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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Masterthesis

The effect of placental hypoxia on endothelial damage in preeclampsia

Iris Kaminski

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Clinical Molecular Sciences

SUPERVISOR :
Dr. Salwan AL-NASIRY

MENTOR :
drs. Philippe VANGRIEKEN
dr. Alexander REMELS

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List of abbreviations

ABTS	2’2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	Analysis of variance
AngII	Angiotensin II
AT_1-AA	Angiotensin II receptor 1 agonistic autoantibodies
BCA	Bicinchonicic acid
BMI	Body mass index
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAT	Catalase
cDNA	Copy dinucleic acid
CM	Conditioned medium
CO_2	Carbon dioxide
DCFH-DA	2’,7’-dichlorodihydrofluorescein diacetate
DMEM	Dulbecco’s modified eagles medium
DMSO	Dimethyl sulfoxide
dNK	Decidual natural killer cells
(D)PBS	(Dulbecco’s) phosphate buffered saline
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
ED	Endothelial damage
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EO-PE	Early-onset preeclampsia
ET-1	Endothelin-1
FBS	Foetal bovine serum
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HAT	Hypoxanthine aminopterin thymidine
HBSS	Hank’s balanced salt solution
HIF	Hypoxia-inducible factor
HRP	Horseradish peroxidase
IL	Interleukin
KCl	Potassium chloride
K_2HPO_4	Dipotassium phosphate

KH_2PO_4	Monopotassium phosphate
KPE	Potassium phosphate buffer with EDTA
LO-PE	Late-onset preeclampsia
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	Disodium phosphate
NO	Nitric oxide
NO_2	Nitric gas
NOX(4)	NAPDH oxidase (4)
Nrf2	Nuclear factor erythroid-related factor 2
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide
$ONOO^-$	Peroxynitrite
PE	Preeclampsia
pHCM	Placental hypoxia-conditioned medium
PlGF	Placental growth factor
P/S	Penicillin-streptomycin
PSMs	Placental secreted messengers
qPCR	Quantitative polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
ROUT	Robust regression and outlier removal
sEng	Soluble endoglin
sFlt-1	Soluble fms-like tyrosine kinase 1
SOD	Copper/zinc superoxide dismutase
SSA	5-Sulfosalicyclic acid
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
tHCM	Trophoblast hypoxia-conditioned medium
TNF-α	Tumour necrosis factor alpha
UV-Vis	Ultraviolet-visible
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1
VP	Vinyl pyridine
XO	Xanthine oxidase

Abstract

Background and hypothesis: Preeclampsia (PE) is a pregnancy-specific disorder that occurs in 5% of all pregnancies. Although the precise pathology is still enigmatic, the placenta plays a central role, as PE is associated with abnormal remodelling of maternal spiral arteries in the uterine wall. Consequently, placental blood flow is reduced, causing local hypoxia and oxidative stress. It is hypothesized that in response to hypoxia, the placenta releases placental secreted messengers (PSMs) in the maternal circulation, which initiate endothelial damage, resulting in the clinical symptoms of PE. Currently, there is a great interest in using dietary interventions including antioxidants like quercetin and tocopherol to prevent PE.

Methods: To examine PSM induced endothelial cell damage, EAhy926 endothelial cells were cultured and incubated with conditioned medium (CM) of placentas which were exposed to 21% O_2 as control or 100% N_2 to simulate severe hypoxia (pHCM). In addition, 1, 3, or 10 µM quercetin or 20 µM tocopherol was introduced prior to aeration of the placenta. Additionally, endothelial cells were exposed to CM of trophoblast cells, which were exposed to 21% O_2 or 1% O_2 (tHCM). First, the viability was assessed by MTT assay. ROS formation and antioxidant capacity were measured by DCFH-DA, glutathione, and TEAC assays. mRNA expression levels of antioxidants and inflammatory markers were measured by qPCR. At last, the secretion of proinflammatory markers (e.g., TNF-α, IL-1β, IL-6, IL-8) and growth factor VEGF were detected using ELISA.

Results: Exposure of endothelial cells to pHCM and tHCM decreased endothelial cell viability and antioxidant capacity, and increased intracellular ROS formation. pHCM decreased endothelial VEGF secretion, while tHCM stimulated endothelial IL-6 and IL-8 secretion and decreased *VEGF* expression. The antioxidant intervention prevented endothelial cell death and oxidative stress and normalised VEGF secretion during exposure to pHCM.

Conclusion: Hypoxic stress in placental tissue and trophoblast cells leads to the release of factors, which induce cell death, free radical formation, inflammation, and a reduction of antioxidant capacity in endothelial cells. These consequences may trigger endothelial damage and explain abnormal endothelial function in PE and may explain some of the clinical symptoms of PE e.g. high blood pressure. In addition, quercetin was able to prevent pHCM induced cell death and ROS formation in endothelial cells and normalises endothelial VEGF secretion, indicating the potentials of a dietary intervention in PE.

1. Introduction

1.1 Preeclampsia

Preeclampsia (PE) is a pregnancy-specific and multisystem disorder with a worldwide prevalence of 5-8% (1). It is defined as new-onset hypertension with systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg and as new-onset proteinuria where urinary excretion exceeds 300 mg protein per 24-hour collection in pregnant women. These symptoms first occur after 20 weeks of gestation (2, 3). However, according to the new American College of Obstetricians and Gynaecologists (ACOG) guidelines, the criteria of new-onset proteinuria is no longer required for the diagnosis of PE. Preeclampsia can also manifest as hypertension in association with new-onset of thrombocytopenia (platelet count < 100.000/µL), impaired liver function (elevated blood levels of liver transaminases to twice the normal concentration), renal insufficiency (elevated serum creatinine > 1.1 mg/dL or a doubling of serum creatinine concentration in absence of other renal disease), pulmonary oedema, or cerebral/visual disturbances (4).

PE is known as one of the leading causes of maternal morbidity and mortality. Worldwide, it is responsible for an estimated 50,000 – 60,000 maternal deaths annually (1, 4). In addition, PE is the main cause of maternal admissions to intensive care units (5-7). This pregnancy condition can result in cardiovascular diseases and systemic inflammation in the mother (8, 9). Furthermore, PE also includes life-threatening risks for the child as it accounts for 500,000 infant deaths annually worldwide due to the risk of prematurity and foetal growth restriction (3, 10, 11). Later in life, they have an increased risk of cardiovascular diseases and PE is also associated with the metabolic syndrome, where there is an occurrence of multiple cardiometabolic risk factors such as hypertension, obesity, insulin resistance, and dyslipidaemia in one individual (12). Moreover, it is known that women with family history of PE will also have a higher risk of developing preeclampsia herself (8). Over the recent years, the number of maternal, foetal, and neonatal deaths from PE has fallen steadily due to universal access to prenatal care, access to timely care, and proper management of PE patients in developed countries. In contrast, these rates remain high in low-income countries (13, 14). Furthermore, even in developed countries, maternal and neonatal morbidity are major contributors to expensive health costs (15). A recent study indicated that the cost per PE patient in the Netherlands is estimated at 8047 euros of which maternal admissions accounted for 57% (16).

1.2 Classification of preeclampsia

PE can be classified according to clinical features, resulting in mild and severe preeclampsia. Mild preeclampsia is defined by a blood pressure of 140/90 mmHg or higher with proteinuria of 0.3 to 3 g/day. Severe preeclampsia is a combination of mild PE with a single additional adverse feature including a systolic blood pressure between 160 and 170 mmHg, a diastolic blood pressure between 100 and 110 mmHg, proteinuria of 3 to 5 g/day, and/or a headache (17, 18). However, this approach does not quantify risk over a broad range of clinical situations, and neglects other potential risk factors, while PE-associated mortality is most commonly due to either hepatic necrosis or acute respiratory distress syndrome as a result of systemic inflammation (9, 19).

The more modern concept of classifying preeclampsia is according to the timing of disease onset, early-onset (EO-PE) and late-onset (LO-PE) (20). Hereby, the gestational age at presentation of PE has been taken into account, as it is the most important clinical variable in predicting both maternal and perinatal outcomes. EO-PE is diagnosed before 34 weeks of pregnancy and is manifest by maternal hypertension and proteinuria progressing to a systemic hypoperfusion of multiple maternal organs. Therefore, EO-PE is commonly associated with abnormal uterine artery development, foetal growth restriction, and adverse maternal and neonatal outcomes (21). In contrast, LO-PE occurs after 37 weeks and has no clear placental pathology compared to EO-PE (22). LO-PE is often associated with normal or slight increased uterine resistance, a low rate of foetal involvement, and more favourable perinatal outcomes (21). Intermediate onset disease is a mixture of both types and occurs between week 34 and 37 of the gestation (22).

1.3 Risk factors

There are many conditions and health risk behaviours that are linked with PE. Women with high risk for the development of PE include those with older age (<35), previously EO-PE, familial predisposition, pre-existing medical conditions, and obesity (10, 23).

Advanced maternal age is an important independent risk factor for preeclampsia (24). Pregnant women aged 40 years or older showed a risk of 6.6% for PE compared to a risk of 3.9% in patients aged from 20 to 29 years (25, 26). Also, women who were previously diagnosed with preeclampsia in a first pregnancy have a seven times higher risk of developing PE in a second pregnancy (23).

A family history of PE increases the risk three times (23). Possible explanations for this predisposition are genetically based hypersensitivity to vasoactive peptides or other conditions that affect placental invasion of the uterus at the time of implantation (27). In addition, the paternal genes play a key role in the placentation and therefore, they can increase the risk of PE. These paternal genes are involved in the renin-angiotensin-aldosterone system (RAAS) and can contribute to the potential risk of hypertension during a pregnancy (27, 28). There is also a strong total increase in risk among mothers who changed partners. Several studies suggest that a new partner contributes to a 30% higher risk (29, 30). A general immunological mechanism can be evoked in response to the new father and increases the risk of developing PE (28). During normal pregnancy, the endometrium of the uterine, which will form the maternal side of the placenta, contains a high number of immune cells, such as macrophages, decidual natural killer (dNK) cells, and regulatory T-cells. During the first trimester, these immune cells infiltrate the endometrium to attract trophoblast cells (31). However, there is a chance that the immune cells will react to the foreign foetal antigen derived from the father's sperm. This response is reduced by prolonged exposure to the paternal antigen through sexual intercourse or from a previous pregnancy. A new male partner indicates a new set of paternal antigens, which can be detected as foreign by the immune system of the women (29).

Pre-existing medical conditions are known risk factors of PE (23). A woman that suffers from diabetes has an overall risk of 21%. Women with underlying chronic hypertension have a 10-25% risk of developing PE compared to the general population. This risk is increased to 31% in women with a severe form of hypertension. For mild renal disease (serum creatinine of less than 1.5 mg/dL), the risk is approximately 20 to 25%. In pregnant women with severe renal disease, it is even higher than 50%. Pregnant women who suffer from autoimmune conditions such as antiphospholipid antibody syndrome are more likely to develop PE (3).

In addition, obesity increases the overall risk of PE by 2- to 3-fold as an elevated body mass index (BMI) is associated with the pregnancy disorder. The risk of PE progressively increases with increasing BMI, even in a normal range (3).

Preeclampsia itself is a risk factor for complications affecting both mother and baby. If not treated, PE may be complicated by seizures (eclampsia), pulmonary oedema, placental abruption, and foetal growth restriction. An increased risk for stroke and hypertension are the long-term health implications for the offspring. In addition, women with PE have 3.7 times higher risk for developing hypertension later in life, 2.2 times increased risk of coronary heart disease and 1.8 times higher risk of stroke (3).

1.4 Pathogenesis of PE

Significant uteroplacental, hemodynamic and vascular adaptations occur in all pregnancies. They are critical to provide sufficient space for the growing foetus and to maintain an adequate blood and nutrient supply to both mother and foetus (32). However, it is known that PE is associated with abnormal placental development caused by inadequate placental cytotrophoblast invasion, followed by widespread maternal endothelial dysfunction (ED) (33).

1.4.1 Failure of trophoblast invasion

The pathogenesis of preeclampsia is thought to commence with the failure of trophoblast invasion in the first trimester of pregnancy. Trophoblast cells are the cells forming the outer layer of the blastocyst, which provides nutrients to the embryo. They are formed during the first stage of pregnancy and there are two subtypes. Fully differentiated syncytiotrophoblasts form a continuous, specialized layer of epithelial cells and are responsible for the complex biomolecular interactions between the foetus and mother. The cells are in direct contact with maternal blood as they cover the entire surface of villous trees (34). In contrast, the undifferentiated cytotrophoblasts form an inner layer of primitive mononuclear cells and are the germinal cells for the syncytium. After implantation is complete, trophoblast cells will further differentiate into villous and extravillous trophoblasts. The villous trophoblasts give rise to the chorionic villi and are responsible for the transport of oxygen, nutrients, and other compounds between foetus and mother. Extravillous trophoblasts will migrate into the myometrium and the decidua, a specialized and highly modified endometrium of pregnancy. Hereby, they will invade the maternal vasculature (35).

During the first half of pregnancy, dNK cells are accumulated in the decidua and have direct contact with trophoblasts. These dNK cells lack cytotoxic functions and are able to dampen inflammatory T-cells. However, they are responsible for attracting and promoting trophoblast invasion and vascular growth in the decidua by expressing interleukin 8 (IL-8), interferon-inducible protein-10, and proangiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). Moreover, trophoblasts also secrete specific chemokines that attract dNK cells to the maternal-foetal interface (35-37). The trophoblast invasion in the maternal vasculature is important for the remodelling of the spiral arteries.

In PE, dNK cell dysfunction results in a lack of adequate cytokines. Consequently, there is a superficial cytotrophoblast invasion, which does not proceed beyond the terminal portions of the spiral arterioles (34, 38).

1.4.2 Impaired remodelling of spiral arteries

The extensive modification of the maternal spiral arteries by the trophoblasts is one of the most remarkable features of human placental development. The extravillous trophoblasts can be subdivided into interstitial and endovascular trophoblasts. The interstitial trophoblasts surround the arteries and they prepare the vessels for endovascular trophoblast invasion (35). Remodelling of spiral arteries is essential for a good uteroplacental blood flow. After the endovascular trophoblasts enter the lumen of spiral arteries, they initially form cellular plugs and destroy the vascular endothelium via an apoptosis mechanism and further invade and modify the vascular media by replacing smooth muscle and connective tissue by fibrinoid tissue. The spiral arteries will, later on, regenerate endothelium. Hereby, the vessels are remodelled into dilated, low-resistance uteroplacental vessels (*Figure 1*) (35). Angiogenic factors such as VEGF and PlGF are thought to be important in the regulation of placental vascular development. Their receptors (e.g. VEGF receptor 1 (VEGFR-1)) are essential for normal placental vascular development (33). Also, other transcription factors, growth factors, and cytokines involved in the regulation of vascular remodelling are secreted by the placenta and are called placental secreted messengers (PSMs) (39).

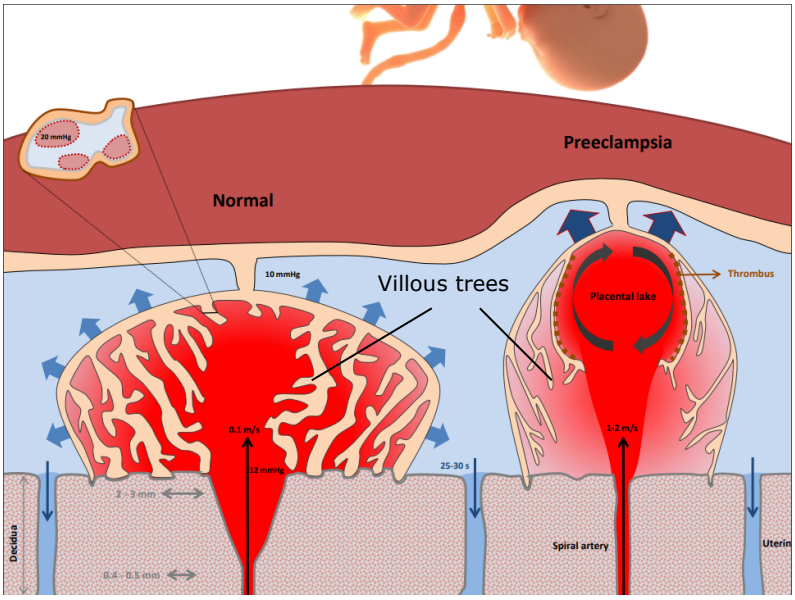

Figure 1: Impaired remodelling of spiral arteries in preeclampsia. Spiral arteries are dilated and low resistant after remodelling, which is required for an adequate uteroplacental blood flow (left side). However, failure of spiral artery remodelling results in non-dilated, high-resistance uteroplacental vessels in PE (right side). Hereby, the momentum of the blood flow is increased and may damage villous trees. In addition, the periphery of the placenta faces local hypoxia as a consequence of local hypoperfusion.

Impaired trophoblast invasion results in an inadequate physiological remodelling of the spiral arteries as they remain small and high-resistance. This will consequently lead to a reduction in uteroplacental blood flow in the periphery of the placenta. This reduced blood flow will affect the foetus and placental oxygen and nutritional status and therefore, a cascade of molecular events will be triggered resulting in hypertension and in peripheral placental hypoxia (*Figure 1*) (40, 41). The hypoxic placenta subsequently triggers the release of anti-angiogenic factors soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), which result in the reduction of VEGF and PlGF. In addition, there are changes in the local and circulating levels of cytokines (e.g., tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6)), hypoxia-inducible factor (HIF), ROS, and angiotensin II (AngII) type I receptor agonistic autoantibodies (AT_1-AA) (42, 43). These factors can cause endothelial dysfunction (ED), severe vasoconstriction and increases in blood pressure observed in PE women (44).

1.4.3 Endothelial dysfunction

Although PE appears to begin in the placenta, the target organ is the maternal endothelium. The reduced placental perfusion observed under PE creates changes in the placental environment. Herein, ROS and endothelial cell activation result in ED through different mechanisms (*Figure 2*). It is hypothesized that the hypoxic placenta releases PSMs that target endothelial cells. This will further cause general damage to the endothelium of the maternal kidneys, liver, and brain resulting in proteinuria, cerebral oedema, and seizures (33, 42, 43). ED is often associated with decreased nitric oxide (NO) bioavailability and changes in NO metabolism could be a factor in PE (44). NO is a potent vasodilator and is generated by endothelial NO synthase (eNOS). The expression of eNOS is often decreased in the umbilical cord of PE compared to normal pregnant women (43). In addition, ED is characterised by increased levels of endothelin-1 (ET-1) and enhanced vascular reactivity to AngII. These are vasopressive factors that increase the blood pressure (33, 42).

The release of sFlt-1, a circulating anti-angiogenic protein and endogenous inhibitor of VEGF, is involved in one of the mechanisms of ED. VEGF is a key factor in the process of vasculogenesis and overall maintenance and endothelial cell health. In PE, levels of sFlt-1 are increased and will antagonise both VEGF and PlGF. As a consequence, high levels of sFlt-1 will contribute to ED (41, 45-47). In addition, placental ischemia may enhance the synthesis of inflammatory cytokines. TNF-α increases vascular permeability, lymphocyte activation, and induces the production of IL-6 and IL-8. TNF-α also downregulates eNOS and mitochondrial biogenesis, leading to mitochondrial dysfunction, oxidative stress, and increased ROS formation (41, 44).

ROS such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) contain highly reactive oxygen (O_2). During normal pregnancy, increased ROS generation is counterbalanced by the presence of abundant antioxidants. However, mRNA expression of antioxidants such as copper/zinc superoxide dismutase (SOD) and catalase (CAT) is decreased and antioxidants fail to counterbalance the increased ROS production. ROS is able to induce lipid peroxidation causing cell damage. An important source of ROS and endothelial damage in PE are neutrophils and monocytes by releasing H_2O_2. In addition, neutrophils produce NO to protect cells from $O_2^{\bullet-}$-induced damage during normal pregnancy. However, the excess of $O_2^{\bullet-}$ will result in the formation of peroxynitrite ($ONOO^-$), thereby effectively reducing NO bioavailability and causing ED. Another important source of ROS is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) such as NOX4. This membrane-bound enzyme complex catalyses the one-electron reduction of oxygen to $O_2^{\bullet-}$ via NADPH (43).

1.5 Management of PE

Currently, no effective treatment exists except for delivery and there is no blood test to diagnose or exclude PE. Diagnosis is only confirmed by the clinical criteria. Once PE diagnosis is suspected, serial blood counts are conducted to monitor the development of thrombocytopenia, haemolysis, liver damage, or renal impairment. Blood pressure is controlled with antihypertensive medication if necessary, while ultrasound surveillance and foetal heart rate testing monitor the foetal status. Patients with PE are often treated with anticonvulsive medication such as magnesium sulphate for 24 hours to decrease the likelihood of developing eclampsia seizures. Also, low-dose aspirin has been used to reduce the risk of foetal or neonatal death by 14%, and the relative risk of preterm birth by 8% (33).

There is a high interest in using dietary supplementation to prevent the development of PE. Calcium supplementation during pregnancy has been suggested to lower the risk of PE as well as reduce the risk of preterm birth (48). Currently, there is a great interest in dietary supplementation with antioxidant properties such as quercetin and α-tocopherol. Quercetin is the most abundant flavonoid in the human diet. Moreover, it is able to exert antioxidant effects by reducing ROS formation and inflammatory markers such as IL-6 (49, 50). In addition, α-tocopherol is also known as vitamin E, which is lipid-soluble. Vitamin E is an antioxidant derived from the diet and prevents the formation of lipid peroxides, hereby protecting cell membranes (51). Thus, both quercetin and α-tocopherol are valuable potential prevention therapies.

1.6 Hypothesis

Placental hypoxia causes the release of PSMs such as sFlt-1, TNF-α, IL-6, and ROS that are able to target endothelial cells. Hereby, they can cause ED, decrease vasodilators, and increase vasoconstrictors. However, whether the changes in PSMs are the cause or consequence of PE remains unclear. Therefore, the hypothesis is that PSMs released under hypoxia initiate endothelial damage, resulting in the clinical symptoms of PE (*Figure 2*). In addition, intervention via quercetin and tocopherol could prevent endothelial damage caused by PSMs released under hypoxia.

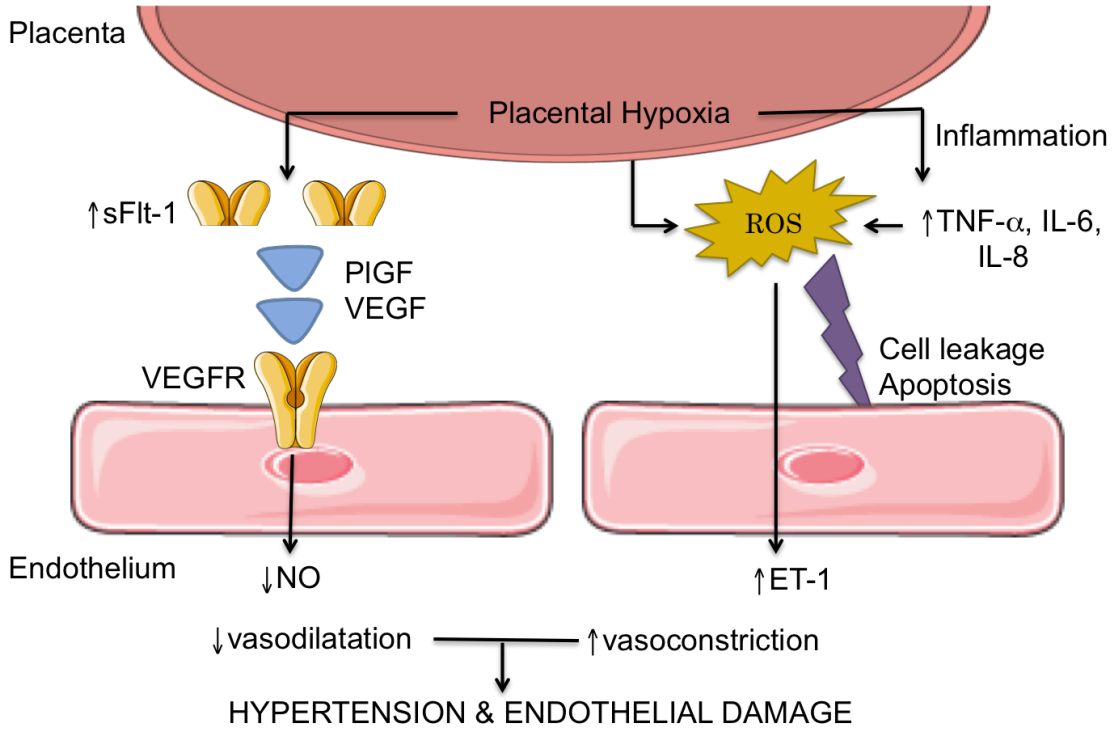

Figure 2: Under hypoxia, the placenta releases PSMs causing endothelial damage and consequently hypertension in preeclampsia. Placental hypoxia causes the release of circulating bioactive factors such as sFlt-1, ROS, TNF-α, IL-6, and IL-8. Excessive sFlt-1 binds VEGF and PlGF and prevents their angiogenic effects mediated by VEGFR binding. In addition, sFlt-1 may also lead to ED through the decreased release of NO. Cytokines such as TNF-α, IL-6, and IL-8 may increase the production of ROS, which decreased the bioavailability of NO and stimulates the production of vasoconstrictor ET-1 by endothelial cells. *ET-1 = endothelin-1, IL-6 = interleukin 6, IL-8 = interleukin 8, NO = nitric oxide, PlGF = placental growth factor, ROS = reactive oxygen species, sFlt-1 = soluble fms-like tyrosine kinase-1, TNF-α = tumour necrosis factor alpha, VEGF = vascular endothelial growth factor, VEGFR = vascular endothelial growth factor receptor (43).*

2. Materials and Methods

2.1 Chemicals and buffer preparation

Potassium chloride (KCl) and Tween*20 were purchased from Acros Organics (Geel, Belgium). Natrium chloride (NaCl) with high purity was obtained from Boom B.V. (Meppel, the Netherlands). Foetal bovine serum (FBS) was available from Bovogen Biologicals (East Keilor, Australia) and trichloroacetic acid (TCA) from Fluka™ from Honeywell (Bucharest, Romania). The following chemicals were purchased from Gibco® by Life Technologies (Rockville, MD, USA): Dulbecco's Modified Eagles Medium (DMEM) modified with 4 mM L-glutamine, 4500 mg/L glucose, 1mM sodium pyruvate, and 1500 mg/L bicarbonate, as well as Dulbecco's Phosphate buffered saline (DPBS), Ham's F12, hypoxanthine-aminopterin-thymidine (HAT) medium (50X, ref. 21060-017), Hank's Balanced Salt Solution (HBSS), Penicillin-Streptomycin (P/S) (10,000 U/mL), and Trypsin-ethylenediaminetetraacetic (EDTA) (0.05%). Dipotassium hydrogen phosphate (K_2HPO_4), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), and triton X-100 were available from Merck KGaA (Darmstadt, Germany). α-Tocopherol nicotinate, β-mercaptoethanol, 2,2'-azino-bis(3-ethylbenzothiazoline-6sulphonic acid) (ABTS), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 2-vinylpyridine (VP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5'5- dithio-bis-(2-nitrobenzoic) (DTNB), 5-sulfosalicyclic acid (SSA), calcium chloride ($CaCl_2$), dimethyl sulfoxide (DMSO), glutathione oxidized (GSH), glutathione reduced (GSSG), GSSG reductase, horseradish peroxidase (HRP), magnesium sulphate ($MgSO_4$), NADPH, quercetin (min. 98%), and Trizma® base were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). EDTA was available from (Thermo Fisher, Landsmeer, the Netherlands) and bovine serum albumin (BSA), H_2O_2 (30%- from VWR International (Leicestershire, UK). HEPES buffer containing in mM: NaCl 143.3, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, glucose 5.5 and HEPES 15 (pH= 7.4). KPE buffer containing in M: H_2KPO_4 0.1, HK_2PO_4 0.1 and EDTA 0.001 (pH 7.5), KPE lysis buffer containing KPE buffer and 1% Triton X-100. PBS buffer containing in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.1, and KH_2PO_4 1.5.

2.2 Culture of EA.hy926

EA.hy926 is a permanent cell line derived by fusing human umbilical vein endothelial cells (HUVECs) with the permanent human cell line A549, which are derived from a human lung carcinoma (52). The EA.hy926 cells were obtained from the American Type Culture Collection (ATCC© CRL-2922™, Manassas, Virginia, USA). The cells were cultured in DMEM, which was modified to contain 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L bicarbonate. In addition, 10% FBS, 1% P/S, and 1X HAT (Gibco® by Life Technologies) were added to the medium. Cultures were maintained in 75 cm^2 U-shape cell culture flasks (Corning®, Merck KGaA, Darmstadt, Germany) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Growth medium was changed three times a week and cells reached 90% confluency after two days in culture. Cells were then washed with HBSS and exposed to a trypsin-EDTA 0.05% solution for 1-2 minutes (Life Technologies).

2.3 Exposure to conditioned medium

In experimental settings, the cells were seeded at a density of 10^5 cells/mL in 96-well plates for 72 hours or in 6-well plates for 24-hours (all from Corning®, Merck KGaA, Darmstadt, Germany). After the seeding period, endothelial cells were exposed for 24 or 72 hours to placental conditioned medium (CM). In case of exposure to trophoblast CM, the exposure duration was 24 hours.

2.3.1 Placental conditioned medium

One placenta from a non-complicated caesarean delivery was collected and processed directly after delivery. Specimens were collected from the central region of the placentas at the maternal side. After removal of the basal plate of the specimens, the remaining tissue was rinsed in a HEPES solution. Six times 30 g minced tissue of the placenta was transferred into 200 mL pre-warmed (37°C) HEPES buffer, which was based on reference values for total maternal blood volume and placental weight. Subsequently, all bottles were kept in a water bath at 37°C where one bottle was constantly aerated with room air (21% O_2) for 3 hours as the control condition and the other bottles were exposed to 100% N_2 to expose the placental tissue to severe hypoxia (placental hypoxia-conditioned medium, pHCM). Prior to 100% N_2 exposure, quercetin (0, 1, 3, or 10 µM) or tocopherol (20 µM) was added to the buffer and the placental tissue explants in additional bottles. After 3h, solutions were centrifuged at 500 *g* for 10 minutes at 4°C. The resulting CM was stored in aliquots of 3 mL at -80°C until used for exposure to the endothelial cells. To achieve 60% placental CM, it was diluted with blank DMEM.

2.3.2 Trophoblast conditioned medium

BeWo cells are a human trophoblastic endocrine cell type and were obtained from the European Collection of Authenticated Cell cultures (ECACC 86082803). These cells were cultured in Ham's F12 growth medium supplemented with 2 nM glutamine according to Kaighn's modification, 10% FBS, and 1% P/S. Cells were maintained in 75 cm^2 flasks (Corning®) under 95% O_2 and 5% CO_2 at 37°C. Growth medium was changed daily and passaged after reaching a 70-80% confluency. For passage, trophoblast cells were washed with HBSS and exposed to 0.05% trypsin-EDTA solution (Life Technologies) for 5 minutes at 37°C. In experimental settings, cells were cultured for 24 hours in 6-well plates under normoxic conditions (21% 0_2/5% CO_2) (control) or hypoxic conditions (1% O_2/5% CO_2) (tHCM). The supernatant was collected and diluted to 60% in DMEM for exposure to the endothelial cells.

2.4 Quantification of cell viability

Endothelial cells were cultured in a 96-well clear polystyrene microplate with a concentration of 10^5 cells per mL for 72 hours. The supernatant was aspirated and cells were exposed for 72 hours to placental CM or 24 hours to trophoblast CM. After exposure, the medium was removed and 150 µL of 0.5 mg MTT/mL DPBS was added to all wells. The plate was incubated in the dark at 37°C for 1 hour. Then all liquids were removed and the same volume DMSO was added and incubated for 10 minutes at room temperature. Next, 100 µL was transferred to a new clear 96 well plate and the absorbance was measured at 540 nm with the iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands). One experiment with 3 replicates was performed to assess endothelial cell viability after exposure to placental or trophoblast CM (N=3). Values were corrected for total protein concentration by a bicinchoninic acid assay (BCA, see 2.9). Data were expressed in percentages compared to positive control DMEM (100% viability).

2.5 Quantification of ROS formation

The intracellular oxidative stress levels of endothelial cells after exposure were quantified via the DCFH assay using a microplate reader. DCFH-DA is a fluorimetric probe that diffuses into the cell and deacetylates to a non-fluorescent compound, which can be oxidized into a fluorescent compound, 2', 7'-dichlorofluorescein (DCF) in the presence of ROS. DCF can be measured by measuring the fluorescence intensity detectable in the cells. The endothelial cells were seeded in a 96 well black polystyrene microplate with clear bottoms with a concentration of 10^5 cells/mL for 72 hours. After this period, cells were washed with HBSS and incubated with 100 µM DCFH-DA in DMEM without FBS in 5% CO_2/21% O_2 at 37°C for 1 hour. As a negative control, cells were incubated without DCFH-DA. After 1-hour incubation, the medium was removed and cells were washed with HBSS after which the cells were exposed to 60% of placental CM for 72 hours or to 60% trophoblast CM for 24 hours. Cells exposed to 100 µM 30% H_2O_2 were included as a positive control. Fluorescence intensity levels were measured with SpectraMax® M2/M2e Microplate Reader (Molecular Devices, Biberach an der Riβ, Germany) at 485 nm (excitation) and 525 nm (emission) after 24 hours. One experiment with 3 replicates was performed to assess ROS formation after exposure to placental CM (N=3). To assess the impact of trophoblast CM on ROS levels of endothelial cells, three experiments each with 3 replicates were performed (N=3). All values were corrected for total protein concentration and expressed in percentages of ROS formation compared to H_2O_2 (100% fluorescence intensity).

2.6 Antioxidant capacity assays

Prior to performing the glutathione assay and the Trolox equivalent antioxidant capacity (TEAC), cells were seeded in 6-well plates (10^5 cells/mL) and incubated with 60% placental or trophoblast CM for 24 hours. After the exposure period, cells were washed HBSS (4°C) and lysed with 500 µL KPE lysis buffer on ice for 30 minutes. Subsequently, cells were scraped and collected for centrifugation at 14,000 rpm for 10 minutes at 4°C. Then, 300 µL of 2.6% SSA was added to 300 µL cell lysate and stored at -80°C for further determination of the reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio by performing the glutathione assay (see 2.6.1). For the determination of the antioxidant capacity by TEAC, 150 µL cell lysate was stored at -80°C (see 2.6.2). In addition, 50 µL cell lysates was used to determine the total protein concentration by BCA (see 2.9).

2.6.1 Glutathione assay

The glutathione assay consists of the measurement of both GSH and GSSG levels in the samples. First, GSH (0.1 µM to 10 µM) and GSSG (0.1 µM to 5 µM) standards were prepared in KPE buffer and 1.3% SSA. Both the GSSG standards and samples for the GSSG measurement were treated with 1:10 VP for 1 hour to form a stable complex with GSH, thereby preventing it from participating in the enzymatic recycling reaction with GSSG reductase. In a 96-well microplate, a 1:1 solution of NADPH (0.8 mM) and DTNB (0.6 mM) in KPE buffer was added to all samples to initiate the reaction. Next, 4 U/mL GSSG reductase in KPE buffer was added after which the colour development of samples and standards were measured kinetically for 3 minutes in 9 reads at 412 nm with the iMark™ Microplate Absorbance Reader at 37°C. Three independent experiments were performed with each three replicates (N=3). GSH and GSSG concentrations were calculated based on slope values compared to the standards and corrected for total protein concentration.

2.6.2 Trolox Equivalent Antioxidant Capacity assay

The TEAC assay is based on the ability of antioxidant molecules to reduce free radicals in the aqueous solution of ABTS-$^{+}$ to ABTS. Consequently, a decolourization can be measured by spectrophotometry at 734 nm. First, a blue-green ABTS solution (5 mM) is produced in sodium phosphate buffer solution (145 mM). Next, an H_2O_2 solution (2 mM) and HRP solution (1/100U) were added to the ABTS solution to form a radical. The mixture is allowed to rest for 10 minutes after which an absorbance of 0.7±0.02 AU at 734 nm at 37°C is measured by Varian Cary® 50 UV-Vis Spectrophotometer (Agilent Technologies, Amstelveen, the Netherlands). The lysates were first deproteinized by adding 10% TCA (1:1) for 5 minutes on ice. Then, they were centrifuged at 15,000 rpm. The radical solution was warmed at 37°C. To start the radical reaction, 50 µL of the sample was added to 950 µL of the radical solution, which was incubated in the warm water bath. After 5 minutes, the absorbance was measured at 734 nm. Three independent experiments were performed with each three replicates (N=3). TEAC values were calculated according to *Equation 1* and they were corrected for total protein concentration per sample.

$$TEAC\ value_{sample} = \left({}^{(absorbance_{blank} - absorbance_{sample})}/_{0.0219} \right) \times 40$$

Equation 1: The equation to calculate the TEAC value of each sample. The TEAC value of 1µM Trolox is 0.0219. The value is multiplied by the total dilution of the sample (dilution = 40).

2.7 Enzyme linked immunosorbent assay

After exposure to CM, the cells were washed and given new blank DMEM for another 24 hours. Then, enzyme-linked immunosorbent assays (ELISAs) were performed on the endothelial supernatant using 5 different DuoSet® ELISA kits for TNF-α (DY210), IL-6 (DY206), IL-8 (DY208), IL-1β (DY201), and VEGF (DY293B) (all from R&D Systems, Abingdon, UK). These assays were all performed according to the manufacturer's instructions. The iMark™ Microplate Absorbance Reader measured the absorbance at 450 nm with a correction at 540 nm. For all 5 ELISAs, 3 individual experiments were performed (N=3). The values were corrected for total protein concentration by BCA (see 2.9).

2.8 RNA isolation, cDNA synthesis, and quantitative PCR

Total RNA was extracted from cell lysates using an on-column RNA isolation kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. The RNA yield was quantified (ng/µL) by NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was reverse transcribed into copy DNA (cDNA) using cDNA synthesis kit (BioRad, Veenendaal, The Netherlands) starting from 400 ng of RNA per sample. The cDNA samples were first diluted (1:50) in nuclease-free water. Hereafter, the samples were diluted in a 384 wells plate (Roche Diagnostics, Mannheim, Germany) with specific primers (Eurofins Genomics, Ebersberg, Germany) (*Supplemental A*) and SYBR green supermix (BioRad). A basic protocol with 45 cycles at an annealing temperature of 60°C was run on a Light Cycler 480 II (Roche Diagnostics, Mannheim, Germany). Data were evaluated using specialised software, LightCycler® 480 software (release 1.5.1.62 SP1) (Roche Diagnostics, Mannheim, Germany) and by LinRegPCR (version 2017.1). Gene expression levels were normalised by calculating a normalisation factor per sample, based on the average of three to four housekeeping genes using GeNorm software. 3 individual experiments with each 3 replicates were carried out for qPCR (N=3).

2.9 Bicinchoninic acid assay

Different values were corrected for total protein concentration, which was measured by using a bicinchoninic acid (BCA™) assay kit (ThermoFisher Scientific, Rockford, IL, USA) according to the provided protocol. The protein concentration was determined in a range of 20 to 2000 µg/mL with the iMark™ Microplate Absorbance Reader.

2.10 Statistical analysis

All data were tested for normality by using Shapiro-Wilk normality test. If it was normally distributed, it was analysed with an unpaired Student *t*-test or One-way analysis of variance (ANOVA) in combination with Tukey's multiple comparisons test. Otherwise, results were analysed by performing a nonparametric Mann-Whitney test or Kruskal-Wallis test in combination with Dunn's multiple comparisons test. In addition, robust regression and outlier removal (ROUT) was used to identify outliers (Q = 1%). The statistical program GraphPad Prism 7 was used to perform all analyses with a significance level of 0.05% (GraphPad Software, California, USA). Data was presented as mean ± standard deviation (SD). Significant differences were presented as # $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

3. Results

3.1 Impact of placental conditioned medium on endothelial cells

Endothelial cells were stimulated with CM of placental explants exposed to normoxia (21% O_2) or hypoxia (100% N_2) for 3 hours in absence or presence of antioxidants (1, 3, and 10 µM quercetin; 20 µM tocopherol). After a 72-hour exposure, endothelial cell viability and ROS formation were measured by MTT and DCFH-DA. In addition, antioxidant levels were measured after 24-hour exposure by glutathione and TEAC assay. Next, mRNA expression levels of cellular antioxidants, proinflammatory cytokines, and VEGF were analysed by qPCR. In addition, ELISA measured secretion of proinflammatory cytokines and VEGF 24h after exposure.

3.1.1 Placental hypoxia-conditioned medium exposure increased endothelial cell death

Exposure of endothelial cells to placental HCM resulted in a significant decrease (51±34%) in cell viability compared to control. Stimulation with placental HCM with 3 µM quercetin prevented a decrease of endothelial cell viability induced by placental HCM. This prevention of endothelial cell death was also observed in cells exposed to placental HCM with 20 µM tocopherol. In case of HCM with 10 µM quercetin, there was only a trend towards an increase of cell viability observed (*Figure 3*).

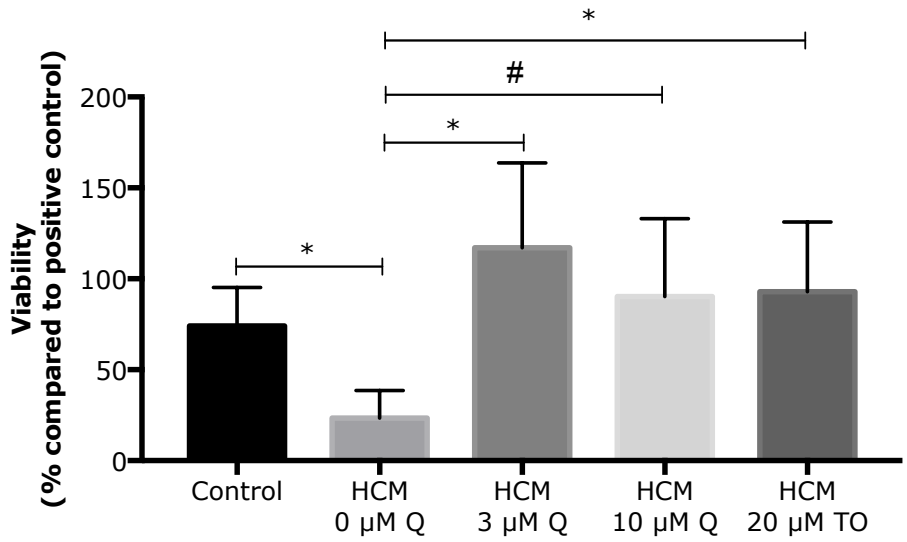

***Figure 3*: Placental hypoxia-conditioned medium exposure increased endothelial cell death.** Endothelial cells were exposed to 60% placental CM for 72h and cell viability was assessed. Data is presented as mean±SD. Significant differences as tested with an unpaired *t*-test are indicated as *: $P \leq 0.05$, #: $P \leq 0.1$. *HCM = hypoxia-conditioned medium, positive control = DMEM growth medium, Q = quercetin, TO = tocopherol.*

3.1.2 Endothelial exposure to placental hypoxia-conditioned medium increased ROS formation and decreased antioxidant levels in endothelial cells

In response to endothelial exposure to placental HCM, endothelial ROS formation was significantly increased (41±22%). Incubation of placental HCM with antioxidants reduced HCM-induced ROS levels by 43±26% (*Figure 4A*). Results from the glutathione assay were expressed in a GSH to GSSG ratio. After exposure to placental HCM, the ratio was significantly decreased (0.42±0.71 μM) in endothelial cells (*Figure 4B*). No significant differences were found in total antioxidant capacity between the conditions (*Figure 4C*). Gene expression levels of cellular antioxidants *CAT*, *SOD*, and *NOX4* were measured by qPCR, but no significant differences were found when endothelial cells were exposed to placental CM (*Figure 4D, E, F*).

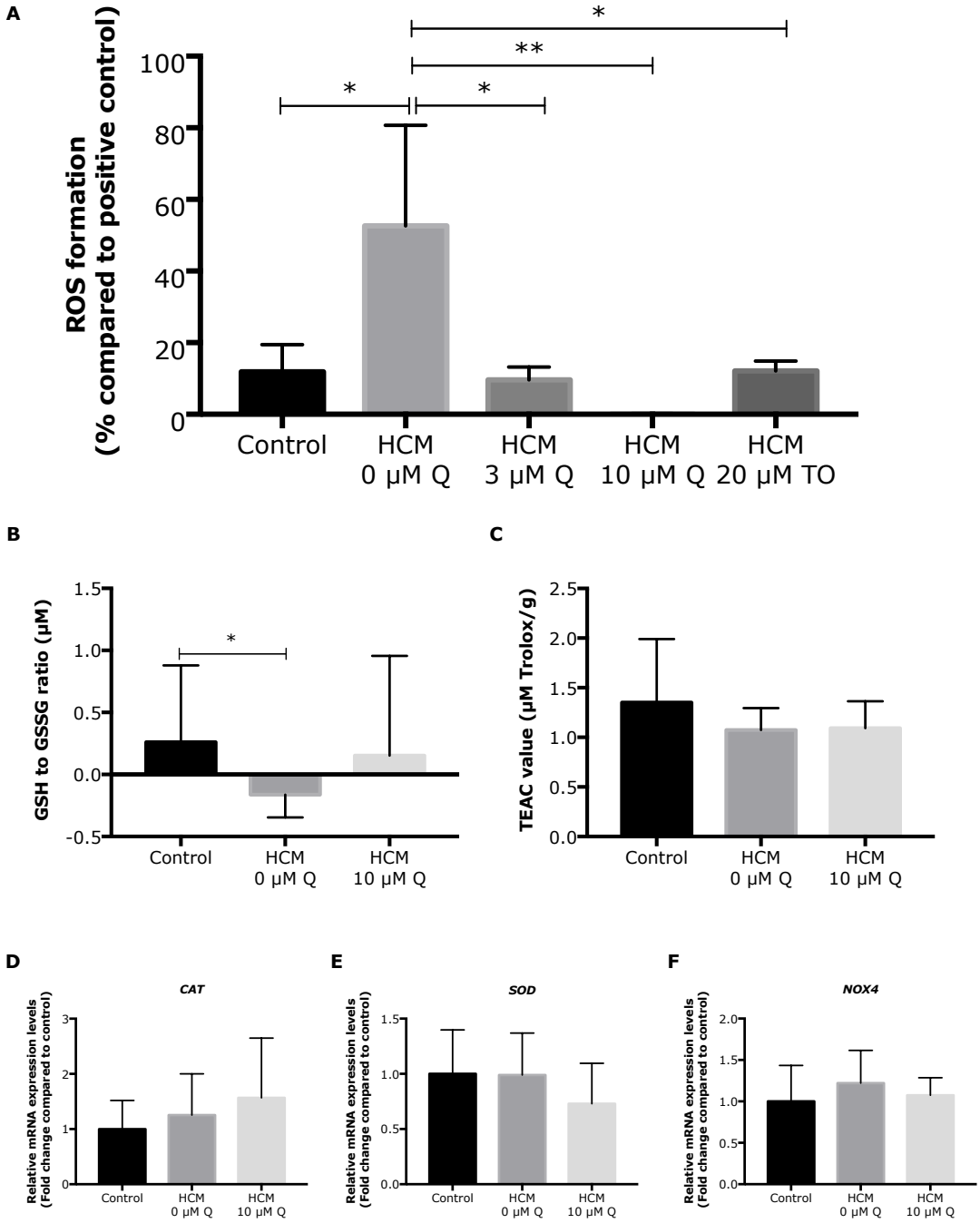

***Figure 4*: Endothelial exposure to placental hypoxia-conditioned medium increased ROS formation and decreased antioxidant levels in endothelial cells. A:** Endothelial cells were 72h exposed to 60% placental CM and ROS formation was assessed by DCFH-DA assay. **B, C:** GSH to GSSG ratio and Trolox equivalent antioxidant capacity of endothelial cells were measured 24h after exposure to 60% placental CM. **D, E, F:** Endothelial mRNA expression levels of antioxidants were analysed by qPCR 24h after exposure to 60% placental CM. Data is presented as mean±SD. Significant differences as tested with One-way ANOVA with multiple comparisons test (**A**), Mann-Whitney (**B**), or an unpaired *t*-test (**rest**) are indicated as *: $P \leq 0.05$, #: $P \leq 0.1$. *CAT = catalase, GSH = oxidized glutathione, GSSG = reduced glutathione, HCM = hypoxia-conditioned medium, mRNA = messenger ribonucleic acid, NOX4 = NADPH oxidase 4, Q = quercetin, ROS = reactive oxygen species, SOD = superoxide dismutase, TEAC = Trolox equivalent antioxidant capacity, TO = tocopherol.*

3.1.3 Placental hypoxia-conditioned medium exposure had no effect on endothelial gene expression or secretion of proinflammatory cytokines

Endothelial exposure to placental HCM did not result in a significant difference in endothelial *IL6* gene expression between the different conditions. The mRNA expression levels of *TNF, IL6, CXCL8, and IL1B* were not detectable after exposure to placental CM (data not shown). Furthermore, the protein levels of TNF-α, IL-6, and IL-1β in the supernatant from endothelial cells exposed to placental CM were under the detection range in both conditions. However, the endothelial secretion of IL-8 was detected, although no significant differences were found between the conditions (data not shown).

3.1.4 Endothelial exposure to placental hypoxia-conditioned medium decreased endothelial VEGF secretion

Endothelial *VEGF* mRNA expression levels were not affected by placental HCM (*Figure 5A*). However, placental HCM exposure resulted in a trend towards decreased endothelial VEGF secretion (0.06±0.03 pg VEGF/µg protein). In addition, endothelial cells exposed to placental HCM with 3 µM quercetin showed a significant increase of 0.07±0.03 pg VEGF/µg protein compared to control (*Figure 5B*).

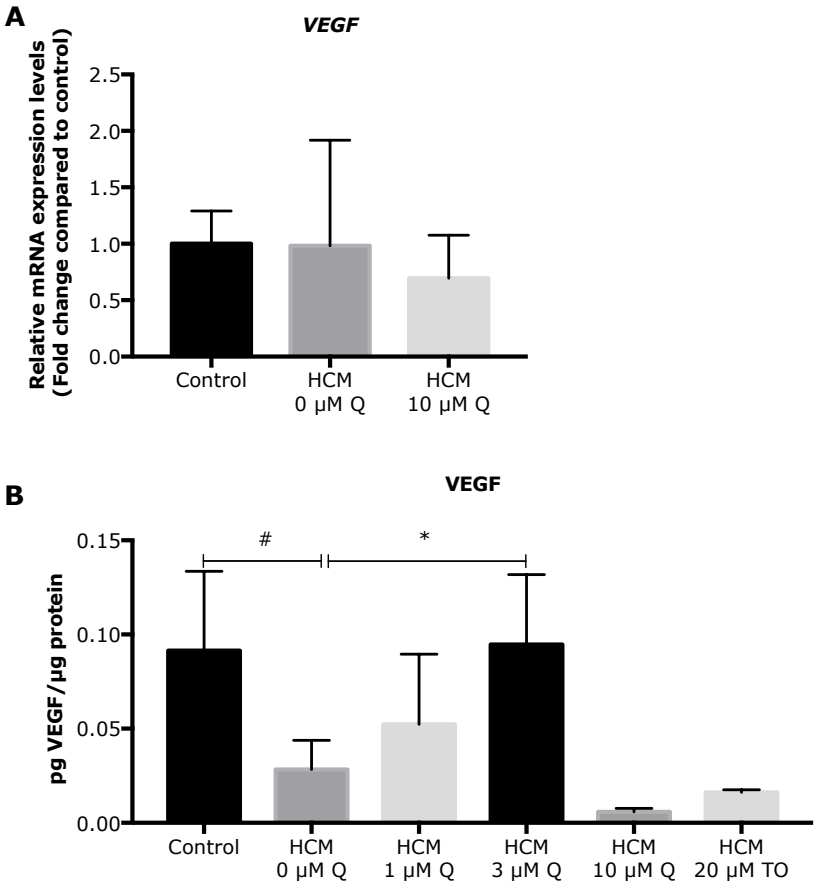

Figure 5: **Endothelial exposure to placental hypoxia-conditioned medium decreased endothelial VEGF secretion. A:** Endothelial cells were exposed to 60% placental CM for 24 hours after which mRNA expression levels of *VEGF* were measured. **B:** The cells were washed after exposure and given new medium for another 24 hours after which an ELISA was performed to measure endothelial VEGF protein levels. Data is presented as mean±SD. Significant differences as tested with Mann-Whitney (**A**) and an unpaired *t*-test (**B**) are indicated as *: $P \leq 0.05$, #: $P \leq 0.1$. *HCM = hypoxia-conditioned medium, mRNA = messenger ribonucleic acid, Q = quercetin, TO = tocopherol, VEGF = vascular endothelial growth factor.*

3.2 Impact of trophoblast conditioned medium on endothelial cells

Endothelial cells were 24h stimulated with CM of trophoblast cells, which were exposed to normoxia (21% O_2) or hypoxia (1% O_2) for 24 hours. After exposure, endothelial cell viability and ROS formation were measured by MTT and DCFH-DA. In addition, antioxidant levels were measured by glutathione and TEAC assay. Subsequently, qPCR was performed to analyse mRNA expression levels of cellular antioxidants, proinflammatory cytokines, and VEGF. In addition, ELISA measured the protein levels of proinflammatory cytokines and VEGF 24 hours after the exposure and washing period.

3.2.1 Exposure of endothelial cells to trophoblast hypoxia-conditioned medium induced endothelial cell death

Endothelial cells showed a significant decrease of approximately 60±26% in viability after exposure to trophoblast HCM (*Figure 6*).

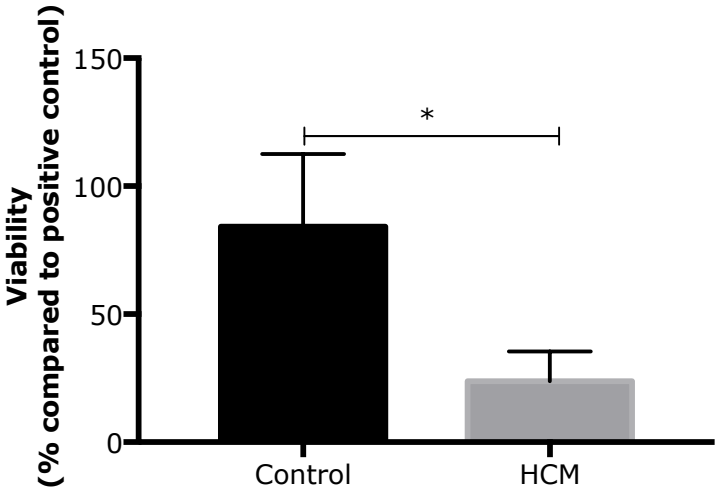

Figure 6: Exposure of endothelial cells to trophoblast hypoxia-conditioned medium induced endothelial cell death. Endothelial cells were exposed to 60% trophoblast CM for 24h after which endothelial cell viability was assessed by MTT. Data is presented as mean±SD. A significant difference as tested by an unpaired *t*-test is indicated as *: $P \leq 0.05$. *HCM = hypoxia-conditioned medium*.

3.2.2 Exposure of endothelial cells to hypoxia-conditioned medium of trophoblasts increased ROS formation and decreased antioxidant capacity

First, a significant elevation in ROS formation (4±4%) was detected in endothelial cells exposed to trophoblast HCM (*Figure 7A*). Next, no difference in the ratio of GSH to GSSG was detected by the glutathione assay after endothelial exposure to trophoblast HCM (*Figure 7B*). In addition, there was a trend towards a decrease in antioxidant capacity (0.24±0.39 µM Trolox/g; 14.9±26.6%) of endothelial cells exposed to HCM (*Figure 7C*). In addition, mRNA expression levels of *CAT*, *SOD*, and *NOX4* were measured in endothelial cells exposed to trophoblast CM; however, there were no significant differences observed (*Figure 7D, E, F).*

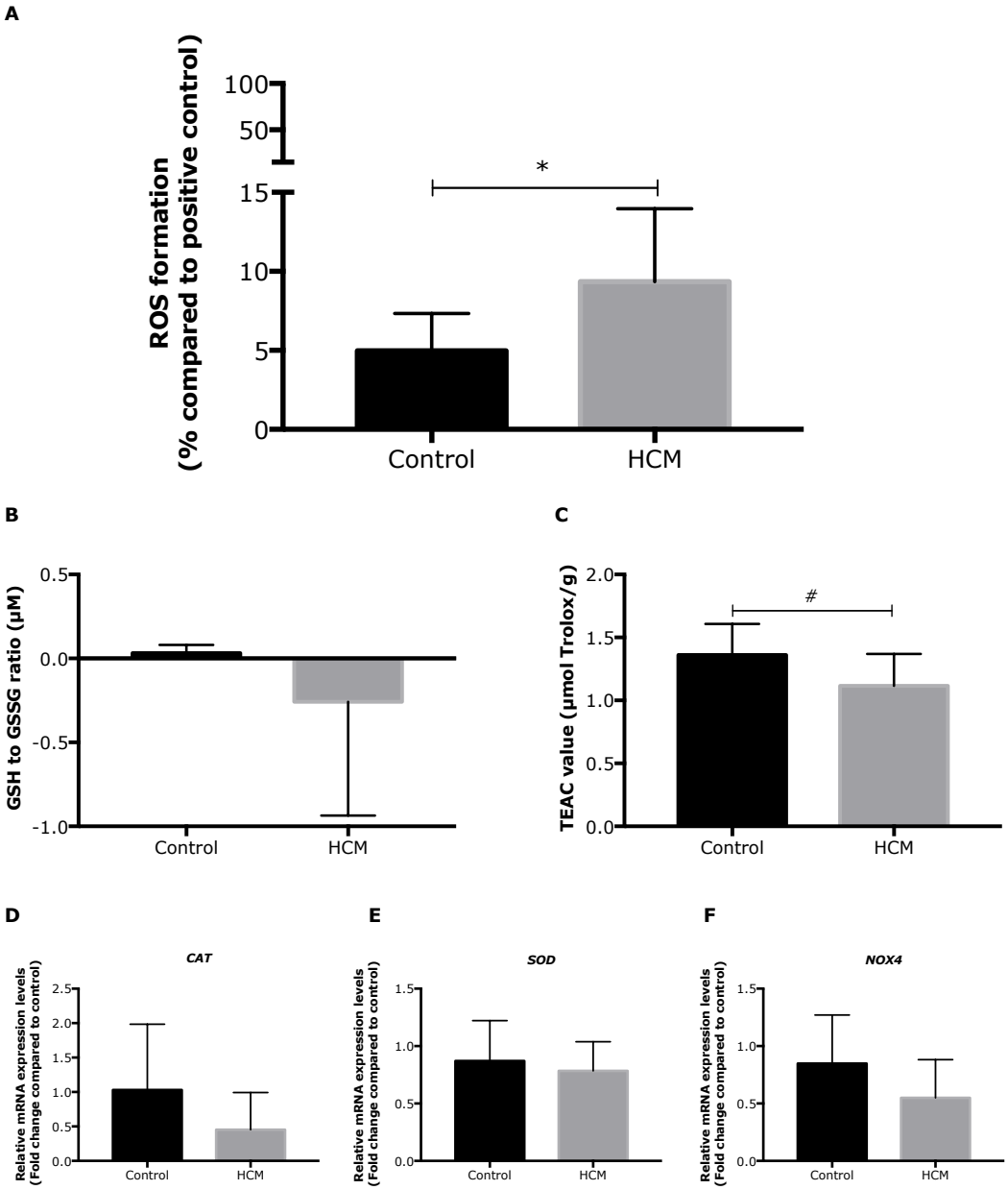

***Figure 7*: Exposure of endothelial cells to hypoxia-conditioned medium of trophoblasts increased ROS formation and decreased antioxidant capacity. A:** Endothelial cells were exposed to 60% trophoblast CM for 24h and ROS formation was assessed by DCFH-DA assay. **B, C:** GSH to GSSG ratio and Trolox equivalent antioxidant capacity of endothelial cells were measured 24h after exposure to 60% trophoblast CM. **D, E, F:** Endothelial mRNA expression levels of antioxidants were analysed by qPCR after 24-hour exposure to 60% trophoblast CM. Data is presented as mean±SD. Significant differences as tested with an unpaired *t*-test (**A, C, D, E, F**) and Mann-Whitney (**B**) are indicated as *: $P \leq 0.05$, #: $P \leq 0.1$. *CAT = catalase, GSH = reduced glutathione, GSSG = oxidized glutathione, HCM = hypoxia-conditioned medium, mRNA = messenger ribonucleic acid, NOX4 = NADPH oxidase 4, ROS = reactive oxygen species, SOD = superoxide dismutase, TEAC = Trolox equivalent antioxidant capacity*.

3.2.3 Exposure of endothelial cells to trophoblast hypoxia-conditioned medium increased the endothelial secretion of IL-6 and IL-8

The endothelial gene expression levels of *TNF*, *CXCL8*, and *IL1B* were not detectable after exposure to trophoblast CM. In addition, endothelial exposure to trophoblast HCM had no effect on endothelial *IL6* gene expression levels (data not shown). The endothelial secretion of these cytokines was also measured after exposure to trophoblast HCM. Concentrations of TNF-α and IL-1β were under the detection range. However, endothelial secretion of IL-6 and IL-8 was significantly increased 24h after cells were exposed to trophoblast HCM by respectively 0.03±0.08 pg IL-6/µg protein and 1.07±0.08 pg IL-8/µg protein (*Figure 8).*

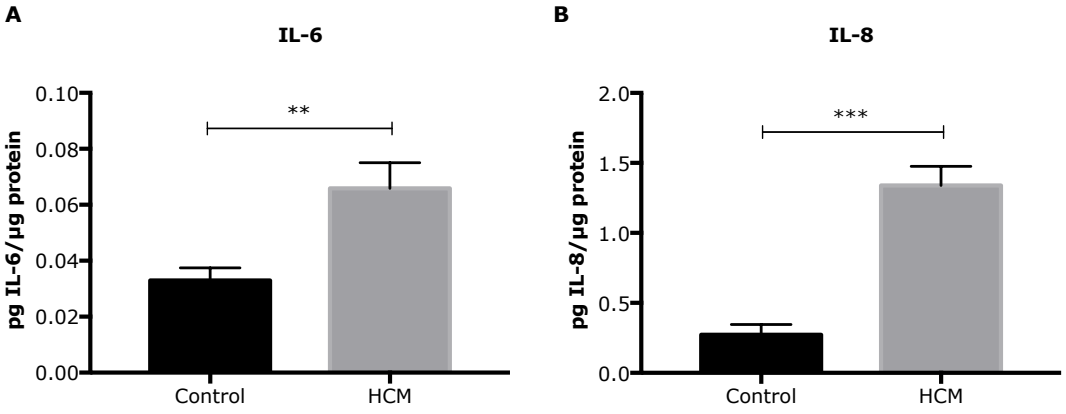

***Figure 8*: Exposure of endothelial cells to trophoblast hypoxia-conditioned medium increased the endothelial secretion of IL-6 and IL-8.** Endothelial cells were exposed to trophoblast CM for 24 hours after which the cells were washed and given new blank medium for another 24 hours. Next, ELISA measured the endothelial secretion of IL-6 (**A**) and IL-8 (**B**). Data is presented as mean±SD. Significant differences as tested with an unpaired *t*-test are indicated as **: $P \leq 0.01$, ***: $P \leq 0.001$. *IL-6 = interleukin 6, IL-8 = interleukin 8, HCM = hypoxia-conditioned medium.*

3.2.4 Exposure of endothelial cells to trophoblast hypoxia-conditioned medium decreased endothelial mRNA expression levels of *VEGF*

Endothelial gene expression of *VEGF* was decreased by 0.66±0.65 fold change after exposure to trophoblast HCM (*Figure 9A*). However, no significant difference between control and trophoblast HCM was observed in endothelial VEGF secretion after exposure (*Figure 9B*).

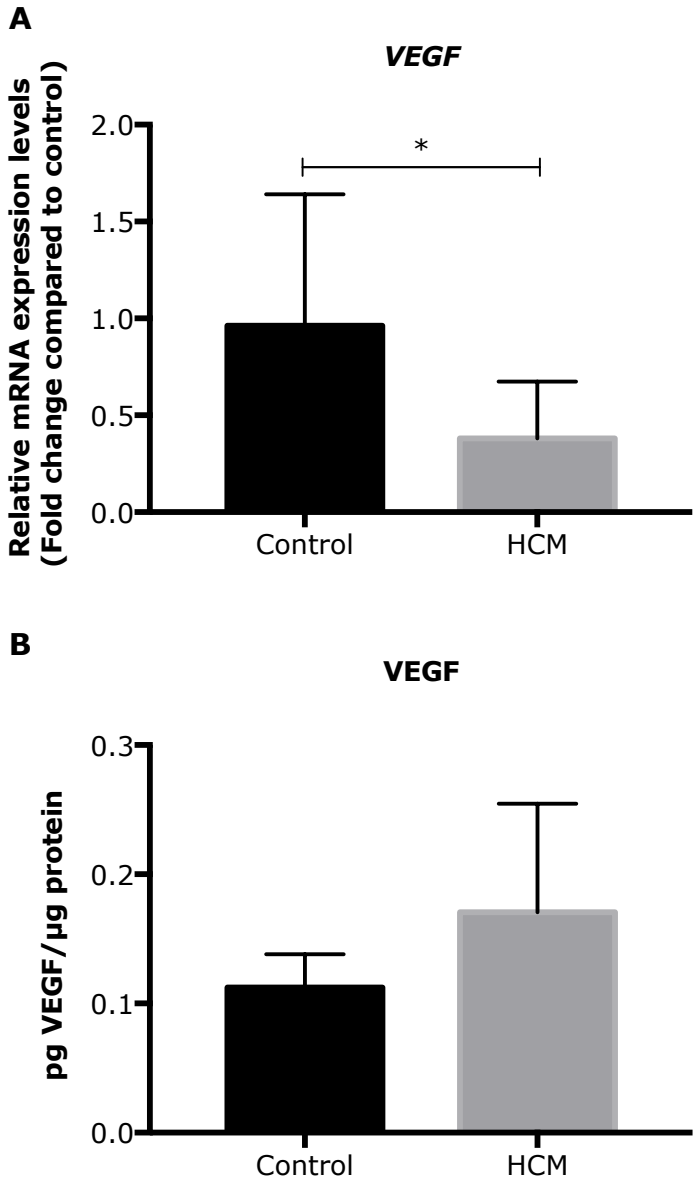

***Figure 9*: Exposure of endothelial cells to trophoblast hypoxia-conditioned medium decreased endothelial mRNA expression levels of *VEGF*. A:** After endothelial cells were exposed to trophoblast HCM for 24h, mRNA expression levels of *VEGF* were measured by qPCR. **B:** The cells were washed and given new blank medium for another 24h after which ELISA was performed to measure VEGF concentrations in the supernatant. Data is presented as mean±SD. Significant differences as tested with Mann-Whitney are indicated as *: $P \leq 0.05$. *HCM = hypoxia-conditioned medium, mRNA = messenger ribonucleic acid, VEGF = vascular endothelial growth factor.*

4. Discussion

In this study, we observed that endothelial exposure to placental or trophoblast HCM induced cell death and ROS formation as well as a decrease in antioxidant capacity. In addition, endothelial VEGF secretion was decreased after exposure to placental HCM while trophoblast HCM increased the endothelial IL-6 and IL-8 secretion and decreased the endothelial *VEGF* expression. These HCM-induced changes were normalised by quercetin and tocopherol intervention.

Inadequate trophoblast invasion and subsequent failure of vascular remodelling are postulated to cause local placental hypoxia (53-55). Hypoxia is a trigger for an increased placental production of proinflammatory cytokines (56, 57). Indeed, in a previous study, we observed that exposure of placental explants to hypoxia for 3h increased the release of IL-1β, IL-6, IL-8, and TNF-α (unpublished data). In addition, we measured higher ROS formation in trophoblast cells, which were exposed to hypoxia for 24h (unpublished data). These studies confirm that placental hypoxia is a trigger for a disturbed release of PSMs such as proinflammatory cytokines and ROS formation.

We demonstrated that endothelial cell viability decreased by more than 50% after exposure to placental or trophoblast HCM. This indicates that in response to hypoxia, the placenta or trophoblasts secrete factors, which are toxic for endothelial cells. Vangrieken *et al.* showed the direct consequences of severe placental hypoxia on vascular contractility in PE. An *in vitro* model for PE was set up, using placental villous explants of non-complicated term placentas and chorionic arteries. Vascular reactivity to PSMs released under hypoxic stress was studied. Chorionic arteries were intraluminal incubated for 2h with placental HCM, which initiated an increase of 47% in vascular contraction compared to the control condition. In addition, it was observed that intraluminal KCl induced a 35% increased vasoconstriction in the arteries exposed to HCM for 2h compared to the control condition (*Supplemental B*, unpublished data). This suggests that PSMs released under hypoxia induce endothelial cell leakage. A possible cause of endothelial cell leakage is oxidative stress. PE placentas produce significantly more lipid peroxides compared to normal pregnancy. Oxidized lipids induce oxidation of endothelial cell membranes resulting in leakage of proteins (58-60). Based on this experiment, our hypothesis is that hypoxia is the trigger for the release of PSMs, which will cause endothelial damage via ROS formation and inflammation.

Our findings indicate that under hypoxia, placental explants and trophoblasts secrete toxic factors resulting in endothelial cell death. To the best of our knowledge, there were no publications that studied endothelial cell death as a consequence of exposure to hypoxic placental or trophoblast HCM. However, PE is commonly accompanied by oedema suggesting a contribution of endothelial cell leakage of proteins (61, 62). Wang *et al.* reported the induction of endothelial cell apoptosis with the involvement of caspase-3 by exposure to foetal plasma from pregnancies with umbilical placental vascular diseases such as PE (63). Furthermore, TNF-α is known to be involved in regulated cell death pathways by activating the NF-κB pathway through the death domain (p60 TNF-receptor) resulting in cell apoptosis by caspase-8 (64). These studies suggest that apoptosis is the potential mechanism of endothelial cell death, however, we were not able to study if the exposure of toxic PSMs resulted in apoptosis or necrosis of endothelial cells. As stated previously, PSMs released under hypoxia may include proinflammatory cytokines. For instance, IL-6 is elevated in PE and has been identified as a promoter of endothelial dysfunction. For example, Yin *et al.* demonstrated that exposure of endothelial cells to IL-6 as well as serum from a PE patient resulted in augmented cell death (65). In addition, TNF-α is able to induce ROS formation, which has also been reported to be involved in cell death (66). These publications together with our observations of endothelial cell death after exposure to both placental and trophoblast HCM and previous detection of proinflammatory cytokines and ROS in HCM suggest a positive correlation between an increased release of proinflammatory cytokines, ROS formation, and cell death.

Placental oxidative stress is still a hot topic in studies that investigate the pathogenesis of PE (41, 67-70). Although it is clear that oxidative stress is present in PE, both the initial trigger and the main source of ROS production are still under investigation. As mentioned above, endothelial cell death was observed after exposure to placental and trophoblast HCM. As oxidative stress is a known trigger for cell death (71) and after observing endothelial cell death, it was investigated if ROS levels in endothelial cells were also elevated in response to HCM. Indeed, we observed that endothelial cells showed an increase in ROS formation after exposure to placental or trophoblast HCM. Hereby, it is confirmed that hypoxic PSMs might be a possible trigger for endothelial ROS production. In line with our findings, several studies suggest that besides trophoblast cells, oxidative stress could also originate from endothelial cells as they detected higher levels of peroxynitrite and superoxide in the maternal vasculature in PE (72-74).

Possible sources of ROS are the mitochondria, endoplasmic reticulum, and nuclear membrane and different enzymes including cyclooxygenase 2, lipoxygenases, xanthine oxidase (XO), and cytochrome P450 also produce ROS. Another important source of ROS is NOX (72). Although NOX4 is the most common NOX in blood vessels and especially in endothelial cells (72), we observed no increase in endothelial mRNA expression of *NOX4* after exposure to placental or trophoblast HCM (72). Our data suggest that NOX4 probably was not the source of endothelial ROS formation. However, more research is required to elucidate the exact source of ROS in endothelial cells during PE.

In addition to elevated ROS formation, the antioxidant capacity was also decreased in endothelial cells exposed to placental or trophoblast HCM. Placental HCM significantly decreased the ratio of GSH to GSSG, indirectly indicating elevated ROS formation. However, no changes in endothelial mRNA expression levels of antioxidants *CAT* or *SOD* were detected. Although it was only a trend, exposure of trophoblast HCM resulted in an 18.4% decrease in antioxidant capacity of endothelial cells. These results indicate that the antioxidant capacity of endothelial cells is failing. To the best of our knowledge, no studies have been conducted where the endothelial antioxidant capacity was lowered due to exposure to hypoxic PSMs. However, it was found that a reduction in GSH levels was associated with endothelial dysfunction and stimulated the formation of ROS (75). In our current study, no differences in mRNA expression levels of antioxidants were detected, while McCarthy *et al.* found significant increases in *SOD* expression levels when endothelial cells were exposed to preeclamptic plasma (76). A possible explanation is that the 24-hour exposure of the endothelial cells to HCM in our experimental set-up was too short or too long to detect changes in gene expression levels of antioxidants. Other studies reported decreased CAT and SOD activity in preeclamptic women, indicating that more research is required (41). Altogether, not much is known about a lowered antioxidant capacity of endothelial cells in PE, however, increased levels of inflammatory markers might be a possible trigger as inflammation is often intertwined with oxidative stress.

In this study, endothelial mRNA expression levels and protein levels of proinflammatory cytokines were measured after exposure to HCM. No differences in gene expression levels of proinflammatory cytokines were detected after exposure to placental or trophoblast HCM. However, exposure of trophoblast HCM induced higher endothelial secretion of IL-6 and IL-8. This data proposes that inflammation is triggered in endothelial cells due to hypoxic PSMs. In addition, it is possible that endothelial secretion of these cytokines contributes to the higher cytokine levels detected in maternal blood and that the placenta might not be the major source (77). Although not much is published about the possible factors that are secreted by endothelial cells in PE, a paper of Chen *et al.* demonstrated that pulmonary endothelial cells secreted IL-6 after phagocytosis of necrotic trophoblast trapped in the pulmonary capillaries. The elevated endothelial IL-6 secretion could activate other endothelial cells in remote vascular beds, contributing to systemic endothelial activation, which is common in PE (78). In addition, our study showed that IL-8 was also elevated in endothelial cells. A recently published study investigated the effect of the hormone leptin on the generation of proinflammatory cytokines by endothelial cells resulting in endothelial dysfunction. They reported that the endothelial IL-8 secretion was elevated in preeclamptic umbilical cord endothelial cells (79).

In our study, exposure of placental HCM caused a decreased endothelial VEGF secretion. In addition, trophoblast HCM decreased endothelial mRNA expression levels of *VEGF*. In line with our observations, multiple papers demonstrated low placental production of VEGF in PE (80-83). VEGF is an important growth factor involved in angiogenesis, vasculogenesis, and thus placental development. A deficit in VEGF can result in poor vascularisation and moreover, contributes to impairment of vascular development during trophoblast invasion (84). Weel *et al.* also observed decreased expression of VEGF and PlGF in the syncytiotrophoblast as well as the endothelial cells from the foetal capillaries in the placenta of pregnant women with PE (84). These data including our own observations indicate that a VEGF deficit is responsible for the failure of the placental vascular remodelling in PE due to abnormalities in growth and formation of new vessels.

In addition to the examination how PSMs secreted under hypoxia affected endothelial cells, we also tested an antioxidant intervention to examine its potential protective effect against placental HCM-induced effects in endothelial cells. Indeed, we observed that quercetin, as well as tocopherol, protected endothelial cells against placental HCM-induced cell death and ROS formation.

As an antioxidant, quercetin has a broad spectrum of bioactive effects including the modulation of mitochondrial biogenesis by reducing ROS production in various cell types. Quercetin can prevent ROS-induced cellular damage by scavenging free radicals (85, 86). Rayamajhi *et al.* demonstrated that quercetin enhances cell survival against oxidative stress by promoting mitochondrial biogenesis (87). There is also evidence that quercetin besides its own antioxidant activity, induces antioxidant enzyme expression in human aortic endothelial cells hereby decreasing ROS levels by activating the nuclear factor erythroid-related factor 2 (Nrf2) pathway. The Nrf2 signalling pathway plays an important role in cellular defence against oxidative stress and protects against inflammation by negatively regulating the expression of proinflammatory cytokines (88). In contrast, we did not detect changes in antioxidant expression; however, ROS levels were significantly decreased and cell viability was increased in endothelial cells exposed to HCM with quercetin or tocopherol compared to endothelial cells exposed to HCM without intervention. This data suggests that endothelial cell death was induced by excessive ROS formation. Quercetin intervention in placental HCM prevented ROS formation in endothelial cells and endothelial cell death was no longer observed after exposure to placental HCM. Although we did not focus on mitochondrial dysfunction and the mitochondria as ROS source, based on literature, there is an indication that mitochondrial dysfunction could be involved in endothelial ROS formation and cell death.

Likewise, tocopherol is able to prevent endothelial dysfunction due to oxidative stress by inhibiting protein kinase C activation (89). However, no publications were found on the effects of quercetin or tocopherol on endothelial dysfunction in relation to PE. In line with the mentioned publications, our data suggest that quercetin and tocopherol may be introduced as a potential therapeutic agent to prevent the development of PE, although more research and human clinical trials are required.

There are some limitations to this study. First of all, it is still unknown which PSMs were present in placental and trophoblast HCM and even more, which factors would be responsible for the induction of cell death and the other effects we have observed such as ROS formation, triggering inflammation, and decreasing antioxidant capacity and secretion and expression of VEGF. We can only hypothesize that proinflammatory and antiangiogenic factors go hand in hand with the formation of ROS culminating in endothelial cell death. Furthermore, the exposure time of placental explants to hypoxia for 3h might be too short. As compensation, endothelial cells were exposed for 72h to placental HCM to measure the effect on endothelial cell death and ROS formation. The short exposure time might also explain why the total antioxidant capacity was not affected after 24h exposure. For the future, it might be more relevant to expose placental explants for longer exposure times (e.g. 24h) to hypoxia and instead of exposing the placenta to 100% use 1% O_2 similar to the trophoblast cell experiments. The most important limitation is the use of cell line experiments. Although *in vitro* studies with human cells, cell lines, and tissue explants provide well-defined experimental models, they are usually limited to one or two cellular players (e.g. trophoblasts, trophoblasts plus endothelial cells). Therefore, this *in vitro* model cannot be extrapolated to human since they cannot mimic the complex interplay of trophoblasts, mesenchyme, various maternal immune cells, and the diverse cellular components of and within the blood vessels. Even the use of placental explants where the foetal and maternal tissue structures are maintained, cannot solve this limitation since not all cellular players remain in the respective state of differentiation during *in vitro* culture (90).

5. Conclusion and future perspectives

To conclude, the hypothesis was that placental hypoxia would lead to a disturbed release of PSMs causing endothelial damage. Both placental and trophoblast HCM induce endothelial cell death, ROS formation, and elevated secretion of IL-6 and IL-8 as well as a decrease in antioxidant capacity and VEGF secretion. All these factors are known to be involved in endothelial damage, thereby resulting in increased vascular reactivity explaining the increased blood pressure observed in PE. Moreover, antioxidant intervention by quercetin or tocopherol was able to prevent ROS formation and cell death indicating its therapeutic potential in PE.

To further improve our experimental set-up, it is required to change the set-up for the placental CM. We will expose placental explants for 24h and even longer to 1% O_2 to mimic hypoxia. In addition, an extensive characterisation of the released factors by the placenta or trophoblast after exposure to hypoxia is necessary to identify which factors are responsible for the observed HCM-induced effects on endothelial cells. This can be achieved by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Also, as we detected endothelial ROS formation after exposure to HCM, it is relevant to reveal the exact source of ROS. To test if mitochondria form the major source of oxidative stress in the exposed endothelial cells, citrate synthase enzyme activity can be measured as well as the use of mitoquinone (mitoQ). In addition, other possible sources of ROS such as different NOX, XO, and eNOS can be measured by qPCR. Furthermore, identifying if endothelial cells die by apoptosis or necrosis is relevant to further unravel the mechanisms of endothelial dysfunction in PE. This is possible by adding pan-caspase inhibitor to block apoptotic cell death. Additionally, it would be interesting to see if antioxidant intervention in trophoblast cells will show the same protective effects in endothelial cells exposed to trophoblast HCM as was observed in placental HCM with intervention. Another future perspective is to use arteries instead of endothelial cells, as the arterial response will be more closely related to the disease condition.

6. Valorisation

This fundamental research is very innovative, as we believe this is one of the first studies to investigate the effect of PSMs released under hypoxia on endothelial cell function. By using this unique combination of *ex vivo* material e.g. placental explants and *in vitro* endothelial cells, we were able to detect endothelial cell death after exposure to placental HCM. In addition, we combined two *in vitro* models of the trophoblast and the endothelial cells, confirming data obtained from the *ex vivo* model. Hereby, we were able to recreate the interactions between trophoblasts and endothelial cells and identify the trophoblasts as the source of PSMs. Besides; this research may also result in clinical applications as we observed that interventions with quercetin or tocopherol, which are antioxidants, were able to prevent the HCM-induced endothelial effects. This led us to believe that there is great potential for the use of these antioxidants as dietary prevention of PE. This is especially of great value since there is currently still no cure for PE, except for (preterm) delivery of the baby and the placenta. The experimental model we have used opens avenues to test both the potentials to prevent a disturbed release of PSMs in response to hypoxia and the effect on maternal targets (e.g. vascular contraction, ED, vascular inflammation).

7. References

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8. Supplemental

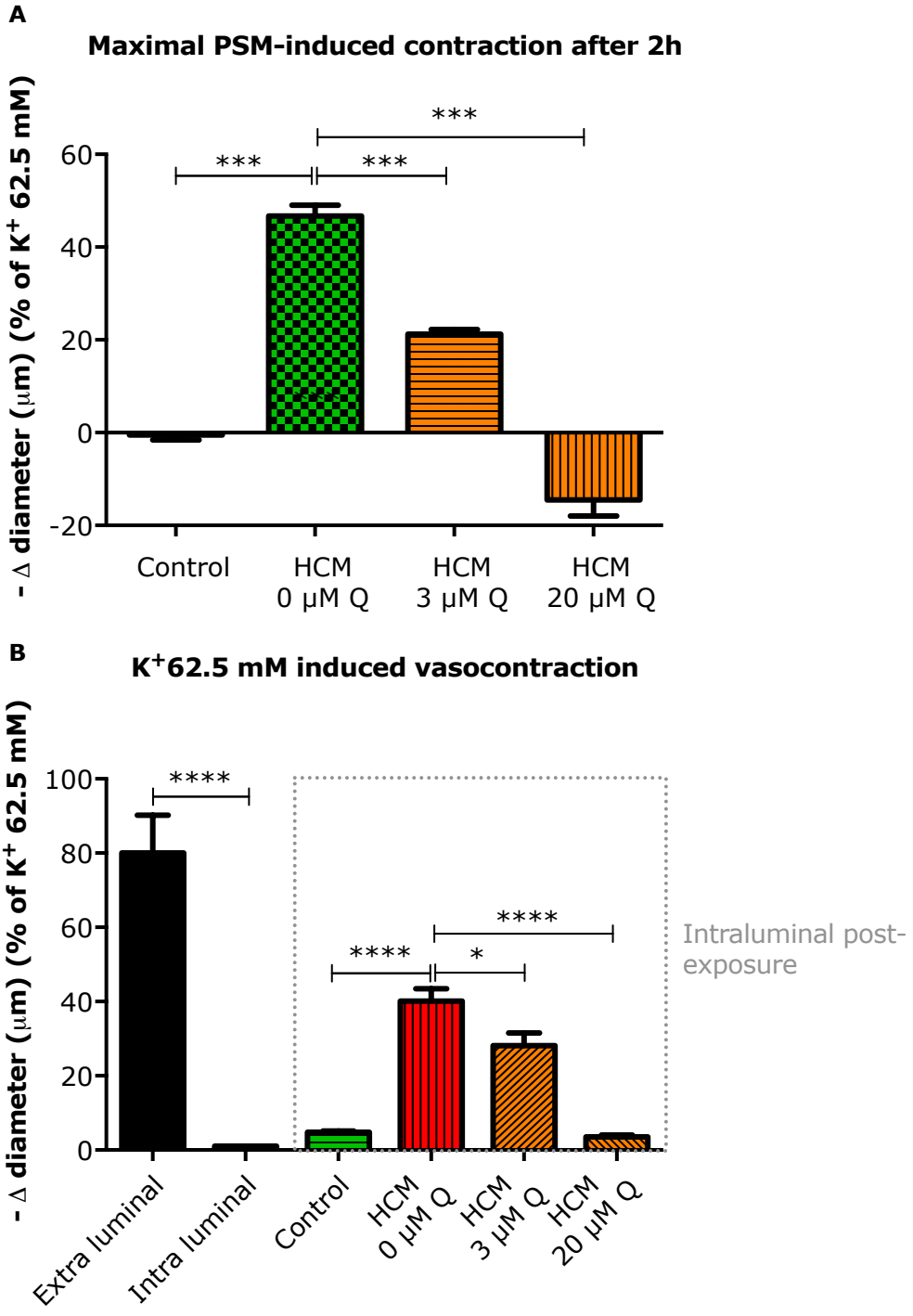
A. List of selected primers

Supplemental A: Sequences of forward and reverse primers of selected genes for qPCR

Gene of interest	Primer	Sequence
CAT	Forward	GATGTGCATGCAGGACAATCAG
	Reverse	GCTTCTCAGCATTGTACTTGTCC
IL1B	Forward	TACCCCCAGGAGAAGATTCC
	Reverse	TTTCAGCCATCTTTGGAAGG
IL6	Forward	AGTGAGGAACAAGCCAGAGC
	Reverse	GTCAGGGGTGGTTATTGCAT
CXCL8	Forward	TTAGAACTATTAAAACAGCCAAAACTCCACA
	Reverse	CAAGTTTCAACCAGCAAGAAATTACTAATATTG
NOX4	Forward	GGATAAGGCTGCAGTTGAGG
	Reverse	AACCAAGGGCCAGAGTATCA
SOD	Forward	ATCAGGATCCACTGCAAGGAA
	Reverse	CGTGCTCCCACACATCAATC
TNF	Forward	CTCGAACCCCGAGTGACAA
	Reverse	AGCTGCCCCTCAGCTTGA
VEGFA	Forward	CCAGGCCCTCGTCATTG
	Reverse	AAGGAGGAGGGCAGAATCAT
Housekeeper genes	**Primer**	**Sequence**
ACTB	Forward	AAGCCACCCCACTTCTCTCTAA
	Reverse	AATGCTATCACCTCCCCTGTGT
B2M	Forward	CTGTGCTCGCGCTACTCTCTCTT
	Reverse	TGAGTAAACCTGAATCTTTGGAGTACGC
GADPH	Forward	GCACCACCAACTGCTTAGCA
	Reverse	TGGCAGTGATGGCATGGA
PPIA	Forward	CATCTGCACTGCCAAGACTGA
	Reverse	TTCATGCCTTCTTTCACTTTGC
RPL13A	Forward	CACTCTGGAGGAGAAACGGAAGG
	Reverse	GCAGGCATGAGGCAAACAGTC

ACTB = beta-actin, B2M = beta-2-microglobulin, CAT = catalase, CXCL8 = C-X-C motif chemokine ligand 8 or interleukin 8, IL1β = interleukin 1 beta, IL6 = interleukin 6, NOX4 = NADPH oxidase 4, PPIA = peptidylprolyl isomerase A or Cyclophilin A, SOD = superoxide dismutase, TNF = tumour necrosis factor, VEGFA = vascular endothelial growth factor A

B. Supplemental figures



Supplemental B: Increased vasoconstriction in placental artery after intraluminal exposure to placental hypoxia-conditioned medium and in addition, K^+-induced vascular contraction suggests endothelial cell leakage. A: Arteries were intraluminal exposed to placental CM and vasoconstriction was measured and compared to maximal contraction induced by K^+. **B:** Afterwards, a K^+ solution was run through the exposed arteries to detect if there was endothelial cell leakage. Significant differences as tested with Mann-Whitney test (**A**) or unpaired *t*-test (**B**) are indicated as *: $P \leq 0.05$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$. *HCM = hypoxia-conditioned medium, K^+ = potassium, PSM = placental secreted messenger, Q = quercetin.*

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Voor akkoord,

Kaminski, Iris

Datum: **7/06/2018**