**

Email: [redacted]

Abstract

Background

It is generally accepted that the widespread maternal endothelial dysfunction in women with preeclampsia is secondary, at least in part, to excessive placental release of anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng), tumour necrosis factor-α (TNF-α) and activin A. This offers opportunities for the development of novel therapies for preeclampsia that target the inflammation and oxidative stress induced by these factors. The antimalarial hydroxychloroquine is an anti-inflammatory that has been shown to improve endothelial health in lupus. Whether it can improve placental and endothelial health in preeclampsia has not been previously explored.

Objective

To assess whether hydroxychloroquine can alter placental production of sFlt-1, sEng, TNF-α, activin A and 8-isoprostane and/or improve endothelial dysfunction *in vitro*.

Study Design

We used *in vitro* term placental explants to assess the effects of hydroxychloroquine on the placental production of sFlt-1, sEng, TNF-α, activin A and 8-isoprostane and human umbilical vein endothelial cells (HUVECs) to assess the effects of hydroxychloroquine on *in vitro* markers of endothelial dysfunction.

Results

Hydroxychloroquine had no effect on the release of sFlt-1, sEng, TNF-α, activin A or 8-isoprostane from *in vitro* placental explants exposed to hypoxic injury or oxidative stress. Hydroxychloroquine significantly mitigated TNF-α and preeclamptic serum induced HUVEC

monolayer permeability and rescued loss of zona occludin-1 (ZO-1). Hydroxychloroquine also mitigated TNF-α induced HUVEC production of 8-isoprostane and NOX2 expression but not that induced by preeclamptic serum.

Discussion

Hydroxychloroquine has no apparent effects on trophoblast function but may be useful as an endothelial protectant in women with established preeclampsia.

Key words: hydroxychloroquine; preeclampsia; 8-isoprostane; activin A; fms-like tyrosine kinase 1; soluble endoglin; tumour necrosis factor-α; NOX2; ZO-1; endothelial dysfunction

Introduction

Preeclampsia complicates about 3-5% of pregnancies and remains one of the leading causes of maternal and perinatal morbidity and mortality [1]. In particular, pregnancies complicated by early-onset preeclampsia at less than 34 weeks gestation are associated with 20-fold increase in maternal mortality [2] and greatly increased rates of maternal and perinatal morbidities [3, 4]. As such, the management of early onset preeclampsia continues to pose significant challenges to the obstetrician who tries to balance maternal risks with the fetal benefits of prolonging the pregnancy.

While not fully understood, the pathophysiology of preeclampsia is generally agreed to originate with poor placentation [5]. Failure of adequate trophoblast invasion and maternal spiral arterial remodeling leads ultimately to impaired placental development and a placenta exposed to chronic progressive ischaemia-reperfusion injury, characterised by evidence of excessive oxidative stress. In turn, this induces excessive placenta release of anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1), and soluble endoglin (sEng) and inflammatory cytokines such as tumour necrosis factor-α (TNF-α), and activin A [6-9]. These various factors target the maternal vasculature system and are thought to be responsible for widespread maternal endothelial dysfunction resulting from oxidative injury [10-13]. The dysfunctional endothelial cells are characterised by increased in endothelial cells permeability, altered distribution of endothelial junctional proteins and reduced endothelium-dependent relaxation [14, 15]. This is depicted in Fig 1.

Fig 1: schematic diagram illustrating the possible pathways involved in the pathophysiology of preeclampsia.

Stage 1 disease involves the first 18 weeks of gestation whereby despite the ongoing

placental insults, which lead to placental ischaemia-reperfusion injury, the patients remain asymptomatic. Stage 2 is a consequent effect of the released placental factors into the maternal circulation that target the maternal vasculature causing widespread endothelial dysfunction. This results in the clinical syndrome of preeclampsia. Abbreviations: 8-IP, 8-isoprostane; TNF-α, tumour necrosis factor-α, sFlt-1, soluble fms-like tyrosine kinase-1; sEng, soluble endoglin.

Antimalarials, such as hydroxychloroquine, were first formally used as a treatment for cutaneous lupus in 1894. Following the observation in the 1940s that they improved inflammatory arthritis they found increasing favour as a therapy in rheumatic diseases. [16]. However, it is only relatively recently that the mechanisms of action of hydroxychloroquine on inflammatory diseases have begun to be understood [17]. Intriguingly, several of the suggested mechanisms of action of hydroxychloroquine in the treatment of lupus could also, theoretically, be effective in the prevention and/or treatment of preeclampsia.

The antimalarial hydroxychloroquine is classified as C under US Food and Drug Administration pregnancy category as it crosses the placenta but has not been reported to cause any teratogenic effects to the fetus [18, 19]. It has both anti-inflammatory and immunomodulatory properties [20-22] and is widely used in autoimmune disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjogren's syndrome. The exact mechanism by which hydroxychloroquine improves the activity of these disorders are still not fully understood but, in women with SLE, it decreases circulating levels of pro-inflammatory cytokines IL-6, IL-8, and TNF-α [23] as well as IL-17, IL-22 which are cytokines produced by the helper T-cells [24]. Recently, in a female mouse model of SLE it was shown that hydroxychloroquine decreased endothelial oxidative stress by reducing

NADPH oxidase activity and that this led to improved endothelial function, lower blood pressure, and a reduction in proteinuria [25].

This may have relevance to preeclampsia because NADPH oxidase dependent oxidative stress is one of the pathways underlying the maternal endothelial dysfunction [13]. NADPH oxidase dependent oxidative stress is thought to contribute to endothelial dysfunction observed in preeclampsia [13]. Accordingly, with a view to assessing hydroxychloroquine as an adjuvant therapy in women diagnosed with preeclampsia, we hypothesized that hydroxychloroquine may confer beneficial effects in preeclampsia by improving the placental and maternal endothelial function. The aim of this study is to investigate the effect of HCQ on the placental and endothelial cell function in preeclampsia.

Methods

Blood and tissue collection

All blood and placental tissues were collected from women after written, informed consent was obtained and with the approval of the Monash Health Human Research Ethics Committee (HREC No: 01067B). Preeclampsia is defined as elevation of blood pressure of 140/90 mmHg or more, and proteinuria of more than 0.3g in a 24 hour urine collection or random urine dipstick test of more than 2+ according to the Society of Obstetric Medicine of Australia and New Zealand guidelines [26]. Venous blood was collected from women with a singleton healthy pregnancy and from women with established preeclampsia, at 24 to 34 weeks of gestation. Women who had received intravenous magnesium sulphate, pre-existing or secondary hypertension, diabetes, or a multiple pregnancy were excluded. None of the women with preeclampsia were in labour at the time of blood sampling. The control (healthy) women were matched for gestation (≤ 34 weeks) and body mass index (BMI). Sera were

129 separated and pooled into two groups: healthy term pregnancy serum and preeclampsia serum.
130 For all *in vitro* experiments, 20% pooled sera from preeclampsia pregnancies were used for
131 treatment of endothelial cells and compared with that of the normotensive sera treated cells.
132 Patient characteristics are summarised in Table 1.
133
134 **Table 1**
135 Clinical characteristics of patient samples used in this study.

Patient characteristics [a]	Normotensive (n=5)	Preeclampsia (n=10)
Gestational age at sampling (weeks)	30.54 ± 2.58	30.42 ± 3.68
Systolic blood pressure (mmHg)	113.60 ± 3.87	164.54 ± 7.51
Diastolic blood pressure (mmHg)	67.20 ± 3.93	112.83 ± 7.12
Proteinuria	None	2+

136 [a] Mann Whitney test was used.

137 Placental explant cultures ex vivo

138 Placental villous explants (n=10) were collected from term uncomplicated pregnancies at
139 elective caesarean section within 20 minutes of delivery of the placenta. Briefly, placental
140 villous tissue was excised by removing maternal decidua. Villous explants (10-70 mg wet
141 weight) were then thoroughly washed with cold Hank's balanced salt solution HBSS (1:10,
142 Life Technologies) and placed in 24-well plates in M199 supplemented with 1% of
143 antibiotics-antimycotics (penicillin G, streptomycin sulphate and amphotericin B) and 1% of
144 L-glutamine (all from Life Technologies).

Assessment of placental function

a) Placental hypoxia

In the early first trimester at 8 weeks of gestation, normally the trophoblast invasion requires a relatively low oxygen concentration as compared to 12 weeks whereby there is a steep rise in the oxygen tension [27]. Placental hypoxia was modeled by incubating placental explants in 1% oxygen, 5% CO_2 at 37°C in the presence or absence of 1 μg/mL hydroxychloroquine (Sigma-Aldrich). Controls were incubated in 5% oxygen. The conditioned media were collected after 24 hours and stored at -80°C for sFlt-1, sEng and TNF-α assay.

b) Placental oxidative stress

Oxidative stress was induced using 2.3 mM xanthine (X) and 0.015 U/mL xanthine oxidase (XO) (Sigma-Aldrich) [9, 28]. Elevated levels of 8-isoprostane has been considered as the best marker for lipid peroxidation due to oxidative stress [29] and in addition, high levels of activin A has been implicated in the pathway of placental oxidative stress [13]. The explants were treated with X/XO in the presence or absence of 1 μg/mL hydroxychloroquine for 48 hours at 37°C in 20% oxygen, 5% CO_2. Untreated cultures served as controls. Conditioned media were collected and stored at -80°C in the presence of 0.005% butylated hydroxytoluene (BHT) (Sigma Aldrich) to prevent autoxidation for activin A and 8-isoprostane assay measurements.

c) Measurement of activin A, sFlt-1, sEng and TNF-α with ELISA

Levels of sFlt-1, sEng**,** TNF-α and activin A were measured in placental explant (n=10) conditioned media using Quantikine immunoassay ELISAs (R&D systems, elisakit.com) according to the manufacturer's protocol. All samples were assayed in duplicates. Briefly, for

the measurement of sFlt-1, sEng, TNF-α and activin A, the conditioned media was diluted 1:40, 1:10, 1:5 and 1:30 respectively with assay diluent. Results were normalized per mg weight of tissue.

Human umbilical vein endothelial cell (HUVEC) isolation

Placentae and umbilical cords were obtained from healthy women with a term singleton pregnancy (n=8) undergoing an elective caesarean section. HUVECs were isolated and cultured, as previously described with minor modifications [9, 30]. Briefly, the umbilical cord was severed from the placenta within an hour after collection. All areas with clamp marks or puncture were removed and the umbilical vein of both ends of the cord were cannulated and tied with thread. After removal of blood, the umbilical veins were infused with type II collagenase (0.5mg/ml, Sigma-Aldrich) and incubated for 10 minutes at 37°C to isolate the endothelial cells. They were maintained in M199 complete media containing 20% heat-inactivated fetal calf serum, 1% of antibiotics-antimycotics (penicillin G, streptomycin sulphate and amphotericin B), 1% of L-glutamine, endothelial and fibroblast growth factor (10 ng/mL each). Only cells at passage 2 to 4 were used for the experiments.

HUVECs viability assay

We first determined the effect of different concentrations of hydroxychloroquine on HUVEC viability. Cells were plated at 2 x 10^4 cells/well in 96-well plates (n=8, Corning) and grown to confluence in 100 µl culture media with hydroxychloroquine added at different concentrations (0.1, 1. 10, 100 µg/mL) and further incubated for 24 hours. Viability was assessed by adding 20 µl MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Promega) to each well. After 1 hour at 37°C, the absorbance at 490 nm was read using a plate reader (SpectraMax i3, Molecular Devices).

10

Assessment of endothelial dysfunction

a) Oxidative stress as assessed by 8-isoprostane

Cells were grown to confluence in 96 well plates (2 x 10^4 cells/well) for 24 hours in M199 complete media. Cells were treated with media (control), 100 ng/mL recombinant tumour necrosis factor-α (TNF-α) (Life Technologies), 20% normal pregnancy sera, or 20% preeclampsia sera, in the presence or absence of hydroxychloroquine (0, 0.1, 1 and 10 μg/mL) for a further 24 hours. Conditioned media were then stored in -80°C in the presence of 0.005% butylated hydroxytoluene (BHT) to prevent autoxidation prior to analysis. Total 8-isoprostane was measured using a commercial enzyme immunoassay (Cayman Chemical) according to the manufacturer's instructions. Samples were assayed in duplicates after diluting 1:5 with assay diluent. Based on the results from this experiment, in all subsequent experiments 1μg/mL hydroxychloroquine was used. The cells were treated with either 100 ng/mL of recombinant TNF-α or 20% preeclampsia sera in combination with either 1 μg/mL hydroxychloroquine or 100 μM apocynin (NADPH oxidase inhibitor) (Sigma Aldrich) for 24 hours.

b) Measurement of NADPH oxidase (NOX2) mRNA expression

Cells were grown to confluence in 6-well plates (1 x 10^5 cells/well) for 48-72 hours in M199 complete media. Cells were treated with 100 ng/mL recombinant TNF-α or 20% preeclampsia serum combined with either 1 μg/mL hydroxychloroquine or 100 μM apocynin for 12 and 6 hours respectively. The treatment groups were compared with untreated HUVECs or cells treated with 20% normotensive sera. Total cellular RNA was isolated with Ambion (Thermo Fisher) according to the manufacturer's protocols. The cDNA was prepared with 1 μg of cellular mRNA, reverse-transcribed using SuperScript®III first strand synthesis

system (Life Technologies). Quantitative PCR was performed on Rotorgene (Qiagen Pty Ltd) in a reaction mixture (20 μl) containing Sensimix SYBR®Green PCR master mix (Bioline). The reactions were performed with the following conditions: 95°C for 10 minutes then for 40 cycles of 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. NOX2 was amplified using primers 5'-TGG CAC CCT TTT ACA CTG-3' and 5'-CCA CTA ACA TCA CCA CCT CA-3'. 18S was amplified using primers 5'-GTC TGT GAT GCC CTT AGA TGT C-3' and 5'-AAG CTT ATG ACC CGC ACT TAC-3'. 18S was used as a house keeping gene. Relative gene expression was determined using delta delta CT.

c) Endothelial permeability assay

An endothelial permeability assay was performed as previously described with minor modifications [31]. Briefly, culture inserts (0.4 μm pore size, 6.5 mm diameter; Corning) were coated with 0.2% gelatin (Sigma-Aldrich) for 30 minutes at room temperature. HUVECs (50,000 cells/well) were plated on the inserts and cultured to form a tight monolayer with 100 μl M199 complete media in the upper chamber and 600 μl in the lower chamber at 37°C, 5% CO2 for 72 hours. Inserts were then transferred to a fresh plate and cell monolayers were treated in fresh media with 100 ng/mL recombinant TNF-α either alone or with 1 μg/mL hydroxychloroquine for 16–22 hours. The treatment groups were compared with untreated HUVECs. The conditioned media were collected and 100 μl fresh media containing fluorescein isothiocyanate (FITC)-conjugated dextran (MW 40000, final concentration 1 mg/mL, Sigma-Aldrich) was added to the upper chamber. The plate was incubated protected from light for 60 minutes. The media from the lower chamber were diluted (1:20) in HBSS for measurement of fluorescence at 485/535 nm using a plate reader (SpectraMax i#, Molecular Devices). Results (fluorescence units) were expressed as percent changes relative to control.

Assessment of cell permeability when treated with 20% normal or preeclampsia sera was performed using in vitro permeability assay kit from Millipore (Merck Millipore) in the absence or presence of 1 μg/mL hydroxychloroquine for 16-22 hours. The treatment groups were compared with HUVECs treated with normal pregnancy (NP) serum. Briefly, the transwells, which were coated with collagen, were rehydrated with 250 μl endothelial growth media (EGM, Lonza) and left at room temperature for 15 minutes. Subsequently, 200 μl of the media removed and replaced with an equal volume of cell stock (1×10^5). Media of 500 μl added to the receiver plate and incubated for 72 hours to form tight monolayer. Following this fresh media was replaced in the receiver plate. The cells were treated accordingly and further incubated for 16-22 hours. Following this, the media in the upper chamber was replaced with fresh media (150 μl) containing fluorescein isothiocyanate (FITC)-conjugated dextran and the plate was incubated for 30 minutes protected from light. The media from the lower chamber was diluted (1:20) with HBSS for measurement of fluorescence at 485/535 nm using a plate reader (SpectraMax i3, Molecular Devices). Results (fluorescence units) were expressed as percent changes relative to control.

d) Zonula occludens (ZO-1) immunohistochemistry for the assessment of endothelial integrity

HUVECs were grown on 14 mm glass coverslips (4×10^4 cells/well) placed in 24 well plates treated with 100 ng/mL recombinant TNF-α or 20% preeclampsia sera in the presence or absence of 1 μg/mL hydroxychloroquine for 16–22 hours prior to fixing with 4% paraformaldehyde (Sigma Aldrich) for 30 minutes at room temperature. The treatment groups were compared with untreated HUVECs or cells treated with 20% normal pregnancy sera. All incubations and washes were carried out at room temperature unless specified otherwise. Cells were blocked with 0.5% bovine serum albumin (BSA, Sigma-Aldrich) for 30 minutes,

incubated first with rabbit anti-ZO-1 (1:50, Zymed) overnight at 4°C, then with donkey anti-rabbit Alexa Fluor 568 (1:100, Invitrogen) for 1 hour in the dark. Cell nuclei were stained with 2 μm 4', 6-diamidino-2-phenyindole dilactate (DAPI, Sigma Aldrich) for 10 minutes and mounted with fluorescent mounting media (DakoCytomation). Staining was examined with an Olympus BX60 fluorescent microscope and images were taken using an Olympus DP70 camera and Olympus CellSens software (Olympus). The primary antibody was replaced with an isotype matched control antibody in the negative controls. The mean intensity of the staining was assessed using Image J software (version 2.0.0-rc-43/1.50i, http://imagej.net/Fiji/Downloads, Bethesda, MD).

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analysis was performed on raw data or percent change relative to control. Unpaired two-tailed t-test was used only for the cell viability and other data were analysed using one-way ANOVA followed by Tukey's post hoc test with PRISM version 6.0 (GraphPad Software). Differences were considered significant where $P < 0.05$. Sample size was chosen based on our previous experience with the methods.

Results

Effects of hydroxychloroquine on placental secretion of angiogenic factors

Figs 2 and 3 depict the effects of 1 μg/mL hydroxychloroquine on the secretion of sFlt-1, sEng, TNF-α, 8-isoprostane, and activin A in placental explant cultures. Compared to normoxic (5% O_2) explant cultures, hypoxia significantly increased the secretion of sFlt-1

(Fig 2A, p=0.01), sEng (Fig 2B, p=0.02) and TNF-α (Fig 2C, p=0.02) after 24 hours. Culture of explants for 48 hours in the presence of X-XO significantly increased 8-isoprostane (Fig 3A, p=0.03) and activin A (Fig 3B, p=0.01) secretion compared to controls. Hydroxychloroquine reduces the hypoxia induced increased secretion of sFlt-1 (Fig 2A), sEng (Fig 2B) and TNF-α (Fig 2C), but it was not statistically significant. The effects of X-XO induced increase in 8-isoprostane (Fig 3A) and activin A (Fig 3B) were not mitigated by hydroxychloroquine.

Fig 2: Effect of hydroxychloroquine on normal term placental explants under hypoxic versus normoxic condition. Release of (A) sFlt-1, (B) sEng and (C) TNF-α by placental explants of human term normal pregnancy placentae after 24 hours incubation at 5% oxygen concentration (normoxia) versus 1% (hypoxia). The explants were incubated in hypoxic environment in the absence or presence of hydroxychloroquine at 1 µg/mL. Data are means ± SEM from ten and twelve independent biological replicates respectively. * denotes $p < 0.05$. NT-non treated, HCQ-hydroxychloroquine.

Fig 3: Effect of hydroxychloroquine on normal term placental explants induced with oxidative stress. Release of (A) 8-isoprostane and (B) activin A by placental explants of human term normal pregnancy placentae after 48 hours incubation at 20% oxygen concentration with 5% CO_2. The explants were incubated in media containing xanthine (2.3 mM)+xanthine oxidase (15 mU/mL) in the absence or presence of hydroxychloroquine at 1 µg/mL. Data are means ± SEM from twelve and eleven independent biological replicates respectively. * denotes $p < 0.05$ and ** $p< 0.005$. X/XO-xanthine/xanthine oxidase, HCQ-hydroxychloroquine.

306

307 Effect of hydroxychloroquine on HUVEC viability

308 Fig 4 summarises the effects of hydroxychloroquine (0.1, 1, 10 and 100 μg/mL) on HUVEC
309 viability in culture. Compared to controls, there was no effect of hydroxychloroquine on cell
310 viability across a dose range of 0.1 μg/mL – 10 μg/mL over 120 hours in culture (Fig 4B-D).
311 At 100 μg/mL hydroxychloroquine significantly reduced cell viability at 24 hours (Fig 4A,
312 $p<0.0001$). Dosing of hydroxychloroquine for all future experiments was based on these
313 results.

314 **Fig 4: Effect of hydroxychloroquine on HUVECs viability.** The effect of
315 hydroxychloroquine on HUVECs viability after 24 hours at 0.1, 1, 10 and 100 1μg/mL (A)
316 and extended incubation for 48, 72, 96 and 120 hours treatment at (B) 0.1μg/mL, (C) 1μg/mL
317 and (D) 10μg/mL. Data are means ± SEM from seven and five independent biological
318 replicates respectively. **** denotes $p<0.0001$.

319 Effects of hydroxychloroquine on endothelial function in vitro

320 Fig 5 summarises the effect of hydroxychloroquine treatment following endothelial
321 dysfunction induced by incubating HUVEC in the presence of (i) TNF-α (100 ng/mL) or (ii)
322 preeclampsia sera (20%) or (iii) normal pregnancy sera (20%) in the presence or absence of
323 hydroxychloroquine (1 μg/mL). Compared to their controls, incubation of HUVEC with
324 either TNF-α (Fig 5A and 5C) or preeclampsia sera (Fig 5B and 5D) significantly increased
325 both NOX2 expression ($p<0.0001$ and $p=0.02$, respectively) and 8-isoprostane secretion
326 ($p=0.003$ and $p=0.04$, respectively). Co-treatment of HUVECs treated with recombinant
327 TNF-α with hydroxychloroquine significantly reduced the increased NOX2 activity (Fig 5A,

p=0.03) and release of 8-isoprostane (Fig 5C, p=0.003). Co-treatment of HUVECs treated with PE serum with hydroxychloroquine did not significantly reduce NOX2 mRNA expression as well as 8-isoprostane releases. However, 100μM apocynin, a NOX inhibitor, significantly reduced the NOX2 mRNA expression and 8-isoprostane release induced by PE serum (Fig 5B and 5D respectively, p=0.01 for both).

Fig 5: Effect of hydrxychloroquine and NOX2 inhibitor on 8-isoprostane release and NOX2 mRNA expression from HUVECs. NOX2 mRNA expression of HUVECs treated with 100 ng/mL TNF-α (A) and 20% preeclampsia (PE) sera (B). Release of 8-isoprostane by HUVECs treated with 100 ng/mL recombinant TNF-α (C) and 20% preeclampsia sera (D). Data are means ± SEM from seven to nine independent biological replicates. * denotes $p < 0.05$ and **** $p<0.0001$.

Effect of hydroxychloroquine on vascular permeability

Fig 6 summarises the effect of hydroxychloroquine on TNF-α and preeclampsia sera induced endothelial permeability. Both TNF-α (Fig 6A) and preeclampsia sera (Fig 6B) significantly increased HUVEC monolayer permeability compared to controls (p=0.02 and p=0.004, respectively), effects mitigated by hydroxychloroquine (p=0.04 and p=0.007, respectively)

Fig 6: The effects of 1μg/mL hydroxychloroquine on HUVECs permeability and ZO-1 (A) HUVECs permeability when treated with 100 ng/mL recombinant TNF-α and (B) 20% preeclampsia sera. (C) Mean ZO-1 fluorescence when treated with 100 ng/mL recombinant TNF-α and (D) 20% preeclampsia sera. Data are means ± SEM from nine to ten independent biological replicates respectively. * denotes $p < 0.05$ * and ** $p<0.005$.

Effect of hydroxychloroquine on zonula occludens (ZO-1) immunohistochemistry

Hydroxychloroquine prevented the significant loss of ZO-1 induced by both TNF-α (Fig 6C, p=0.003 and p=0.002) and preeclampsia sera (Fig 6D, p=0.005 and p=0.02). Fig 7 showed representative images of ZO-1 immunostaining. There is normal ZO-1 immunostaining in untreated or normal pregnancy sera treated HUVEC (Fig 7A and 7D) and loss of immunostaining in cells treated with either TNF-α or preeclampsia sera (Fig 7B and 7E). Hydroxychloroquine rescued the loss of ZO-1 induced by both TNF-α (Fig 7C) and preeclampsia sera (Fig 7F).

Fig 7: Images of ZO-1 staining of HUVEC. Immunofluorescent staining of ZO-1 on HUVECs treated with 100 ng/mL recombinant TNF-α or 20% preeclampsia sera for 16-22 hours. Representative images from one of five experiments are shown. (A) Control-untreated HUVECs, (B) TNF-α 100 ng/mL, (C) TNF-α 100 ng/mL with hydroxychloroquine 1 μg/mL, (D) control-HUVECs treated with 20% normal pregnancy sera, (E) 20% preeclampsia sera and (F) preeclampsia sera with hydroxychloroquine 1 μg/mL. Arrows show the ZO-1 staining of the endothelial cells border.

Immunofluorescent staining of ZO-1 on HUVECs treated with 100 ng/mL recombinant TNF-α or 20% preeclampsia sera for 16-22 hours. Representative images from one of six experiments are shown. (A) Control-untreated HUVECs, (B) TNF-α 100 ng/mL, (C) TNF-α 100 ng/mL with hydroxychloroquine 1 μg/mL, (D) control-HUVECs treated with 20% normal pregnancy sera, (E) 20% preeclampsia sera and (F) preeclampsia sera with hydroxychloroquine 1 μg/mL. Arrows show the ZO-1 staining on the endothelial cells border.

Discussion

To our knowledge, this is the first study to report the effects of hydroxychloroquine on HUVEC and placental explant function. We undertook the study with a view to exploring the potential of hydroxychloroquine as a novel targeted therapy addressing key pathophysiological pathways in preeclampsia. We have shown that it affords no apparent protection against hypoxia or oxidative stress in placental explants but that it does have some endothelial protective effects. These observations suggest that hydroxychloroquine may be worth exploring further as an adjuvant therapy for women with preeclampsia but that it is unlikely to be useful as a primary preventative therapy.

Effects of hydroxychloroquine on placental hypoxic injury and oxidative stress

We had hypothesised that hydroxychloroquine might be able to protect the hypoxia and hyperoxia induced injury in the placenta *ex-vivo*. Specifically, we sought to show that hydroxychloroquine could mitigate the effects of hypoxia and hyperoxia on the placental release of the anti-angiogenic factors sFlt-1 and sEng and on the release of the pro-inflammatory cytokines TNF-α, activin A, respectively. However, we found this not to be the case. Hydroxychloroquine had no effect on modulating either hypoxia or hyperoxia induced placental injury. These findings support those of others who tested hydroxychloroquine in a trophoblast-derived cell line exposed to antiphospholipid antibodies as a model of antiphospholipid syndrome [32]. They found that while hydroxychloroquine was able to mitigate trophoblast secretion of IL-6, it had no effect on sEng release [32]. Specific insults believed to be involved in the pathophysiology of preeclampsia were simulated in normal term human placenta. This is important to investigate which pathway or injury can be

reversed by hydroxychloroquine. Collectively, this suggests that in an established diagnosis of preeclampsia, the use of hydroxychloroquine may not confer any beneficial effects.

Effects of hydroxychloroquine on endothelial cells dysfunction

The maternal signs and symptoms of preeclampsia are due to widespread maternal endothelial dysfunction [33, 34]. Lupus shares this feature as the key mechanism underlying hypertension, renal dysfunction, and other organ injury [35]. Indeed, the endothelial dysfunction in both preeclampsia and lupus have also been shown to be due, at least in part, to excessive oxidative stress secondary to NOX activation [13, 36, 37]. Recently, in murine models of lupus hydroxychloroquine has been shown to reverse endothelial dysfunction via the downregulation of NOX and subsequent oxidative stress [25, 38]. Here we show that hydroxychloroquine may have similar effects in an *in vitro* model of preeclampsia-like endothelial dysfunction. Specifically, hydroxychloroquine was able to prevent the TNF-α induction of NOX2 and subsequent oxidative stress in HUVECs but, importantly, was not able to block similar effects induced by preeclampsia sera. Interestingly, apocynin, which is a NOX inhibitor, was able to prevent the effects of both TNF-α and preeclamptic sera on NOX and oxidative stress. This confirms that the pro-oxidative effects of preeclamptic serum are mediated via NOX2 [13] but that the inducer(s) of NOX present in the maternal circulation must be in addition to or other than those blocked by hydroxychoroquine. We have shown before that follistatin, an activin binding protein, can wholly block the endothelial effects of preeclamptic serum [13, 39]. Compared to women with a normal pregnancy, maternal circulating levels of activin are increased about 10-fold in women with preeclampsia [40]. We have not yet explored whether hydroxychloroquine can block activin-mediated effects but that would be worthwhile. Certainly, the current studies suggested that preeclampsia

preeclampsia serum suggests that these effects may be exerted mainly through TNF-α dependent NOX upregulation. Whether this is so would require further evaluation, perhaps using TNF-α receptor antagonists to block effects of preeclamptic sera.

Hydroxychloroquine for treatment of established preeclampsia

There are still other pathways in preeclampsia that may be targeted by hydroxychloroquine that had not been explored in this study. For example, it is now thought that a key mechanism of action of antimalarial drugs is the antagonism of Toll-like receptor (TLR) signaling and subsequent downstream activation of pro-inflammatory cytokines [17, 44]. With regard to preeclampsia this is promising because the placental expression of TLR 3, 7, and 8 is upregulated in the preeclamptic placenta compared to the normal healthy placenta and the treatment of pregnant rodents with TLR agonists induces a preeclampsia-like phenotype [45, 46].

In addition to the effects of antimalarial agents on TLRs, these drugs have other benefits such as inhibition of phospholipase A2 (PLA2) enzyme. PLA2 has been implicated in the pathogenesis of preeclampsia and is found to be elevated in both decidual tissue and serum of preeclamptic women [47, 48]. Similarly, in patients with active SLE, there is 4.6 fold increase in the mean activity of PLA2 [49]. Lipid peroxidation occurs because of oxidative stress induced by the elevated levels of reactive oxygen species. This leads to membrane phospholipid degradation and hence release of arachidonic acid [50]. In turn, arachidonic acid stimulates release of superoxide from neutrophils and macrophages [51]. Antimalarial drugs have been shown to inhibit the PLA2 activity and therefore reduces the generation of superoxides, which will be beneficial for preeclamptic patients in regards to improvement in endothelial dysfunction [50, 52].

21

Conclusion

While hydroxychloroquine has some protective effects on endothelial function, acting via the suppression of NOX-induced oxidative stress, it is unable to mitigate all the effects of preeclampsia sera-induced injury *in vitro* or to mitigate *ex-vivo* placental injury. Further evaluation is warranted to determine other molecular pathways by which hydroxychloroquine may protect endothelial function in preeclampsia. From this study, hydroxychloroquine appears unlikely to be effective as primary prevention of preeclampsia but offers some promise as an adjuvant therapy in established disease.

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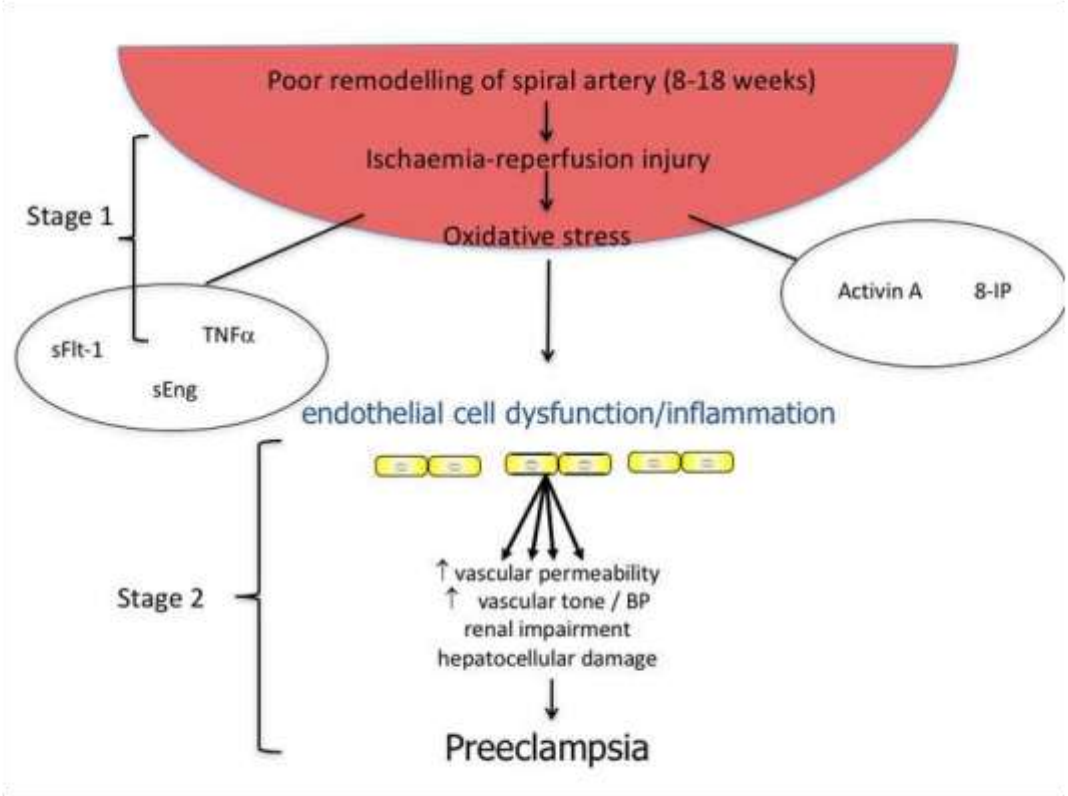
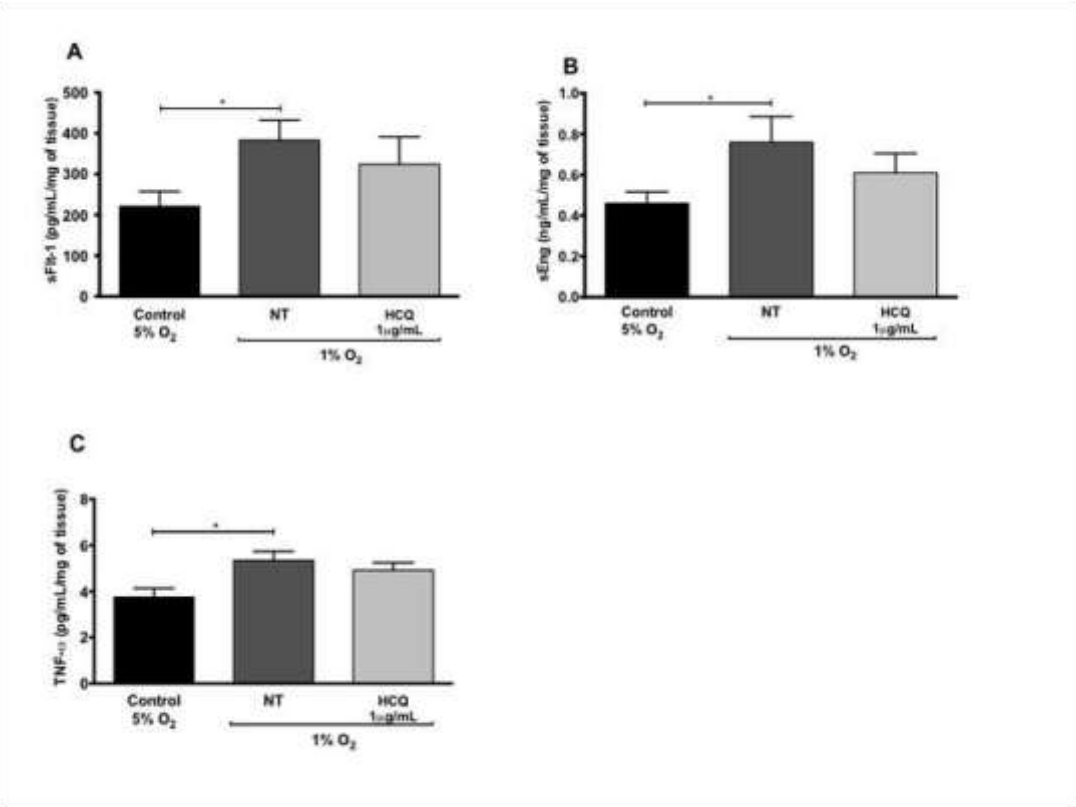

Poor remodelling of spiral artery (8-18 weeks)
Ischaemia-reperfusion injury
Oxidative stress
Stage 1
sFlt-1
TNFα
sEng
Activin A
8-IP
endothelial cell dysfunction/inflammation
Stage 2
↑ vascular permeability
↑ vascular tone / BP
renal impairment
hepatocellular damage
Preeclampsia

Figure 2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0.8
0.6
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5% O2
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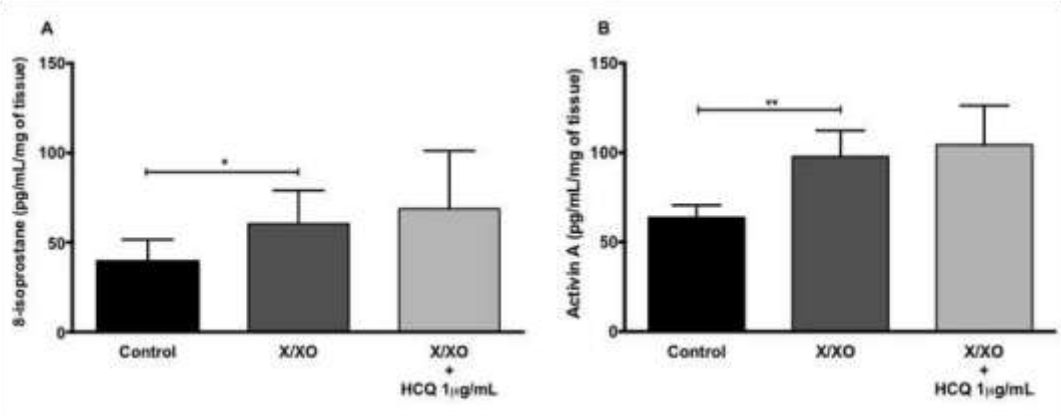
1% O2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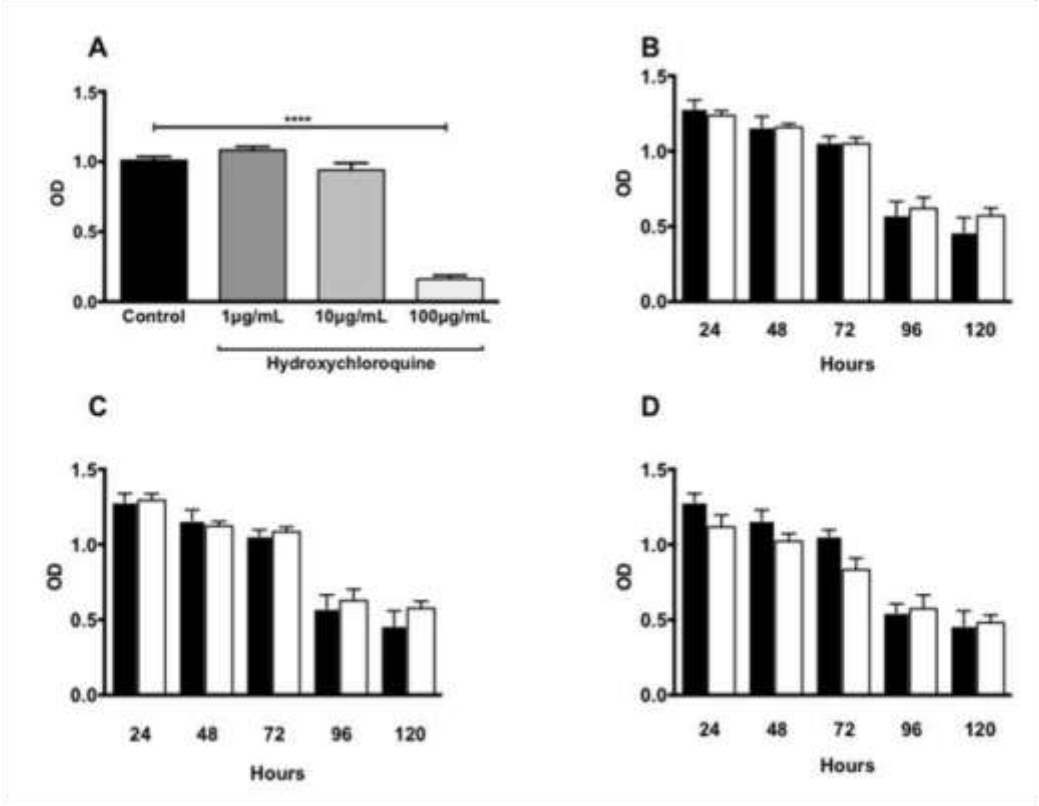
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Figure 5

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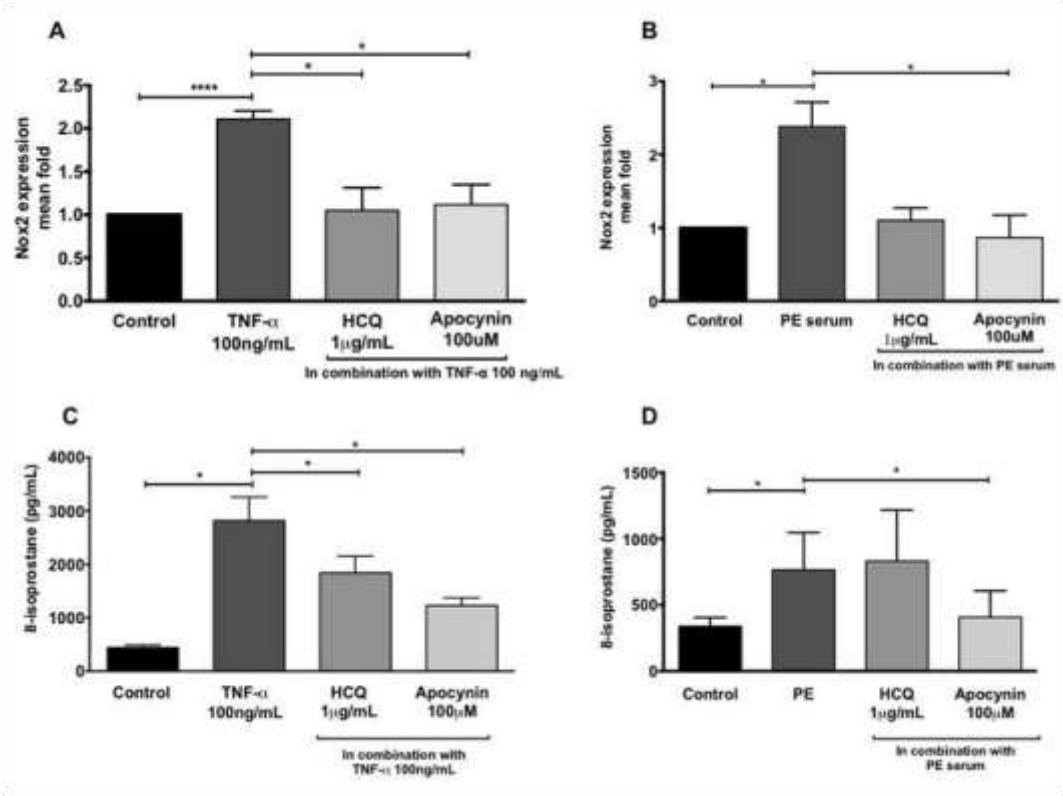

Figure 6

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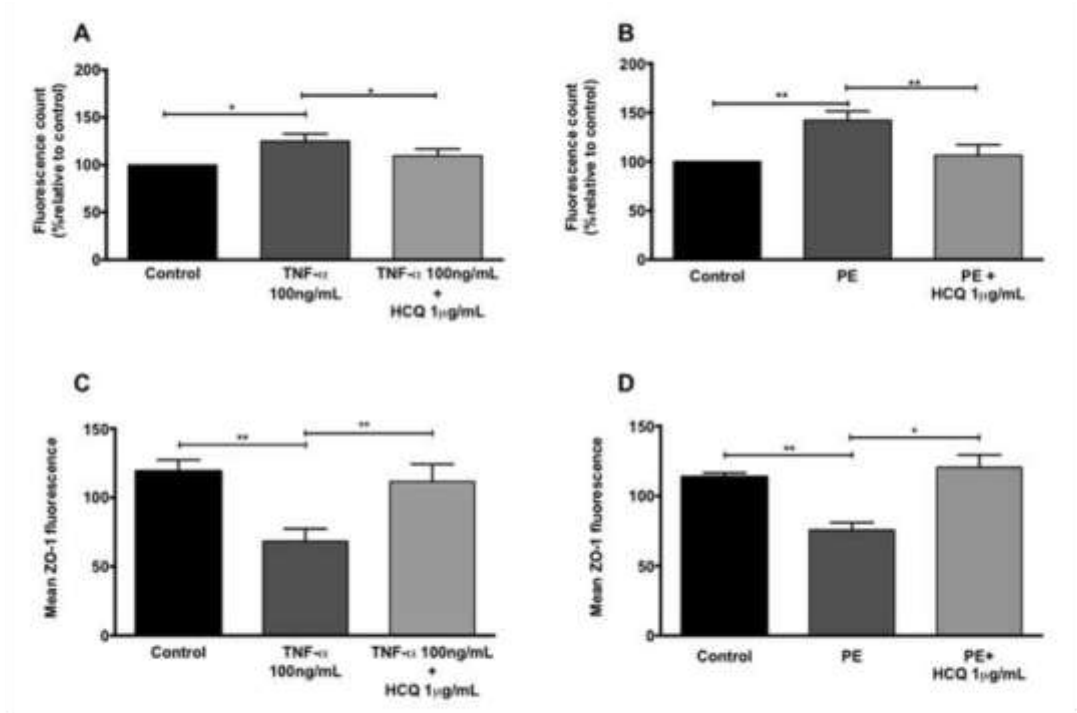

Figure 7

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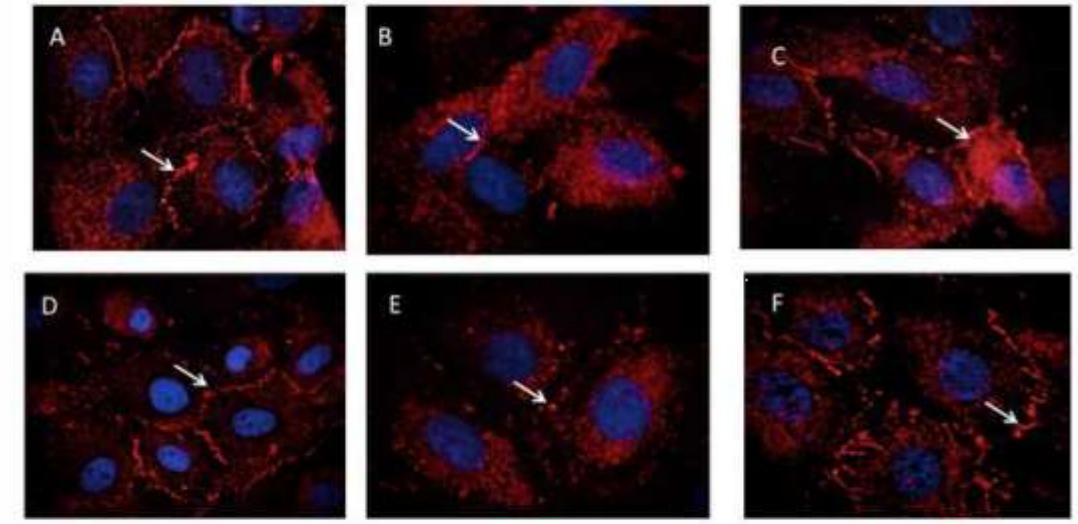

CHAPTER FOUR

The effects of hydroxychloroquine on endothelial dysfunction

4.1 Preamble

Endothelial cells have been recognised to be an important structure that plays a vital role in many diseases. These cells behave like sensors detecting both physical and chemical stimuli in the vessels to modify the shape or produce agents that are necessary to maintain hemostasis and overcome the insults. The agents produced consist of a balanced vasodilatory and vasoconstrictor substances along with other various molecules to modulate hemostasis. In the presence of overwhelming injury to the endothelial cells by excessive inflammation and oxidative stress, the endothelial cells are activated and this may lead to endothelial dysfunction.

Endothelial dysfunction has a complex pathophysiology which involves multiple mechanisms. It serves as an important link between diseases such as hypertension, diabetes mellitus and atherosclerosis (101). Most importantly, it was proposed to be an early event in the pathophysiology of these diseases. Therefore, many researchers have attempted to use drugs that target endothelial cells to improve or prevent endothelial dysfunction and hence improve the clinical outcome of these diseases.

Preeclampsia is a clinical syndrome originating from widespread endothelial dysfunction based on considerable evidence. There are various biomarkers or assay that can be used to detect this such as serum levels of ET-1 which is a potent vasoconstrictor that is mainly produced by the endothelial cells (124). It has been implicated in the elevation of blood pressure in the rat model of preeclampsia (RUPP) (125). On the other hand, the angiogenic potentials of endothelial cells in preeclampsia has not been well established. Only one published *in vitro* study had shown that there is increased branching angiogenesis in human umbilical vein endothelial cells from preeclamptic women (126).

Therefore, this chapter further investigates the effect of hydroxychloroquine on the endothelial dysfunction in preeclampsia.

4.2 The effects of hydroxychloroquine on endothelial dysfunction

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Short communication

The effects of hydroxychloroquine on endothelial dysfunction

Rahana Rahman [a,b,c], Padma Murthi [d], Harmeet Singh [a], Seshini Gurusinghe [a,b], Joanne C. Mockler [a,b], Rebecca Lim [a], Euan M. Wallace [a,b,*]

[a] The Ritchie Centre, Hudson Institute of Medical Research, 27-31 Wright Street, Clayton 3168, Melbourne, VIC, Australia
[b] Department of Obstetrics and Gynaecology, School of Clinical Sciences, Monash University, Monash Medical Centre, 246 Clayton Road, Clayton 3168, Melbourne, VIC, Australia
[c] Department of Obstetrics and Gynaecology, Faculty of Medicine, National University of Malaysia, Jalan Yaacob Latiff, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia
[d] Department of Medicine, School of Clinical Sciences, Monash University, Monash Medical Centre, 246 Clayton Road, Clayton 3168, Melbourne, VIC, Australia

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ABSTRACT

Hydroxychloroquine is an anti-malarial drug which, due to its anti-inflammatory and immunomodulatory effects, is widely used for the treatment of autoimmune diseases. In a model of systemic lupus erythematosus hydroxychloroquine has been shown to exert protective endothelial effects. In this study, we aimed to investigate whether hydroxychloroquine was endothelial protective in an *in vitro* model of TNF-α and preeclamptic serum induced dysfunction. We showed that hydroxychloroquine significantly reduced the production of TNF-α and preeclamptic serum induced endothelin-1 (ET-1). Hydroxychloroquine also significantly mitigated TNF-α induced impairment of angiogenesis. These findings support the further assessment of hydroxychloroquine as an adjuvant therapy in preeclampsia.

1. Introduction

Preeclampsia is a multi-systemic disorder affecting about 5% of pregnancies [1]. It is associated with increased risks of maternal and perinatal mortality and morbidity and remains a leading cause of iatrogenic preterm birth [1,2]. While the pathophysiology of preeclampsia is yet to be fully elucidated there is growing evidence that excessive placental and systemic oxidative stress and widespread maternal endothelial dysfunction are the two main pathologies contributing to the signs and symptoms of the clinical syndrome [1,3–6].

Specifically, it is currently thought that the endothelial dysfunction is, at least in part, secondary to excessive placental release of pro-inflammatory and anti-angiogenic factors, such as tumour necrosis factor-α (TNF-α), soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng) and activin A into the maternal circulation [6–13]. In particular, women with established preeclampsia have significantly higher levels of TNF-α than women with a healthy pregnancy [13]. Maternal levels of TNF-α are also increased in other pregnancy complications associated with altered placental function such as fetal growth restriction and diabetes [14,15]. It has been shown that TNF-α induces endothelial dysfunction with many of the features seen in women with preeclampsia including increased endothelin-1 (ET-1) release, down-regulated endothelial nitric oxide synthase (eNOS) expression, increased NADPH oxidase activity and impaired angiogenesis [16].

Systemic lupus erythematosus (SLE), an autoimmune disease, shares many features with preeclampsia including elevated levels of TNF-α and endothelial dysfunction [17,18]. Recently, hydroxychloroquine, an antimalarial drug commonly used in the treatment of SLE, was shown to improve endothelial function in mice model of severe SLE [19]. Treatment with hydroxychloroquine is also associated with a decline in serum ET-1 levels in patients with SLE [20].

Accordingly, we aimed to determine whether hydroxychloroquine was able to mitigate the *in vitro* features of endothelial dysfunction induced by recombinant TNF-α or preeclamptic serum specifically to changes in endothelin-1 (ET-1) release and angiogenesis. To our knowledge, this is the first study to investigate the potential of hydroxychloroquine to improve TNF-α and preeclamptic serum induced endothelial dysfunction.

2. Materials and methods

Maternal sera were collected from 10 women with established preeclampsia and from five gestation-matched normotensive

* Corresponding author at: Department of Obstetrics and Gynaecology, Monash University, Level 5, Monash Medical Centre, 246 Clayton Road, Clayton, VIC 3168, Australia.
E-mail address: euan.wallace@monash.edu (E.M. Wallace).

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2210-7789/

Table 1
Characteristics of pregnant women from whom serum pools were derived.

	Normotensive (n = 5)	Preeclampsia (n = 10)
Mean (±SEM) gestation at sampling (weeks)	30.5 ± 2.6	30.4 ± 3.7
Mean (±SEM) systolic blood pressure (mmHg)	107.3 ± 2.2	164.5 ± 7.5
Mean (±SEM) diastolic blood pressure (mmHg)	62.4 ± 1.6	112.8 ± 7.1
Proteinuria, g/mL 24 h	0 ± 0	1.0 ± 0.3

pregnant women, with the approval of the Monash Health Human Research Ethics Committee following written, informed consent. Sera were separated and pooled into two groups: preeclampsia and normotensive pregnancy. The patient characteristics are summarised in Table 1. Preeclampsia was defined new onset of hypertension (⩾140/90 mmHg) after 20 weeks of pregnancy with one or more of the following: renal involvement (proteinuria > 300 mg 24 h), haematological involvement (low platelets, haemolysis, DIC), liver involvement (raised transaminases), neurological involvement (seizures, headache, visual disturbance, stroke), pulmonary oedema, fetal growth restriction, or placental abruption, as per Society of Obstetric Medicine of Australia and New Zealand guidelines [21]. Exclusion criteria were pre-existing hypertension, diabetes mellitus, multiple pregnancy and treatment with magnesium sulphate.

Human umbilical vein endothelial cells (HUVECs) were isolated from term uncomplicated pregnancies (n = 8) and expanded as previously described [22]. Experiments were conducted in 96-well plates. The effect of different concentrations of hydroxychloroquine (1, 10, 100 μg/mL) (Sigma-Aldrich, Missouri, USA) on cell viability was first determined using the MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H* tetrazolium) (Promega, Victoria, Australia). The absorbance at 490 nm was recorded using an ELISA plate reader (SpectraMax i3, Molecular Devices, California, USA).

HUVECs were grown to confluence in 96-well plates (2×10^4 cells/well, Corning, New York, USA) and incubated with recombinant TNF-α (100 ng/mL, Life Technologies, Carlsbad, CA) or 20% preeclamptic serum in the absence or presence of hydroxychloroquine at 1 and 10 μg/mL for 24 h. The conditioned media were collected and stored at −80 °C. The levels of ET-1 in the conditioned media were measured by ELISA (R&D systems, Minneapolis, MN) according to the manufacturer's protocols.

Endothelial tube formation was performed as previously described [23], with minor modifications Briefly, pre-chilled angiogenesis μ-slides (Ibidi, Victoria, Australia) were coated with 10 μL/well growth factor reduced Matrigel (Corning, New York, USA). HUVEC cells (20,000 cells) in 50 μL complete endothelial growth media (EGM, Lonza, Victoria, Australia) were placed in the wells, treated with recombinant TNF-α (10 ng/mL, Life Technologies, Carlsbad, CA) or 5% pre-eclamptic serum in the absence or presence of hydroxychloroquine (1 and 10 μg/mL, Sigma-Aldrich, Missouri, USA) for six hours at 37 °C, 5% CO_2. The culture medium was removed from the wells, and Calcein AM fluorescent dye (Millipore, Victoria, Australia) diluted 1:500 with Hank's Balanced Salt Solution (HBSS 1:10, Gibco, Waltham, USA) was added (40 μL/well). Tubes were assessed immediately through an inverted fluorescent microscope at 4x magnification (Olympus) and quantitatively analysed (total tube lengths, branch points) using image J software (http://rsbweb.nih.gov/ij/; National Institutes of Health, Bethesda, MD).

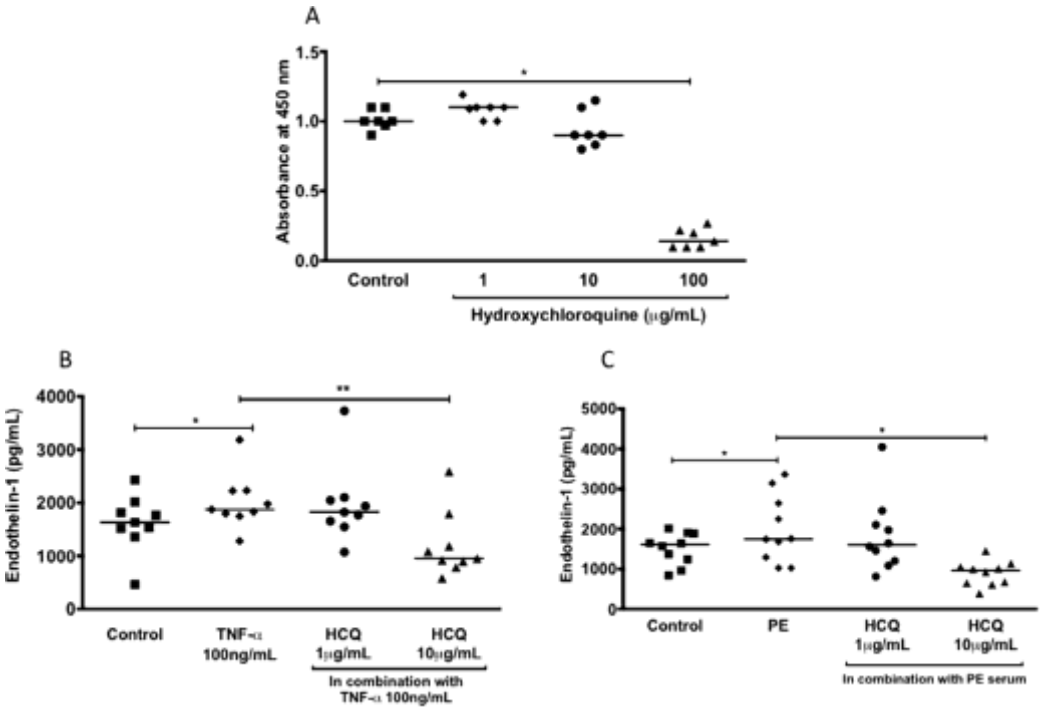

Fig. 1. (A) Hydroxychloroquine did not alter HUVEC endothelial viability at 0.1, 1 and 10 μg/mL, but reduced viability at 100 μg/mL. Data are median from seven independent biological replicates. *Denotes $p < 0.05$. (B) Recombinant TNF-α (100 ng/mL) and (C) pre-eclamptic serum (PE) increased HUVEC secretion of endothelin-1, effects mitigated hydroxychloroquine (1 and 10 μg/mL). Data are median from eight independent biological replicates and *denotes $p < 0.05$ and **$p < 0.005$.

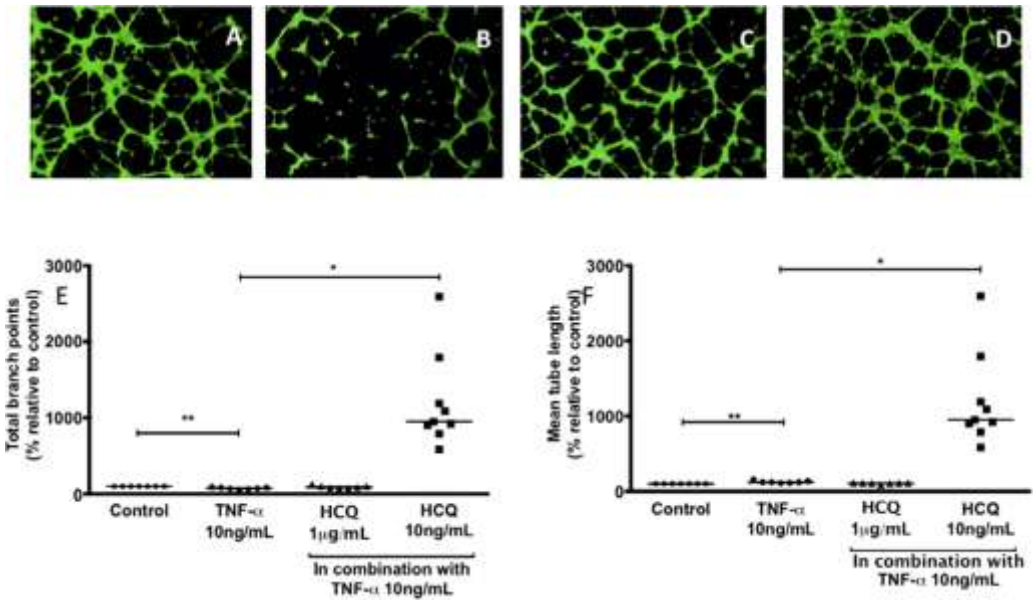

Fig. 2. Images of one from eight experiments are shown: (A) control, (B) TNF-α 10 ng/mL alone, (C) TNF-α 10 ng/mL and hydroxychloroquine 1 μg/mL, (D) TNF-α 10 ng/mL and hydroxychloroquine 10 μg/mL. Recombinant TNF-α (10 ng/mL) impaired HUVEC angiogenesis as assessed by (E) decreased total branching points and (F) increased mean tube length of neo-capillaries. These effects were mitigated by (1 and 10 μg/mL). Data are shown as median from eight independent biological replicates. *Denotes $p < 0.05$ and **$p < 0.005$.

2.1. Statistical analysis

All data are expressed as medians. Statistical analysis was performed on raw data or percent change relative to control using Friedman non-parametric analysis followed by Dunn's post hoc test with PRISM version 6.0 (GraphPad Software). Differences were considered significant where $P < 0.05$.

3. Results and discussion

The cell viability assay was first performed to determine the optimum concentration of hydroxychloroquine to be used in subsequent experiments. Fig. 1A shows that at 100 μg/mL hydroxychloroquine significantly reduced HUVECs viability compared to the untreated control. In view of this, all subsequent experiments were undertaken using 1 and 10 μg/mL of hydroxychloroquine.

We next examined the effect of hydroxychloroquine on ET-1 production by HUVECs. Compared to controls, recombinant TNF-α (Fig. 1B) and preeclamptic serum (Fig. 1C) significantly increased ET-1 secretion by HUVECs ($p = 0.01$, $p = 0.01$, respectively). The addition of 10 μg/mL, but not 1 μg/mL, hydroxychloroquine significantly reduced the TNF-α and preeclamptic serum induced ET-1 increase ($p = 0.03$, $p < 0.0001$, respectively). It is likely that the hypertension of preeclampsia is due, at least in part, to increased ET-1, as evidenced by the observation that circulating ET-1 levels are increased in the reduced uterine perfusion pressure (RUPP) rat model of PE and the administration of endothelin receptor antagonist mitigates the hypertension [24,25]. We have shown that hydroxychloroquine can decrease TNF-α and PE serum induced ET-1 secretion from endothelial cells, albeit *in vitro*. This offers promise that hydroxychloroquine may be able to reduce ET-1 related hypertension. Evaluation of this in the RUPP model would be worthwhile.

We investigated whether hydroxychloroquine could exert other pro-angiogenic effects. HUVECs spontaneously form capillary tube-like structures in culture (Fig. 2A), which is disrupted in the presence of 10 ng/mL recombinant TNF-α (Fig. 2B). Here, we show that this disruptive effect of TNF-α is mitigated by treatment of HUVECs with 10 μg/mL hydroxychloroquine (Fig. 2D) but not by1 μg/mL hydroxychloroquine (Fig. 2C). Specifically, 10 μg/mL hydroxychloroquine mitigated the effect of TNF-α on the number of branching points (Fig. 2E, $p = 0.02$) and on mean tube length of neo-capillaries (Fig. 2F, $p = 0.03$). In our hands, compared to controls, preeclamptic serum did not alter tube formation and so there was no further effect of hydroxychloroquine (data not shown).

To our knowledge this is the first study to show the ability of hydroxychloroquine to improve endothelial cell function in an *in vitro* model of preeclampsia. Our observations support the findings of Gomez-Guzman and colleagues that hydroxychloroquine has endothelial protective effects in an SLE mice model [19]. Together, these findings suggest that there is merit in further assessing hydroxychloroquine, a drug that has a proven safety profile in pregnancy, as an adjuvant therapy for preeclampsia.

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CHAPTER FIVE

Hydroxychloroquine and pregnancy outcomes in women with systemic lupus erythematosus

5.1 Preamble

In view of the previous findings in Chapter 3 and 4, it was necessary to evaluate the effects of HCQ when used during pregnancy. The only cohort of pregnant women known to use this drug are those with systemic lupus erythematosus (SLE). The aim of this retrospective clinical study was to compare the pregnancy outcomes of women who took HCQ to those who didn't. This study is aimed to be published in Obstetric Medicine Journal once the manuscript has been completely reviewed and edited by the co-authors.

5.2 Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects multiple organ systems characterised by malar rash, photosensitivity, oral ulcers and non erosive arthritis. A diagnosis of SLE is based on the presence of four or more criteria as per the standard defined by the American College of Rheumatology (ACR)(127). SLE is much more common in women than men with a relative prevalence of 7:1(128). The average of first diagnosis of SLE in women is 32 years old and hence it is not surprising that obstetricians and rheumatologists commonly attend women with SLE in pregnancy (129, 130).

Pregnancy in women with SLE is considered high risk. It is associated with increased risks of a number of serious maternal complications such as preeclampsia, venous thromboembolism, stroke, renal impairment, sepsis and pneumonia (131, 132), with 20 fold increase in maternal mortality. It is also associated with a 2-4 fold increase in the rate of preterm birth and fetal growth restriction (131). The rate of pregnancy complications is known to be associated with the disease reactivation or flare and the strongest predictor is the number of flares before conception experienced by the women (133, 134). Although a long standing disease has lower risk of disease flare, during pregnancy 50% of women have disease reactivation which occur mostly in the second trimester and during the postpartum period (134).

The mainstay of the management of SLE consists of a combination of steroids, low dose aspirin (LDA), low molecular weight heparin (LMWH) and disease-modifying anti-rheumatic drugs (DMARDs) such as hydroxychloroquine (HCQ).

The choice of treatment depends on the disease activity and organ manifestations. For example, patients with concurrent antiphospholipid syndrome in pregnancy should be treated with low dose aspirin and low molecular weight heparin to reduce the risk of pregnancy loss as a consequence of thrombo-occlusive incidence. On the other hand, the treatment of lupus nephritis requires immunosuppressive therapy such as cyclophosphamide and azathioprine in combination with steroids (116). Additionally, antimalarial drugs particularly HCQ has been recommended to be used for long term treatment of all SLE patients due to its protective effect on survival (135).

Pregnancy outcomes in SLE women depends on several factors. Amongst the predictors of poor obstetric outcomes are disease activity in the six to twelve months prior to pregnancy, number of hospital admissions, use of immunosuppressive drugs, presence of anti-SSA/Ro and anti-SSB/La and lupus nephritis (136). Therefore pre-pregnancy counselling and optimisation of disease control is central to improving pregnancy outcomes in this high risk group of women. Most of the drugs used in SLE are safe in pregnancy except for cyclophosphamide. Given that good disease control improves pregnancy outcomes it is important that medication is continued throughout pregnancy and breastfeeding. However, there are limited published data on the pregnancy outcomes in women treated with HCQ compared to those who were not (137, 138). There is lack of data in particular, the incidence and severity of hypertensive disease in pregnant SLE women treated with HCQ as compared to those who don't.

This study aimed to assess the impact of HCQ on pregnancy outcomes in women with SLE attending a single, academic obstetric service including the incidence of hypertensive disease.

5.3 Methods

Patients

We conducted a retrospective, single centre cohort study of pregnant women with SLE. The records of all women with lupus who gave birth beyond 20 weeks of gestation at Monash Health from January 2001 to December 2015 were accessed and analysed. All patients fulfilled the 1997 American College of Rheumatology (ACR) classification criteria for SLE (139). The gestational age of pregnancies in the women was determined from their menstrual history as well as dating scan in the first trimester. Patients were classified according to their HCQ use in pregnancy. For each patient, demographic data comprising maternal age, parity and ethnicity were collected. We also collected the clinical characteristics of each pregnancy including mean disease duration, activity of disease at conception, use of more than one immunosuppressive drugs, previous thromboembolic events and recurrent miscarriages, smoking status, concurrent medical illness, type of disease and treatment during pregnancy. We collected the following pregnancy outcomes: miscarriage (pregnancy loss before 20 completed weeks), hypertensive disease (7), stillbirth (fetal loss more than 20 weeks of gestation), gestation at birth (140), birth weight and birthweight centile, mode of birth, admission to neonatal intensive or special care unit (NICU). A composite adverse pregnancy outcome was defined as any pregnancy complicated by one or more of pregnancy hypertension, stillbirth,

preterm birth (less than 37 weeks), and fetal birth weight of less than 10th percentile for gestation and sex. Cases of multiple pregnancies were excluded from the study due to their association with preterm birth, fetal birth weight of less than 10th percentile, and hypertensive disease in pregnancy.

5.4 Statistical analysis

Statistical analysis was performed with SPSS software (version 23; SPSS Inc, Chicago, IL). Continuous data were presented using mean ± SD or median with interquartile range (IQR) and compared using the Student-t or Mann–Whitney tests, depending on whether they followed normal distribution, or otherwise. Pearson chi-square or Fisher's exact test were used for categorical variables with statistical significance level of $p<0.05$.

Odds ratio (ORs) of concurrent medical illness, smoking, type of disease and use of more than one immunosuppressive drug, which are the confounding factors for preterm birth less than 37 weeks, were estimated in simple logistic regression models. In the multiple logistic regression models, adjustments were made for concurrent medical illness, smoking, disease type, and use of more than one immunosuppressive drug. The final model was determined using a stepwise forward selection approach. Two-sided p-values of less than 0.05 were considered statistically significant.

5.5 Results

Table 1 summarises demographic information of all women and pregnancies. In total there were 244 pregnancies involving 159 women at our centre in 2001-2015, inclusive. Following exclusion of multiple pregnancies, the final cohort of

105 women was 155 with 238 pregnancies. Of the 57 (36.8%) women who took
106 hydroxychloroquine throughout their pregnancy they had all taken it for more
107 than six months prior to conception. Of the 104 (63.2%) women who did not
108 take hydroxychloroquine throughout their pregnancy, two had conceived while
109 taking it but ceased taking it of their own accord without consultation with their
110 physician or obstetrician and did not re-start. Two other women had been taking
111 hydroxychloroquine but ceased taking it six months prior to their planned
112 pregnancy on the advice of their family physician. Six women with more than
113 one pregnancy in the series had been treated in one pregnancy with
114 hydroxychloroquine and not treated in another, mainly due to changes in
115 disease activity. Overall, there were no differences in demographics between
116 those women who took hydroxychloroquine and those who didn't.

117 Table 2 summarises information on disease status for the two groups of
118 women. There were no differences between the two groups in diagnosis, mean
119 disease duration, disease activity at conception and associated complications
120 namely previous thromboembolic events, recurrent miscarriages, smoking and
121 type of disease. Significantly more women in the hydroxychloroquine treated
122 group had a history of use of more than one imuunosuppressive drug (34.6% vs
123 13.6%, p<0.001), concurrent medical illness (42.9% vs 29.9%, p=0.047) which
124 comprised, mainly of other autoimmune disorders such as rheumatoid arthritis,
125 Grave's disease, autoimmune thyroiditis, Crohn's disease and idiopathic
126 thrombocytopenia. There were significant differences in the use of prednisolone
127 (72.2% vs 52.0%, p=0.011) and azathioprine (40.3% vs 20.0%, p=0.006)
128 between the two groups with those women who took hydroxychloroquine were

more likely to take prednisolone and azathioprine than the women who did not take hydroxychloroquine throughout.

Table 3 summarises the outcomes of the 238 pregnancies. Whilst the overall mode of delivery, NICU admission, livebirth rate of less than 10th percentile were similar between the two groups, the rate of term livebirths was significantly lower in the women who had taken hydroxychloroquine (59.8% vs 79.9%) with a correspondingly higher rate of preterm birth (39.0% vs 20.1%), particularly iatrogenic preterm birth (53.1% vs 46.9%). As expected, the higher rate of preterm birth in the hydroxychloroquine treated group, have significantly earlier gestation at birth (median=37, IQR=35-38, p=0.003) and lower overall mean birthweights (median=2.8, IQR=2.3-3.1, p<0.001). Interestingly, although there is no statistical significance in the incidence of hypertensive disease in pregnancy, more women who were not on HCQ throughout pregnancy were diagnosed with gestational hypertension. Otherwise the numbers of preeclampsia, HELLP syndrome and secondary hypertension with superimposed preeclampsia were similar in both groups.

Table 4 summarises the findings of the logistic regression analysis which was performed to assess the risks of preterm birth in these two groups of women. Women treated with HCQ had significantly a higher risk of preterm delivery. The use of multiple immunosuppressive agents was significantly associated with the risk of preterm birth. The simple logistic regression shows non significant association with concurrent medical illness and disease type.

5.6 Discussion

This retrospective study reported the findings of pregnancy outcomes in women

with SLE depending on whether they took HCQ during pregnancy or otherwise. Notwithstanding the inherent limitations of retrospective methodology, there are some interesting observations that may be useful in informing future prospective studies, whether cohort studies or randomised controlled trials.

There is no strong evidence to show that the incidence of hypertensive disease in pregnancy is reduced in women who received HCQ treatment. However, in this study I have shown that the incidence of hypertensive disease in pregnancy was higher in the group of women who were not treated with HCQ, suggestive, though not definitively proving, that HCQ may be protective. This supports previous published findings that suggested a trend towards lower rates of hypertensive disease(141, 142). Others have observed an increasing trend of preeclampsia in DMARD users (112). This was thought to be principally due to increased disease severity in those women rather than the medication itself(115).

I found that the rate of preterm birth was significantly higher among women taking HCQ than those not taking it. This is the opposite finding to that of Leroux and colleagues who reported a significantly lower rate of preterm birth, albeit spontaneous or iatrogenic, in women taking HCQ during pregnancy compared to those who were not(142). While I observed no differences in the rate of spontaneous preterm birth, I did observe a higher rate of iatrogenic preterm birth in the women taking HCQ. The majority of these women were delivered prematurely because of non-reassuring fetal well-being, principally fetal growth restriction as determined by an estimated fetal growth less than 10th percentile, or abnormal fetal surveillance (CTG, AFI, Dopplers). A further

analysis of this apparent increased risk of preterm birth associated with HCQ revealed that the risk was significantly associated with the use of multiple immunosuppressive drugs. As with the risk of preeclampsia (112) this is most likely a reflection of disease severity rather than a direct effect of the medication itself. Further studies taking into account disease activity scores would assist in unraveling this.

It is also important to note that there was a significantly higher percentage of those women taking HCQ who had other associated autoimmune disorders. This is in keeping with other studies, which had also recognised a higher prevalence of other autoimmune diseases including SLE in patients with autoimmune thyroiditis (115, 143). This association is important for obstetricians to be aware of so that other conditions can be screened for and pregnancy outcomes optimised.

Perhaps not surprisingly the use of HCQ has been associated with more use of corticosteroids. Significantly, more HCQ treated women were also taking prednisolone. This is similar and consistent with previous published studies(144, 145). On the other hand, others have shown that using HCQ during pregnancy with SLE allowed the overall use and doses of corticosteroids to be reduced (114, 146). The reduced need for high dose steroids is beneficial to patients, both pregnant and non-pregnant, as it is associated with a reduction in the rate of long-term complications. In particular, pregnancy with higher intake of corticosteroid is associated with lower birth weight and delay in the milestone development (147).

There were some important weaknesses in the current study. First, due to the

retrospective nature of this study, it was not possible to ascertain all the medical information required to adjust for disease duration and severity, particularly during the pregnancy. This compromised our ability to account for all confounding factors adequately. Nonetheless, strength of the study was it is a relatively large study, involving155 pregnant women. Taking into account the findings and observations in this study, it would certainly be a worthwhile effort exploring interactions between use of HCQ and the incidence of PE. A larger prospective clinical study is needed to investigate the pregnancy outcomes of women with early onset established PE when given a combination treatment of antihypertensive agents and HCQ. Use of HCQ seemed to be associated with an improved kidney function (113). Perhaps administration of HCQ in patients with severe PE may improve the clinical outcome because of improvement in the kidney function.

In conclusion, in this relatively large cohort of pregnant women with SLE the use of the use of multiple immunosuppressive drugs including HCQ for active SLE disease is associated with higher rate of preterm birth. Hydroxychloroquine was associated with a higher rate of iatrogenic preterm birth, along with birth weight of less than 10th percentile, and concurrent medical illness such as hypertension and other autoimmune disorders. These observations are likely to be of benefit for future studies of pregnancy outcome in early onset preeclampsia and association with HCQ therapy.

Table 1: *Demographic information of pregnant women with SLE grouped by use of hydroxychloroquine.*

	Hydroxychloroquine		
	Yes n (%)	**No n (%)**	**p value**
Patients (n=155)	57 (36.8)	104 (63.2)	NA
Pregnancies (n=238)	84 (35.3)	154 (64.7)	NA
Mean ± SD age (years)	30.9 ± 4.3	31.4 ± 4.7	0.498
Ethnicity			0.157
Caucasian	32 (56.1)	74 (71.2)	
South East/East Asian	19 (33.3)	21 (20.2)	
Others	6 (10.6)	9 (8.7)	
Parity			1.000
Primipara	25 (29.7)	47 (30.5)	
Multipara	59 (70.3)	107 (69.5)	

228 ***Table 2:*** *Clinical characteristics of women with SLE, grouped by use of hydroxychloroquine.*

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	Hydroxychloroquine		
	Yes **n (%)**	**No** **n (%)**	**p value**
Disease duration (years) median (IQR)	6.0 (4.0-11.0)	7.0 (3.0-11.0)	0.293
Disease activity at conception			0.285
Remission	82 (97.6)	153 (99.3)	
Active	2 (2.4)	1 (0.7)	
Use of mutiple immunosuppressive drugs			<0.001
No	55 (65.4)	133 (86.4)	
Yes	29 (34.6)	21 (13.6)	
No. (%) prior thromboembolic event	1 (1.2)	8 (5.2)	0.165
No. (%)recurrent miscarriages	2 (2.4)	7 (4.5)	0.499
No. (%) smoking			0.960
No	62 (73.8)	114 (74.0)	
Yes, stop during pregnancy	14 (16.7)	24 (15.6)	
Yes, continue during pregnancy	8 (9.5)	16 (10.4)	
No. (%) concurrent medical illness	36 (42.9)	46 (29.9)	0.047
Hypertension	7 (19.4)	14 (30.4)	

Autoimmune diseases	14 (38.9)	12 (26.1)	
Others	10 (27.8)	16 (34.8)	
No. (%) type of disease			0.682
SLE without antiphospholipid antibody	71 (84.5)	129 (83.8)	
SLE with antiphospholipid antibody	11 (13.1)	18 (11.7)	
Primary APS	2 (2.4)	7 (4.5)	
No. (%) treatment during pregnancy:			
Prednisolone	52 (72.2)	52 (52.0)	0.011
Azathioprine	29 (40.3)	20 (20.0)	0.006
Low dose aspirin	51 (70.8)	66 (66.0)	0.619
Low molecular weight heparin	22 (30.6)	38 (38.0)	0.335

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230

232 ***Table 3:*** *Pregnancy outcomes in women with SLE, grouped by use of hydroxychloroquine.*

	Hydroxychloroquine		
	Yes n=84	**No n=154**	**p value**
No. (%) with pregnancy induced hypertension	11 (13.1)	19 (12.3)	0.866
Gestational hypertension	0	7 (36.8)	
Preeclampsia	5 (45.5)	6 (31.6)	
HELLP syndrome	1 (9.1)	1 (5.3)	
Secondary hypertension with superimposed PE	5 (45.5)	5 (26.3)	
No. (%) fetal growth restriction*	6 (54.5)	5 (26.3)	0.238
No. (%) abnormal fetal surveillance*	2 (18.2)	1 (5.3)	0.537
No. (%) other fetal complications*	3 (27.3)	1 (5.3)	0.126
Gestation at birth (wks) median (IQR)	37 (35-38)	38 (37-39)	0.003
No. (%) total livebirth	82 (97.6)	149 (96.8)	1.000
No. (%) term livebirth	49 (59.8)	119 (79.9)	0.003
No. (%) preterm livebirth (<37 wks)	32 (39.0)	30 (20.1)	
Spontaneous	15 (46.9)	16 (53.3)	
Induced	17 (53.1)	14 (46.7)	
Fetal loss > 20 weeks	2 (2.3)	5 (3.2)	1.000
Termination of pregnancy	2 (2.3)	3 (1.9)	
Fetal death in utero (stillbirth)	0	2 (1.3)	
Birthweight (kg) median (IQR)	2.8 (2.3-3.1)	3.1 (2.5-3.4)	<0.001
No (%) livebirth <10thpercentile	20 (24.4)	27 (18.1)	0.257
No. (%) NICU admission	5 (6.1)	14 (9.4)	0.383
Mode of birth			0.133
No. (%) normal vaginal birth	32 (38.1)	77 (50.0)	
No. (%) assisted vaginal	8 (9.5)	17 (11.0)	
No. (%) caesarean section	44 (52.4)	60 (39.0)	

Table 5: *Risks of preterm birth after adjustment of confounding factors.*

Confounding factors	Crude OR (95% CI) [a]	p value	Adjusted OR (95% CI) [b]	p value
Concurrent medical illness	1.363 (0.748-2.484)	0.311		
Smoking	0.706 (0.428-1.164)	0.172		
Disease type	1.131 (0.636-2.013)	0.675		
Use of multiple immunosuppressive drugs	3.039 (1.358-6.801)	0.007	3.622 (1.587-8.267)	0.002

[a] Simple logistic regression [b] multiple logistic regression. The model was based on forward method. No multicollinearity and interaction.

CHAPTER SIX

General discussion

Preeclampsia is a multifactorial disorder that, in its most severe manifestations, involves mutiple organ systems. It remains a major cause of maternal mortality and morbidity worldwide and the major cause of iatrogenic preterm birth in Australia. For over 50 years the mainstay of the management of PE has been to manage the hypertension to allow prolongation of pregnancy for fetal maturation while preventing serious maternal complications. Over the last ten years or so, improved insights into the mechanisms of the disease process, particularly the recognition that excessive placental release of anti-angiogenic factors is central to the maternal syndrome, has opened up new opportunities for (i) screening, (ii) secondary prevention, and (iii) treatment. In the studies in this thesis, using *in vitro* approaches, I sought to explore whether HCQ was able to mitigate placental and/or endothelial injury with a view to using HCQ as either a secondary preventative agent or a novel therapy for women with established disease.

The main focus of my thesis was an assessment of the effects of HCQ in regards to the placental function in women with established disease. Although HCQ was unable to significantly mitigate the effect of hypoxic injury to the placenta demonstrated by the modest reduction levels of sFlt-1, sEng and TNF-α release, there was a downward trend observed. On the other hand no reduction in the

levels of markers for oxidative stress injury to the placenta i.e 8-isoprostane and activin A was observed. It was not possible for me to investigate the effect of HCQ on other factors released by the placenta, such as placental growth factor (PlGF), vascular endothelial growth factor (VEGF), IL-1β and NADPH oxidase, but this might be worth exploring in future studies. Additionally, it would be worthwhile to investigate the effect of HCQ in first trimester placenta, specifically looking at trophoblastic invasion. This will provide insights in to whether HCQ could be effective as a secondary preventative therapy for PE. Additionally, I had also evaluated the effects on the maternal endothelial dysfunction which is known to be the main complication that leads to the clinical manifestation of PE. *In vitro* experiments showed that HCQ effectively mitigated the effects of endothelial dysfunction induced by TNF-α alone, but with PE serum there was no effect. This is most probably due to the presence of various molecular pathways that give rise to endothelial dysfunction and HCQ may target only one specific pathway. However, there are many other pathways that are targeted by HCQ such as toll like receptor (TLR) that were not investigated. This may be worthwhile to be pursued in future studies. The results of this thesis in combination with improvement in the kidney function in murine model of SLE by Gomez et al. showed that HCQ has potential to improve the clinical outcome of early onset PE by delaying the time of delivery as a consequence of improved edothelial, renal and placental function to some extent.

In order to have a better idea on how much benefits HCQ confers to patients who are treated with this drug, chapter 5 explored its impact on pregnant SLE women as this is by far the only cohort of women who are treated with HCQ during

pregnancy. There is a similar trend seen in majority of women who were treated with HCQ which is a decrease in the incident of hypertensive disease particularly gestational hypertension in pregnancy. A clinical study on the SLE cohort of women is not ideal though as the incidence of PE is definitely higher in more severe disease. The use of HCQ in preeclamptic women may improve the severity of the disease to gestational hypertension due to the endoprotective effect.

In future, more robust clinical studies are needed to assess whether HCQ can be used as a therapy in early onset established PE. A prospective clinical study can be performed in women with early onset PE to investigate whether administration of HCQ will improve the degree of proteinuria as reflected in the in vivo study by Gomez et al. This will prolong the pregnancy and hence improve both the maternal and perinatal outcomes.

On the other hand, HCQ therapy may be commenced in patients at high risk of PE as a preventative therapy. This can be predicted using the sFlt-1/PlGF ratio(148). Measurement of the ratio of these two biomarkers has good negative predictive value for at least a week whether a patient will develop PE. However, it is not early enough to allow the decision to start aspirin as prophylactic therapy. Its usefulness is limited to deciding whether these patients need close monitoring, admission, or even antenatal corticosteroids and magnesium sulphate infusion for fetal neuroprotection in anticipation of early delivery.

In regards to preventive therapy, HCQ has also been shown to reverse the binding of antiphospholipid antibodies to the placental syncytiotrophoblast and hence

improves trophoblast function in patients with APS (149, 150). Likewise, an *in vivo* study involving mice model of APS found that HCQ improves placental insufficiency by inhibiting thrombosis, restores trophoblast invasion and reduces inflammatory cells activation early in pregnancy (151). Therefore, HCQ may have minimal positive effects on placental function in established PE but the effects in the first trimester pregnancies of women at high risk of PE is unknown. Therefore, it will be exciting to see whether it is able to improve the maternal spiral artery remodelling in the first and early second trimester.

With this in mind, a retrospective clinical study on SLE women treated with HCQ was performed. The retrospective design was chosen mainly due to the time constraint. The cohort of SLE women was chosen as HCQ is used commonly amongst these patients. Although it is known that they are already at high risk of developing PE, but the results will provide some idea on the impact of HCQ on the severity and incidence of PE. The results of this study is classified as grade 2 evidence and therefore the results cannot be applied to all patients but instead need to be individualised. The clinical outcome although was suggestive of the lower numbers of women with SLE complicated by hypertensive disease in pregnancy, will need a larger clinical study for further clarification albeit there was statistical significance.

In conclusion, this thesis has opened up a new discovery on the benefits of HCQ in regards to the treatment of PE. More data will be required to look at other pathways in PE that may be targeted by HCQ both as a treatment and prevention. Similarly, more clinical studies are needed to show whether HCQ has any benefits

to prevent the incidence of PE or to arrest the progress of the disease in those with established PE. I believe both *in vitro* and clinical studies would reveal promising results that is highly warranted.

CHAPTER SEVEN

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