

**New approaches in the management of preeclampsia
with cerium oxide nanoparticles**

Joana Ramis García

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New approaches in the management of preeclampsia with cerium oxide nanoparticles

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Doctoral Thesis

Programa de doctorat en Bioquímica, Biologia Molecular i Biomedicina

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“Science and everyday life cannot and should not be separated”

Rosalind Franklin

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Thesis overview

Preeclampsia is a severe pregnancy-related disorder affecting 2-8% of pregnancies worldwide. It is characterized by hypertension, systemic inflammation, endothelial dysfunction, and placental oxidative stress, and remains a major cause of maternal and fetal morbidity and mortality. Current preventive strategies, such as low-dose aspirin, provide only modest benefit (15-17% risk reduction). In addition, large clinical trials evaluating classical antioxidant vitamins C and E failed to prevent preeclampsia and were associated with adverse neonatal outcomes. These findings highlight a critical unmet need for innovative interventions targeting disease-specific mechanisms.

This doctoral thesis investigates cerium oxide nanoparticles (CeO_2 NPs) as a novel antioxidant strategy for the multimodal management of preeclampsia. The central hypothesis is that biocompatible, redox-active CeO_2 NPs, owing to their ability to reversibly cycle between Ce^{3+} and Ce^{4+} oxidation states, act as self-regenerating antioxidants capable of mitigating oxidative stress and inflammation associated with preeclampsia.

The thesis comprises four main sections, followed by a dedicated experimental section and final conclusions. Chapter 1 provides the conceptual framework by introducing preeclampsia, distinguishing early- and late-onset forms, and summarizing the underlying pathophysiology. This chapter highlights the contribution of reactive oxygen species (ROS) to PE, with particular emphasis on mitochondrial dysfunction and the dual role of ROS in both cellular damage and redox signaling. It also reviews endogenous antioxidant defenses and introduces CeO_2 NPs as redox-active, enzyme-mimetic materials with potential relevance for PE. Building on this rationale, Chapter 2 provides a detailed examination of the critical parameters and key steps involved in the CeO_2 NPs synthesis, with particular attention to the factors that govern nanoparticle formation, stability, and reproducibility. This section presents an extensive physicochemical characterization of the resulting CeO_2 NPs and evaluates their antioxidant capacity and redox behavior, establishing the foundation for subsequent biological studies. Next, Chapter 3 investigates the therapeutic

antioxidant effects of CeO₂ NPs in a severe-hypoxia *in vitro* model of preeclampsia. Using HTR-8/SVneo immortalized trophoblasts cultured under 0.5% O₂, this chapter examines how CeO₂ NPs modulate trophoblast immunometabolism under hypoxic stress. Finally, Chapter 4 translates these findings to *in vivo* conditions by exploring the pharmacokinetics, biodistribution, and therapeutic potential of CeO₂ NPs in a low-dose LPS-induced preeclampsia-like rat model. This chapter evaluates whether CeO₂ NPs administration, either prophylactically, before lipopolysaccharide (LPS) challenge (pre-LPS), or as a treatment after LPS (post-LPS), can improve maternal and fetal disease-related parameters. In parallel, it provides a detailed biodistribution analysis, with particular emphasis on placental transfer and fetal ceria exposure. Therefore, this chapter addresses both the efficacy and safety of CeO₂ NPs.

List of Abbreviations

A

ABTS / ABTS•⁺ — 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (reduced/oxidized)
ADME — Absorption, Distribution, Metabolism, and Excretion
αKG (2-oxoglutarate) — Alpha-ketoglutarate
AKT — Protein Kinase B
AP-1 — Activator Protein-1
AQP — Aquaporin
Asn — Asparagine
ATP — Adenosine Triphosphate
A1M — α-1-Microglobulin

B

BH₄ — Tetrahydrobiopterin
bw — Body Weight
BSA — Bovine Serum Albumin

C

Ca²⁺ — Calcium (II) Cation
CAT — Catalase
CO₂ — Carbon Dioxide
CBP — CREB-binding Protein
CCL2 (MCP-1) — C-C motif Chemokine Ligand 2 (Monocyte Chemoattractant Protein-1)
Ce⁺³ / Ce⁺⁴ — Cerium Cation (III reduced/IV oxidized)
Ce(NO₃)₃ — Cerium (III) Nitrate
CeCl₃ — Cerium (III) Chloride
CeO₂ — Cerium Oxide
CeO₂ NPs — Cerium Oxide Nanoparticles
CeO₂ NPs@RSA — Cerium Oxide Nanoparticles Coated with Rat Serum Albumin
CI — Confidence Interval
Complex I — NADH:ubiquinone Oxidoreductase
Complex III — Ubiquinol-cytochrome C oxidoreductase
Complex IV — Cytochrome c oxidase
COO⁻ / COOH — Carboxylate/Carboxyl (oxidized/reduced)
CoCl₂ — Cobalt (II) Chloride
CoQ / CoQH₂ — Ubiquinone/Ubiquinol (oxidized/reduced)
COX — Cyclooxygenases
CTAD — C-terminal Transactivation Domain (of HIF)
Cu⁺ / Cu²⁺ — Copper Cation (I reduced/II oxidized)
CYP — Cytochrome P450

D

DLS — Dynamic Light Scattering

DRP1 — Dynamin-Related Protein 1

E

ECM — Extracellular Matrix

EDX — Energy-Dispersive X-Ray Spectroscopy

eNOS — Endothelial Nitric Oxide Synthase

EPO — Eosinophil Peroxidase

ER — Endoplasmic Reticulum

ERK — Extracellular Signal-regulated Kinase

ERO1 — Oxidoreductin-1

ETC — Electron Transport Chain

EVT — Extravillous Trophoblast

E° — Electrode Potential

F

FAD — Flavin Adenine Dinucleotide

FAO — Fatty Acid Oxidation

FBS — Fetal Bovine Serum

fcc — Face-Centered Cubic

FcRn — Neonatal Fc Receptor

Fe(IV)=O — Ferryl Intermediate

Fe²⁺ / Fe³⁺ — Iron Cation ((II) reduced/(III) oxidized)

FGR — Fetal Growth Restriction

FIH — Factor Inhibiting HIF

FMN/FMNH₂ — Flavin Mononucleotide (oxidized/reduced)

FWHM — Full Width at Half Maximum

G

GD — Gestational Day

GLUT1 (protein) — Glucose Transporter 1

gMFI — Geometric Mean Fluorescence Intensity

GMP — Good Manufacturing Practices

GPx — Glutathione Peroxidase

gp60 — Glycoprotein 60

GR — Glutathione Reductase

GSH — Reduced Glutathione

GSSG — Oxidized Glutathione (Glutathione Disulfide)

H

HAADF-STEM — High-Angle Annular Dark-Field Scanning Transmission Microscopy

H&E — Hematoxylin-Eosin

HIF-1 α (protein) / *HIF1A* (mRNA) — Hypoxia-Inducible Factor-1 α

HIF-2 α — Hypoxia-Inducible Factor-2 α

HLA-G — Human Leukocyte Antigen-G

HOCl — Hypochlorous Acid

HRE — Hypoxia-Response Element

HR-TEM — High-Resolution Transmission Electron Microscopy

H₂O — Water

H₂O₂ — Hydrogen Peroxide

I

ICP-MS — Inductively Coupled Plasma Mass Spectrometry

ID — Injected Dose (%ID = percent of injected dose)

IKK — IκB Kinase

IL — Interleukin

iLNPs — Ionizable Lipid Nanoparticles

IMS — Intermembrane Space

INF — Interferon

iNOS — Inducible Nitric Oxide Synthase

I.P. — Intraperitoneal

IRE1 — Inositol-Requiring Kinase 1

IRF3 — Interferon Regulatory Factor 3

IRF7 — Interferon Regulatory Factor 7

I.V. — Intravenous

J

JAK — Janus Kinase

JNK — c-Jun N-terminal Kinase

K

kDa — Kilo Dalton

K₂S₂O₈ — Potassium Persulfate

L

LBP — LPS-Binding Protein

LH — Luteinizing Hormone

LOX — Lipoxygenases

LPO — Lactoperoxidase

LPS — Lipopolysaccharide

M

MAPK — Mitogen-Activated Protein Kinase

MD-2 — Myeloid Differentiation Factor 2

MFN — Mitofusin

MIF — Macrophage Migration Inhibitory Factor

mitCOX — Complex IV Cytochrome C Oxidase

Mn²⁺ / Mn³⁺ — Manganese Cation (II reduced/III oxidized)

MMP — Matrix Metalloproteinase or Matrix Metallopeptidase

MPO — Myeloperoxidase

MPS — Mononuclear Phagocyte System

mtROS — Mitochondrial Reactive Oxygen Species

MyD88 — Myeloid Differentiation Primary Response 88

N

NAC — N-Acetylcysteine

NADH / NAD⁺ — Nicotinamide Adenine Dinucleotide (reduced/oxidized)

NADPH / NADP⁺ — Nicotinamide Adenine Dinucleotide Phosphate (reduced/oxidized)

NF- κ B — Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NLRP3 — NOD-, LRR- and Pyrin Domain-Containing Protein 3

NO — Nitric Oxide

NOXs — NADPH Oxidases

NPs — Nanoparticles

O

O/N — Over Night

ODD — Oxygen-dependent Degradation Domain (of HIF)

OH• — Hydroxyl Radical

OH⁻ — Hydroxide Ion

OMA1 — Metalloendopeptidase 1

ONOO⁻ — Peroxynitrite

OPA1 — Optic Atrophy Protein 1

Ovs — Oxygen Vacancies

OXPHOS — Oxidative Phosphorylation

O₂•⁻ — Superoxide Anion Radical

P

PAMP — Pathogen-Associated Molecular Pattern

PB — Phosphate Buffer

PBS — Phosphate-Buffered Saline

PC-DCA — Phosphocholine-Docosanoic Acid

PDH — Pyruvate Dehydrogenase Complex

PDK — Pyruvate Dehydrogenase Kinase

PE — Preeclampsia

PGF/PIGF — Placental Growth Factor

PHD — Protein Prolyl Hydroxylase Domain Enzymes

PI3K — Phosphoinositide 3-Kinase

Poly I:C — Polyinosinic-Polycytidylic Acid

PPAR γ (protein) / **PPARG** (mRNA) — Peroxisome Proliferator-activated Receptor Gamma

Prx — Peroxiredoxins

Pro — Proline

pVHL — Von Hippel-Lindau Tumor Suppressor Protein

p300 — E1A-Binding Protein p300

Q

qRT-PCR — Quantitative Reverse Transcription Polymerase Chain Reaction

R

RET — Reverse Electron Transport
RHS — Reactive Halogen Species
RIG-I — Retinoic Acid-Inducible Gene
ROOH — Organic Peroxides
ROS — Reactive Oxygen Species
RPMI — Roswell Park Memorial Institute
RSA — Rat Serum Albumin
RT — Room Temperature
RUPP — Reduced Uterine Perfusion Pressure
RyR — Ryanodine Receptor

S

SC — Sodium Citrate
sEng — Soluble Endoglin
Ser — Serine
sFlt-1 — Soluble Fms-Like Tyrosine Kinase
SLC2A1 (mRNA) — Solute Carrier Family 2 Member 1
SOD — Superoxide Dismutase
SPARC — Secreted Protein Acidic and Rich in Cysteine
STAT — Signal Transducer and Activator of Transcription
STEM — Scanning Transmission Electron Microscopy
SBP — Systolic Blood Pressure

T

TAK1 — Transforming Growth Factor β -activated Kinase 1
TCA — Tricarboxylic Acid
TEM — Transmission Electron Microscopy
Th1 — T helper 1
Th2 — T helper 2
TLR3 — Toll-Like Receptor 3
TLR4 — Toll-Like Receptor 4
TMA-OH — Tetramethylammonium Hydroxide
TNF- α — Tumor Necrosis Factor- α
Tregs — Regulatory T cells
TRIF — TIR-Domain-Containing Adapter-inducing Interferon- β
Trx — Thioredoxin
TrxR — Thioredoxin Reductase

U

UPR — Unfolded Protein Response
UV — Ultra Violet

V

VEGF-A (protein) / ***VEGFA*** (mRNA) — Vascular endothelial growth factor A

VEGFR-1— Vascular Endothelial Growth Factor Receptor-1

VEGFR-2— Vascular Endothelial Growth Factor Receptor-2

X

XRD — X-ray Diffraction

Z

Zn²⁺ — Zinc (II) Cation

Symbols & numerals

¹O₂ — Singlet Oxygen

°C — Degrees Celsius

Hypothesis and objectives

Scalable and reproducible synthesis of biocompatible CeO₂ nanoparticles (Chapter 2)

General objective

To establish a scalable, robust, and reproducible synthesis of biocompatible CeO₂ NPs under good manufacturing practices (GMP)-oriented standards (GMP available reagents, scalable and industrializable, traceable).

Specific objectives

- I. Identify the critical steps and process parameters that govern the synthesis of 3 nm CeO₂ NPs
- II. Precisely control the role of each reactant and experimental condition in defining the physicochemical properties of the NPs
- III. Thoroughly characterize the physicochemical properties of the resulting CeO₂ NPs across different batches and large volumes.
- IV. Prove the antioxidant properties of the synthesized CeO₂ NPs.
- V. Assess the catalytic activity of the synthesized CeO₂ NPs.

Cytoprotective effect of CeO₂ NPs on extravillous trophoblasts in a severe hypoxia preeclampsia-like model (Chapter 3)

General objective

The main objective is to investigate the pharmacodynamics and mechanism of action of CeO₂ NPs on hypoxia-induced inflammation by evaluating their (i) biological interactions, (ii) anti-inflammatory properties, (iii) metabolic effects, (iv) mitochondrial adaptations, and (v) functional responses in a trophoblast-based *in vitro* model of severe hypoxia relevant to preeclampsia.

Specific objectives

- I. Characterize the colloidal stability of the CeO₂ NPs-protein conjugates in trophoblast living media.
- II. Define a safe and effective dose of CeO₂ NPs by determining their biocompatibility and concentration-dependent effects on trophoblast viability under normoxic and hypoxic conditions.
- III. Assess the capacity of CeO₂ NPs to modulate hypoxia-induced inflammatory, metabolic, migratory, and angiogenic signaling pathways in trophoblasts.
- IV. Evaluate the impact of CeO₂ NPs on mitochondrial morphology, function, and adaptive responses during sustained hypoxic stress.

Biodistribution and protective effects of CeO₂ NPs in an LPS-induced pre-eclampsia model: Preliminary pilot study (Chapter 4)

General objective

The general objective is to evaluate the protective effects and biodistribution of CeO₂ NPs in a pregnant rat model of LPS-induced preeclampsia, comparing prophylactic and therapeutic administration schemes.

Specific objectives

- I. Establish and characterize a low-dose LPS-induced preeclampsia-like model in pregnant Sprague-Dawley rats, based on maternal clinical signs, systolic blood pressure, proteinuria, and fetal/maternal weight outcomes.
- II. Assess the prophylactic and therapeutic effects of CeO₂ NPs on maternal well-being by analyzing behavioral and physical clinical scores and systolic blood pressure in LPS-challenged dams.
- III. Evaluate the impact of CeO₂ NPs on renal and placental damage by analyzing kidney histology, proteinuria, and placental alterations in LPS-challenged dams.

- IV. Determine the anti-inflammatory potential of CeO₂ NPs by quantifying their impact on maternal serum cytokines (CCL-2, IL-6, TNF- α) in the LPS-induced preeclampsia-like model.
- V. Characterize the maternal and maternofetal biodistribution and clearance of CeO₂ NPs, focusing on placental deposition, potential fetal exposure, and the comparative effects of prophylactic versus therapeutic administration

1.1 Introduction

1.1.1 Normal pregnancy: physiological adaptation and placental development

Normal pregnancy in humans encompasses an approximately 40-week gestation period (280 days from the last menstrual period), divided into three trimesters: the first (weeks 1-12/13), second (weeks 14-27), and third (weeks 28-40). These phases are characterized by profound maternal physiological adaptations and the development of a complex fetal-placental unit which becomes the interface between the mother and the child.¹⁻³ Beginning at implantation (approximately 6-12 days post-conception), the blastocyst invades the decidualized endometrium, initiating the critical process of placentation.⁴ During this phase, extravillous trophoblasts (EVTs) migrate into the decidua and acquire an endothelial-like phenotype, progressively remodeling the uterine spiral arteries. This process involves replacement of the muscular tunica media by EVT cells, transforming these vessels into low-resistance, high-capacitance conduits capable of accommodating an approximately tenfold increase in uteroplacental blood flow and supporting the rising fetoplacental oxygen demand by the second trimester.^{4,5} This vascular remodeling, which occurs predominantly during the first 18 weeks of pregnancy, is essential for adequate placental function and is regulated by balanced angiogenic signaling, including vascular endothelial growth factor A (VEGF-A) and placental growth factor (PGF), which promote endothelial cell proliferation, migration, and vascular stability.^{6,7} **Figure 1.1** illustrates this critical placentation process and the associated oxygen tension transition.

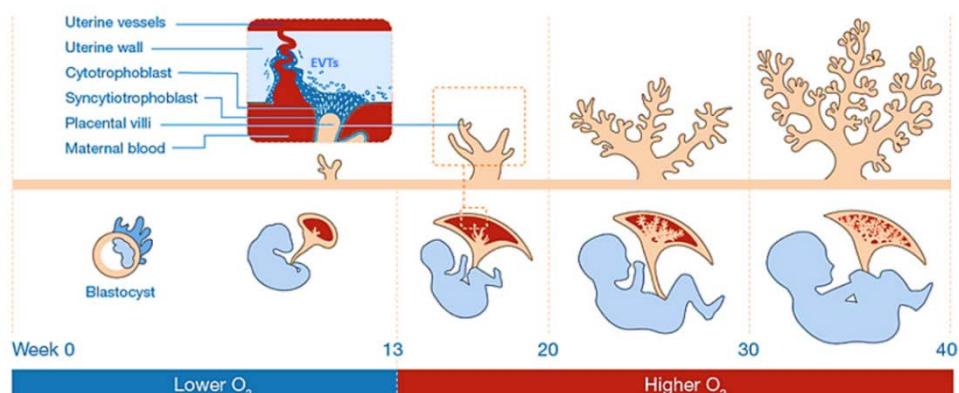


Figure 1.1 Placental development and the transition in oxygen tension during early pregnancy. From blastocyst following implantation (week 0) through sequential stages of spiral artery remodeling by EVTs, plugs dissolution (between weeks 10-14), and onset of maternal blood flow into intervillous space, elevating pO₂ from <20 mmHg (low O₂, blue) to 40-60 mmHg (higher O₂, red). Week 20: Complete spiral artery remodeling (aprox. 90% transformed) and stable maternal flow with pO₂ around 50 mmHg constant. Week 30: Maximum villous growth and optimal O₂/nutrient diffusion. Week 40: Mature placenta that undergoes a 10% flow reduction preparing for delivery. Adapted from: National Institute of Child Health and Human Development (NICHD).⁸

Simultaneously, the maternal immune system undergoes profound tolerance mechanisms to prevent rejection of the semi-allogeneic fetus.⁹⁻¹¹ This adaptation is characterized by an expansion of regulatory T cells (Tregs) in the decidua, a shift toward anti-inflammatory Th2 cytokines, and the production of immunomodulatory molecules such as human leukocyte antigen-G (HLA-G).¹⁰⁻¹³ Concurrently, maternal cardiovascular adaptation includes progressive decreases in systemic vascular resistance and blood pressure (reaching a minimum at approximately 20 weeks), expansion of plasma volume (approximately 50% increase by the third trimester), and increased cardiac output to support the heightened metabolic demands of pregnancy.¹⁴

The placenta develops into a hemochorial structure with intimate maternal-fetal contact, facilitating the bidirectional exchange of (i) gases, (ii) nutrients, (iii) metabolic waste, and (iv) signaling molecules.¹⁵ Crucially, despite the high energy demand, the placenta maintains maternal antioxidant homeostasis through robust antioxidant defenses, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which preserve redox homeostasis and endothelial integrity throughout gestation.^{16,17}

Fetal growth proceeds at an exponential rate during the second (weeks 14-27) and third trimesters (weeks 28-40), with the placenta reaching maximum villous growth and optimal oxygen/nutrient diffusion capacity by week 30, ultimately resulting in the delivery of a term infant at approximately week 40.^{1,18,19} These coordinated maternal-placental-fetal interactions define a successful pregnancy. Disruption of placental development, maternal vascular adaptation, or redox balance, particularly during the critical early placentation phase (weeks 1-18) or in response to maternal constitutional vulnerability, can precipitate pregnancy complications, including preeclampsia (PE).^{20,21}

1.1.2 Placental dysfunctions: the interconnected great obstetrical syndromes

The cascade of placental dysfunction extends beyond isolated pathologies to create a complex web of interdependent pregnancy complications, encompassing disorders ranging from preeclampsia and gestational diabetes to preterm labor, infections, and stillbirth (**Figure 1.2**).²¹ These conditions share common mechanistic pathways rooted in abnormal placentation, maternal vascular dysfunction, and systemic inflammation.^{20,22} PE frequently coexists with fetal growth restriction (FGR) and preterm birth. The presence of one complication increases susceptibility to additional pregnancy disorders through shared biological mechanisms, including endothelial dysfunction, inadequate trophoblastic invasion, and impaired placental nutrient and gas exchange,^{22,23} while infectious and inflammatory insults can independently or synergistically compromise placental function and fetal oxygenation.²¹

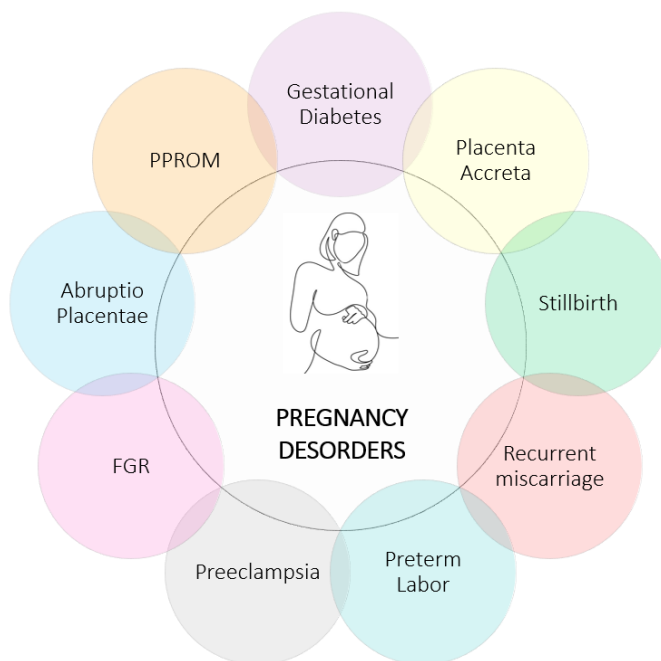


Figure 1.2 The spectrum of placental-related pregnancy disorders. Schematic representation of major clinical entities arising from placental dysfunction, including preeclampsia, fetal growth restriction (FGR), preterm labor, preterm premature rupture of membranes (PPROM), placental abruption, placenta accreta, gestational diabetes, recurrent miscarriage, and stillbirth.

Failure of proper placentation, represents a fundamental defect that underpins a spectrum of obstetric pathologies collectively termed the “Great Obstetrical Syndromes”.^{4,21} The extent of impaired spiral artery remodeling correlates with clinical phenotype: partial failure manifests as fetal growth restriction, whereas severe or absent remodeling precipitates preeclampsia, and in the presence of obstructive lesions, results in preeclampsia with concurrent FGR.^{4,23} Molecular evidence demonstrates that both PE and FGR are characterized by heightened inflammatory signaling and immune dysregulation, including an imbalance of T helper 1 (Th1) and T helper 2 (Th2) cells and altered macrophage polarization.^{12,20}

This interconnectedness indicates that pregnancy complications should not be viewed as isolated entities, but rather as clinical manifestations of a shared underlying pathology: placental insufficiency leading to inadequate support of maternal adaptation and fetal

development.²¹ Elucidating these shared pathways is essential for early identification of high-risk pregnancies, implementation of preventive strategies, and optimization of maternal and fetal outcomes.²⁴

1.1.3 Preeclampsia: clinical definition and early vs. late forms

PE is a pregnancy-specific hypertensive disorder affecting approximately 2-8% of pregnancies worldwide and remains a leading cause of maternal and perinatal morbidity and mortality.^{25,26} It is a placenta-mediated, multisystem syndrome that originates at the maternal-fetal interface, where abnormal placentation and uteroplacental malperfusion lead to the release of circulating factors that precipitate systemic endothelial dysfunction (see **Section 1.1.4** for detailed pathophysiology description). In severe cases, preeclampsia can be fatal for both the mother and the fetus.²⁰ From a clinical point of view, PE is defined by the onset of systolic hypertension ≥ 140 mmHg and/or diastolic hypertension ≥ 90 mmHg on two occasions separated by at least 4 hours, or severe hypertension ($\geq 160/110$ mmHg) confirmed on a single occasion, occurring after 20 weeks of gestation in a previously normotensive woman. To this criterion, significant proteinuria must be added, or in its absence, any of the following: thrombocytopenia, renal insufficiency, hepatic dysfunction, acute pulmonary edema, neurological symptoms, and/or signs of uteroplacental dysfunction such as FGR or abnormal uterine artery Doppler findings indicative of increased resistance in the uteroplacental circulation.²⁷

Two main phenotypes are clinically recognized: early-onset PE (occurring before 34 weeks) and late-onset PE (occurring at or after 34 weeks).²⁸ The early-onset form is associated with primary placental disease and marked fetal involvement, with frequent FGR and Doppler abnormalities.²⁹ In this context, the defect is localized to the early phases of implantation and the deciduo-myometrial junction, where EVT's fail to complete the transformation of spiral arteries into low-resistance vessels (**Figure 1.3**), leading sequentially to placental hypoperfusion, chronic hypoxia, sustained activation of hypoxia-inducible factor-1 α (HIF-1 α), and a dysregulated angiogenic milieu, including increased release of soluble

factors such as sFlt-1 and soluble endoglin (sEng).^{4,13,24} Associated comorbidities in early-onset PE includes a family history of preeclampsia, inherited or acquired thrombophilia, autoimmune diseases (systemic lupus erythematosus, antiphospholipid syndrome), chronic nephropathies, and genetic polymorphisms affecting angiogenic, oxidative, or immune pathways.³⁰ The clinical phenotype is more severe, with increased risk of FGR, eclampsia, and cerebral edema, and often requires preterm delivery for maternal-fetal indications.³¹

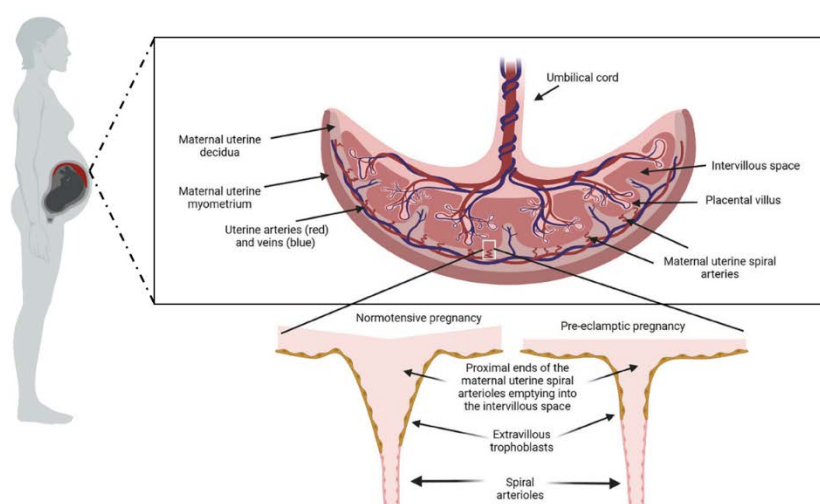


Figure 1.3 Uteroplacental circulation and spiral artery remodeling in normotensive and preeclamptic pregnancy. Schematic representation of the uteroplacental unit showing maternal uterine arteries and veins, the intervillous space, and placental villi. In normotensive pregnancy, EVT invasion remodels the uterine spiral arteries by replacing the muscular tunica media, converting these vessels into low-resistance, high-capacitance conduits that ensure adequate placental perfusion. In preeclamptic pregnancy, particularly in early-onset PE, incomplete EVT invasion results in poorly remodeled spiral arteries, leading to increased vascular resistance and reduced uteroplacental blood flow. *Adapted from:*³²

In late-onset PE, placentation is typically relatively well preserved, and the main problem lies in how the maternal cardiovascular system adapts to the stress of pregnancy.²⁴ The placenta acts as a "mirror" of preexisting susceptibility: maternal endothelial dysfunction and hemodynamic decompensation emerge when cardiovascular reserve is insufficient to tolerate the physiological increase in blood volume, cardiac output, and metabolic burden.^{33,34} Typical risk factors in late-onset PE include obesity, metabolic syndrome, type 2 or

gestational diabetes mellitus, chronic hypertension, dyslipidemia, and advanced maternal age.^{35,36} In these patients, the risk is centered primarily on the mother (hypertensive crises, pulmonary edema, cardiovascular events), while fetal compromise is typically mild and the disease develops near term with better neonatal prognosis but increased risk of long-term maternal cardiovascular disease.^{37,38}

Furthermore, both phenotypes share a clinical spectrum that includes headache, visual disturbances (scotomata), epigastric or right upper quadrant pain, acute pulmonary edema, proteinuria, renal and hepatic abnormalities, thrombocytopenia, and in the most severe cases, HELLP syndrome and eclampsia.³⁹ The early-onset form is more frequently associated with pathological uterine Doppler, FGR, and findings of damage placenta, whereas the late-onset form presents a profile more overlapping with gestational hypertension in women with pre-existing metabolic and cardiovascular risk factors.⁴⁰

1.1.4 Pathophysiology of preeclampsia: placental stress, metabolic reprogramming, and systemic endothelial dysfunction

PE is fundamentally a placenta-driven disorder in which placental hypoxia and ischemia act as central triggers, promoting oxidative stress, angiogenic imbalance, inflammation, and ultimately systemic maternal endothelial dysfunction that together drive disease development and progression^{34,39}

To contextualize, early placental development occurs, as previously mentioned in **Section 1.1.1**, within a physiologically low-oxygen environment (<20 mmHg), maintained by EVT plugs that transiently occlude spiral arteries and establish an oxygen gradient between the maternal placental bed and the developing placenta and embryo.^{41,42} This hypoxic milieu is not pathological but essential, as reduced oxygen tension stabilizes HIF-1 α and hypoxia-inducible factor-2 α (HIF-2 α) (see full HIF-1 α mechanism in **Sections 3.1.2.1** and **Section 3.1.2.2**), which in turn promote the transcription of multiple genes involved in key developmental programs, including vasculogenesis, cardiogenesis, and hematopoiesis,

together with erythropoietin production, thereby expanding the fetal erythroid compartment and enhancing vascularization and, consequently, increasing the oxygen-carrying capacity of the fetal circulation. Altogether, these mechanisms position the placenta as a dynamic oxygen sensor that integrates local oxygen tension with fetal and uteroplacental development.^{42,43} By the end of the first trimester (weeks 12-13), EVT^s progressively dissolve the trophoblastic plugs in a process spanning weeks 10-14, allowing maternal blood flow to gradually increase into the intervillous space and pO₂ to rise (40-60 mmHg).⁴⁴⁻⁴⁶ In parallel, EVT^s invade the decidua and proximal myometrium, transforming spiral arteries into low-resistance, high-capacitance vessels through coordinated interactions with uterine natural killer (NK) cells and M2-like macrophages.^{47,48} This extensive arterial remodeling progressively replaces the muscular-elastic wall with a fibrinoid, trophoblast-rich layer, resulting in dilated, low-resistance vessels capable of delivering large volumes of maternal blood at low pressure. As a consequence, uteroplacental perfusion increases creating a hemodynamically stable environment that supports efficient maternal-fetal gas and nutrient exchange and sustains the developmental gains established during the early hypoxic phase.⁴⁷

However, when this tightly regulated oxygen-sensing and vascular remodeling system fails to adapt appropriately, the obstetric pathophysiology, such as, preeclampsia emerges. It exists two main types of PE. In early-onset disease, shallow EVT invasion and incomplete spiral artery remodeling leave these vessels narrow and high-resistant, resulting in higher risk of placental ischemia when oxygen levels increase.^{16,49} In late-onset PE, vascular remodeling is largely preserved; nevertheless, continued placental growth or senescence creates a mismatch between placental size and perfusion capacity. Villous overcrowding and microvascular stress thereby induce relative hypoxia and oxidative stress, even in the presence of apparently adequate macroscopic blood flow.⁵⁰ In all scenarios, the placenta senses local hypoxia and oxidative stress, driving persistent stabilization of HIF-1 α , the central transcriptional effector of oxygen and reactive oxygen species (ROS) sensing.^{51,52}

Stabilized HIF-1 α in both villous and extravillous trophoblasts binds to hypoxia-response elements (HREs), thereby reprogramming gene expression and upregulating metabolic targets such as solute carrier family 2 member 1 (SLC2A1), which encodes GLUT1, ultimately promoting a predominantly glycolytic trophoblast phenotype.⁵³ HIF-1 α upregulates pyruvate dehydrogenase kinase-1 (PDK1), which inhibits the pyruvate dehydrogenase complex and diverts pyruvate toward lactate production via LDHA, reinforcing dependence on anaerobic glycolysis.⁵⁴ This metabolic reprogramming is not merely a response to energy deprivation but actively precedes and sustains inflammation, as accumulation of lactate and succinate further stabilizes HIF-1 α and supports continued cytokine production by trophoblasts and immune cells.

Hypoxia also augments mitochondrial and non-mitochondrial ROS production, which reinforces HIF-1 α stabilization through inhibition of its negative regulators, prolyl hydroxylase domain enzymes (PHDs) and factor inhibiting HIF-1 α (FIH). Moreover, HIF-1 α , ROS, and accumulated hypoxia-associated metabolites (such as lactate, succinate, and fumarate) collectively activate canonical inflammatory pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), p38 mitogen-activated protein kinase (MAPK), janus kinase (JAK)/STAT, and phosphoinositide 3-Kinase (PI3K)/protein kinase B (AKT), amplifying transcription of pro-inflammatory cytokines (IL-6, IL-1 β , tumor necrosis factor- α (TNF- α)), chemokines (C-C motif chemokine ligand 2 (CCL2)), and matrix metalloproteinases (MMP-2 and MMP-9) in trophoblasts and decidual immune cells.⁵⁵ Thus, the pathological sequence proceeds from placental hypoxia to HIF-1 α stabilization, metabolic reprogramming, and sustained inflammatory signaling, collectively leading to excessive oxidative stress and tissue damage. Within this inflammatory milieu, macrophages act as key amplifiers. Elevated CCL2 recruits CCR2⁺ monocytes from the circulation, while resident decidual M2-like macrophages are reprogrammed toward an M1-like phenotype characterized by secretion of TNF- α , IL-6, ROS, and MMPs.⁵⁶ Together with stressed trophoblast populations, these macrophages drive excessive MMP-2 and MMP-9 activity, aberrant extracellular matrix remodeling, and disordered trophoblast

migration, hallmarks observed under severe hypoxia in experimental models of preeclampsia.^{57,58} In parallel, HIF-1 α -dependent signaling and inflammation induce a profound angiogenic imbalance, marked by increased release of sFlt-1 and sEng from syncytiotrophoblasts, Hofbauer cells, and decidual macrophages.^{6,59} These soluble receptors sequester VEGF-A and PGF, reducing their bioavailability and impairing endothelial survival and angiogenesis. Elevated sFlt-1 and reduced PGF thus represent mechanistic consequences of placental stress rather than passive biomarkers, directly contributing to systemic vasoconstriction, endothelial dysfunction, and glomerular endotheliosis.^{39,49}

Placental factors released into the maternal circulation, including sFlt-1, sEng, pro-inflammatory cytokines (e.g. IL-8, IL-6, IL-1 β , CCL-2), ROS, DAMPs, and syncytial debris, reprogram the maternal endothelium toward an activated, pro-inflammatory, procoagulant, and vasoconstrictive state, with reduced nitric oxide bioavailability and altered vascular tone. Infiltrating monocytes differentiate into tissue macrophages within target organs such as the kidney, liver, and brain, perpetuating local inflammation in these organs. Clinically, these processes manifest as hypertension, proteinuria, and multi-organ dysfunction, and in severe cases progress to HELLP syndrome and eclampsia, reflecting the transition from a placenta-restricted disorder to a systemic vasculopathy driven by maladaptive oxygen sensing and metabolic reprogramming.^{39,51,60}

1.1.5 Biomarkers and therapeutic landscape in preeclampsia: current research and emerging nanotherapies

Despite the considerable clinical burden represented by placental disorders, including preeclampsia, investment in obstetric pathology research remains disproportionately low compared to other medical areas with similar mortality impact.^{24,61} In recent years, moreover, the development of placental biomarkers has transformed the understanding and clinical management of the disease.⁶² Angiogenic factors such as PGF and sFlt-1 have redefined PE, shifting from a primarily hypertensive syndrome to a disorder characterized

by angiogenic imbalance.^{39,63} The sFlt-1/PGF ratio is now a central diagnostic and prognostic tool, precisely identifying women at high risk when the ratio is >85 before 34 weeks or >110 thereafter.⁶² Additionally, sEng acts as an antiangiogenic receptor interfering with TGF- β signaling and rises parallel to sFlt-1, especially in severe and early-onset forms.^{24,64} Consequently, combining the sFlt-1/PGF ratio with sEng levels improves severity stratification.³⁹ Furthermore, the clinical management of PE remains fundamentally supportive, with placental delivery as the only definitive curative intervention.^{63,64} Since therapeutic decisions aim to balance maternal stabilization with risks associated with preterm birth, preventive strategies like low-dose aspirin administration (75-81 mg daily before 16-20 weeks), which improves placental vascular function by inhibiting thromboxane, have shown only modest efficacy, offering risk reductions of 15-62% limited to high-risk women but providing no therapeutic benefits once the disease is established.⁶⁵ Similarly, magnesium sulfate is currently used to prevent eclamptic seizures; although it reduces neurological morbidity, it does not halt disease progression and requires close monitoring due to maternal toxicity potential, including hypotension, bradycardia, and respiratory depression.⁶⁴ For its part, severe hypertension ($\geq 160/110$ mmHg) is routinely controlled with labetalol, hydralazine, or nifedipine, drugs that limit hypertensive symptoms without modifying the underlying pathogenesis and may cause maternal-fetal adverse effects.³⁹ Finally, betamethasone is used to induce fetal lung maturation before 34 weeks in cases where early delivery is necessary.⁶³

Turning to current research in clinical phases, various pharmacological agents with potential to modify the course of PE are under development. Among candidates in early phases (Phase I), CBP-4888 stands out, a nanoformulation with two siRNAs conjugated with phosphocholine-docosanoic acid (PC-DCA) that silences sFlt-1, decreasing the sFlt-1/PGF ratio and improving placental perfusion and viability,⁶⁶ as well as α -1-microglobulin (A1M), an endogenous protein with antioxidant properties protective of the kidney and placenta. In intermediate development phases (Phase II), compounds like DM199, a recombinant form of kallikrein-1, a vasodilator that acts by reducing blood pressure and improving

placental blood flow and endothelial function,⁶⁷ stand out, while metformin has demonstrated prolongation of gestation by approximately one week in women with PE via AMPK reducing sFlt-1/PGF ratio.⁶⁸ Among other candidates, hydroxychloroquine attenuates placental inflammation and complement activation;⁶⁹ L-citrulline and dydrogesterone exert vasodilatory and hormonal protective effects;⁷⁰ and ozagrel, a thromboxane synthase inhibitor, improves uteroplacental perfusion.⁷¹ In advanced phases (Phase III), pravastatin may restore VEGF-mediated angiogenic signaling, although its efficacy has been inconsistent;^{72,73} esomeprazole shows potential in preventing late-onset PE by reducing sFlt-1 and exerting antioxidant properties;⁷⁴ and natural compounds like sulforaphane and resveratrol activate endogenous antioxidant and cytoprotective pathways. Additionally, vitamin D supplementation is associated with lower recurrence risk in subsequent pregnancies, and high-dose aspirin (>150 mg) use in multiple pregnancies (e.g. duplets or triplets) is being investigated as a prophylactic strategy.⁶⁵ However, none of these alternatives has yet achieved regulatory approval or demonstrated consistent disease-modifying capacity in large clinical trials.

In this context, nanotechnology represents a paradigm shift in the PE approach by enabling selective, localized, and efficient delivery of therapies targeted to placental tissue. Currently, the only therapy in clinical phases is CBP-4888, which, as mentioned earlier, is an siRNA decreasing the sFlt-1/PGF ratio.⁶⁶ Similarly, studies are testing siRNAs encapsulated in PLGA, silica, or lipopolymers, as well as antihypertensives such as labetalol, nifedipine, or hydralazine, in nanoparticle systems under 150 nm, thereby facilitating placental uptake and reducing transplacental transfer by over 90% in ex vivo models.⁷⁵ In parallel, ionizable lipid nanoparticles (iLNPs) loaded with mRNA encoding proangiogenic factors such as PGF or VEGF have shown promising results in hypoxic murine models, achieving placental accumulation over 100-fold greater than conventional formulations, with reduction of maternal hypertension and improvement of fetal weight and flow after a single dose. Finally, there is emerging interest in CeO₂ NPs for PE, although the few published articles show mixed results, some reporting positive effects and others negative

ones.^{76,77} Notably, doses are highly variable, and many studies fail to coat the nanoparticles (NPs), thus not controlling aggregation. As will be demonstrated in the present thesis, doses >400 µg/ml that form aggregates prove toxic in *in vitro* HTR-8/SVneo trophoblasts.

1.1.6 CeO₂ NPs as a therapeutic strategy for PE: ROS scavenging and redox homeostasis

In this study, CeO₂ NPs are investigated as a therapeutic strategy for PE because their antioxidant and anti-inflammatory activities target core disease mechanisms. A key feature of CeO₂ NPs is the reversible switching of cerium cations between Ce³⁺ and Ce⁴⁺ valence states, which confers enzyme-mimetic activity, catalase- and superoxide dismutase-like, and enables ROS scavenging through sustained surface redox Ce³⁺/Ce⁴⁺ cycling.^{78–82} This catalytic behavior, distinguishes CeO₂ NPs from stoichiometric antioxidants, such as vitamins C and E, whose activity depends on finite molecular consumption and is further limited by suboptimal bioavailability, as only a fraction of the orally administered dose is effectively absorbed, reaches target tissues, and is retained long enough to exert a durable antioxidant effect.^{83,84} Moreover, high-dose vitamin C and E supplementation has not consistently improved clinical outcomes in PE trials and the reasons for this failure remain incompletely understood, reinforcing the need for alternative antioxidant strategies.^{85,86} By acting through a regenerative, catalyst-like mechanism and exhibiting a distinct pharmacokinetic and bioavailability profile compared with classical antioxidants, CeO₂ NPs emerge as promising candidates for the treatment of PE.

1.1.7 Reactive oxygen species and their production under physiological conditions: Focus on mitochondrial electron transport chain

ROS are chemically reactive molecules derived from oxygen produced during cellular respiration that play dual roles in human physiology and pathology. At basal levels, ROS act as secondary messengers in intracellular signaling, immune defense, and redox homeostasis. However, excessive ROS accumulation disrupts cellular balance, damaging

proteins, lipids, and nucleic acids, and contributing to inflammation, such as in PE, and to aging. The main ROS species are summarized in **Table 1.1**.⁸⁷ Among these, the superoxide anion radical ($O_2\bullet^-$) is a primary source of ROS, generated predominantly by the mitochondrial electron transport chain (ETC), whereas the hydroxyl radical ($\bullet OH$) is the most reactive and damaging ROS^{88,89}.

Reactive oxygen species
$O_2\bullet^-$ (Superoxide anion)
H_2O_2 (Hydrogen peroxide)
$\bullet OH$ (Hydroxyl radical)
$ONOO^-$ (Peroxynitrite)
1O_2 (Singlet oxygen)
$HOCl$ (Reactive chlorine species)
$ROOH$ (Lipid hydroperoxides)
$ROO\bullet$ (Peroxi radical)
$RO\bullet$ (Alcoxi radical)

$O_2\bullet^-$ = primary ROS source, mainly from ETC mitochondria
 $\bullet OH$ = most reactive radical, mainly from Fenton reaction

Table 1.1 Main reactive oxygen species relevant in human biology and pathophysiology. *Information obtained from:*⁹⁰

ROS in humans is generated by multiple (i) enzymatic and (ii) organellar sources. Among organellar sources, the mitochondrial ETC, located in the inner mitochondrial membrane, represents the principal site of ROS formation, predominantly $O_2\bullet^-$, and is estimated to account for up to ~90% of cellular ROS under physiological conditions.^{91,92}

The electron transport chain (ETC) consists of five protein complexes (I-V), whose main function is the synthesis of adenosine triphosphate (ATP) (**Figure 1.4**).^{93,94} Complexes I (NADH:ubiquinone oxidoreductase) and III (ubiquinol:cytochrome c oxidoreductase or bc1 complex) are the main sites of mitochondrial ROS (mtROS) production, with Complex III considered the predominant source under physiological conditions and in intact mitochondria.^{90,93,95}

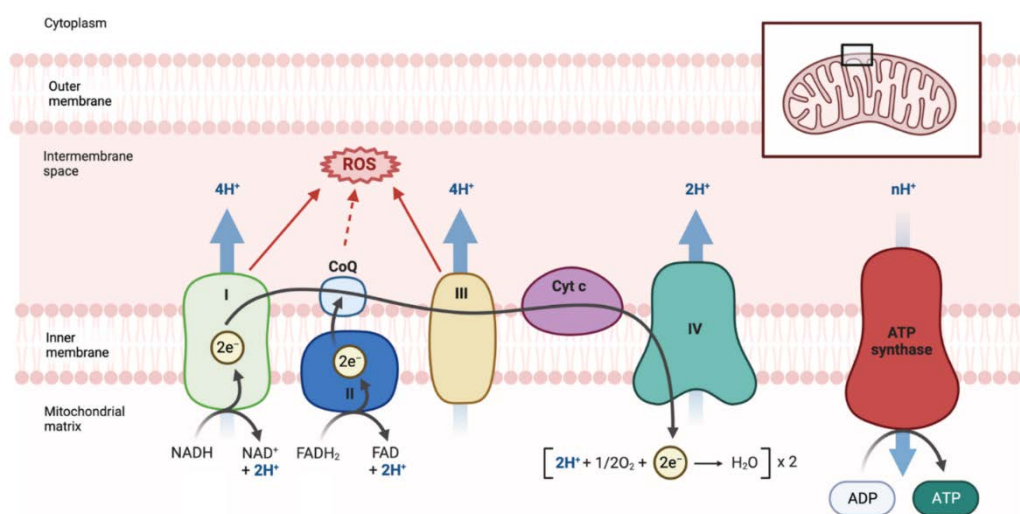


Figure 1.4 Schematic representation of the mitochondrial electron transport chain (ETC) and reactive oxygen species (ROS) production sites. The diagram illustrates the inner mitochondrial membrane with Complexes I-V embedded, showing electron flow. *Adapted from:*⁹⁶

The image represents the normal process of the electron transport chain (ETC) in the mitochondrion (**Figure 1.4**), showing how NADH and succinate (primarily originating from the tricarboxylic acid (TCA) cycle) donate electrons to Complexes I and II, respectively, to generate a proton gradient that drives ATP synthesis while minimizing ROS production.^{90,93,94,97,98} Under normal operation, NADH reduces flavin mononucleotide (FMN) to FMNH₂, which transfers electrons through a series of iron-sulfur (Fe-S) clusters before finally reducing ubiquinone (CoQ) to ubiquinol (CoQH₂), pumping 4H⁺ into the intermembrane space.^{93,94,99} In parallel, in Complex II (succinate dehydrogenase), succinate reduces the covalently bound FAD to FADH₂, which passes electrons via Fe-S clusters directly to CoQ, without FMN and with lower redox potential, also minimizing ROS.^{90,97,98}

Succulently, electrons from both complexes converge on CoQH₂, which is oxidized at Complex III through the Q cycle, where one electron goes to the Rieske Fe-S cluster, then to cytochrome c1 and finally to cytochrome c, while the second electron goes via cytochromes b to a semiquinone ubiquinone at the Qo and Qi sites, allowing the pumping of 4H⁺.^{90,95} Finally, at Complex IV, electrons from cytochrome c pass sequentially through CuA, heme a, and heme a3-CuB to O₂ and 4H⁺, forming 2H₂O and pumping 2 additional H⁺.^{93,94} Then, protons flow through ATP synthase (Complex V), converting ADP + Pi into ATP.⁹⁴ Under normal conditions, efficient electron flow prevents electron accumulation, reducing ROS levels.^{93,99,100}

1.1.7.1 Complexes I and III as major mitochondrial ROS sources during hypoxia-reoxygenation in PE and hypoxia

Pathological alterations in electron flow during hypoxia-reoxygenation cycles, such as in PE or sustained hypoxia, dramatically enhance ROS production at Complexes I and III.^{93,101} In Complex I, the main sites of electron leakage are flavin mononucleotide (FMN/FMNH₂) and the ubiquinone binding site (CoQ/CoQH₂).^{99,100} When (i) NADH/NAD⁺ is high or (ii) electron flow slows, electrons can be diverted from FMNH₂ to oxygen, generating O₂•⁻ in the mitochondrial matrix even during "forward" transport (forward electron leak).^{93,99} Under conditions of high proton motive force (Δp) and a highly reduced CoQ pool (CoQH₂), electrons can flow backward from CoQH₂ to FMN (reverse electron transport (RET)), triggering massive bursts of O₂•⁻ at the same flavin site.^{99,100} In preeclampsia, placental perfusion is typically intermittent, with repeated episodes of hypoxia followed by reoxygenation (mitochondrial ischemia-reperfusion).^{101,102} During hypoxic periods, succinate accumulates due to the functional reversal of Complex II, also called succinate dehydrogenase, which under normal conditions oxidizes succinate to fumarate by transferring electrons to FAD and reducing it to FADH₂.^{97,98} Upon reintroduction of oxygen, this succinate is rapidly oxidized, massively reducing the CoQ pool to CoQH₂ and, together with elevated Δp, triggers RET in Complex I and intense O₂•⁻ production at the flavin site

of Complex I,^{97,99,100} as explained in the previous paragraph. In parallel, the excess CoQH₂ entering Complex III fuels the Q cycle at the Qo site, generating unstable semiquinones that escape to oxygen and produce O₂•⁻ and H₂O₂ toward both the matrix and the intermembrane space during the reoxygenation phase (**Figure 1.5**).^{95,103} This repeated pattern of hypoxia-reoxygenation in preeclamptic placenta maintains a state of chronic oxidative stress, contributes to persistent HIF-1α activation, which is also sustained not only by oxygen but also by ROS, and favors the release of antiangiogenic and proinflammatory factors into the maternal circulation.^{101,104,105}

In sustained hypoxia (without intermittent reoxygenation), the situation is different. Low O₂ availability limits Complex IV activity, as O₂ is its direct substrate, causing the CoQ pool to remain more reduced and prolonging the half-life of semiquinone species at the Qo site of Complex III (**Figure 1.5**).^{106,107} Under these conditions, Complex III (Qo site) becomes the main source of mtROS, and O₂•⁻ and H₂O₂ production from this site serves as a signal to stabilize HIF-1α and initiate hypoxia-adaptive transcriptional reprogramming.^{103,104,108} At the same time, RET in Complex I tends to be limited in sustained hypoxia because the proton motive force does not recover sufficiently to sustain robust backward electron flow from CoQH₂.¹⁰⁰ In this context, Complex I generates ROS mainly through "forward" leakage at the FMN site, driven by NADH accumulation as the TCA cycle slows, while Complex III ROS at Qo remains the dominant signal for the HIF pathway.^{93,98,99}

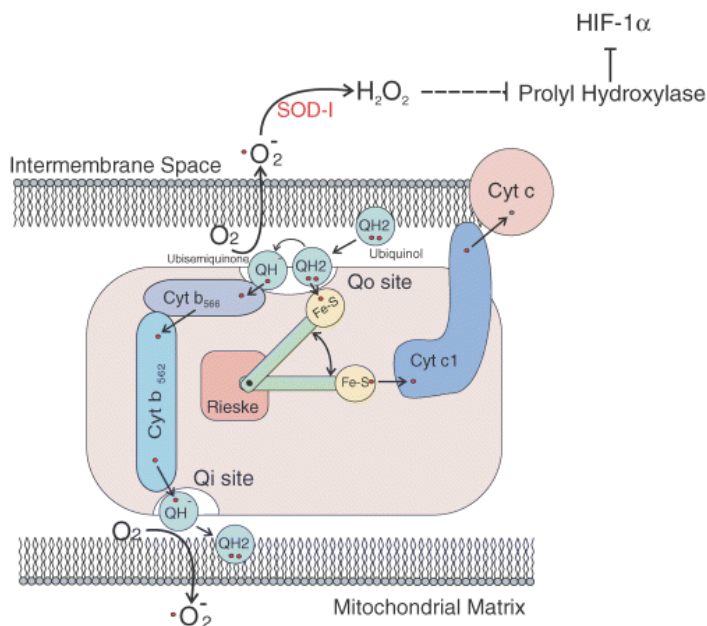


Figure 1.5 Simplified schematic illustrating the role of mitochondrial Complex III-derived ROS in hypoxia-induced HIF-1 α stabilization. From:¹⁰⁶

Beyond complexes I and III, additional mitochondrial matrix enzymes such as glycerol-3-phosphate dehydrogenase, 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase, and electron transfer flavoprotein Q oxidoreductase also contribute to mtROS generation.¹⁰⁹

1.1.7.2 Enzymatic and other organellar sources of reactive oxygen species under hypoxia and hypoxia-reoxygenation conditions

Another major enzymatic source of ROS is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOXs) family, which produces either O₂^{•-} or H₂O₂ in plasma membranes, endoplasmic reticulum (ER), and phagosomal membranes. ROS formation in NOXs begins when NADPH binds to the dehydrogenase domain of the enzyme. Electrons are sequentially transferred from NADPH to the flavin adenine dinucleotide (FAD) cofactor and then to the two heme groups located in the transmembrane domain. Finally, molecular O₂ is reduced to O₂^{•-} or H₂O₂, depending on the NOX isoform.¹¹⁰ Notably, NOX activity is enhanced under hypoxic conditions, particularly NOX4, making it a significant contributor to ROS in both sustained and intermittent hypoxia, and also has been observed in PE

pathology.^{111,112} Similarly, xanthine oxidase (XO), a key enzyme in cytosol and peroxisomes, is another ROS source in hypoxic conditions.¹¹³ XO catalyzes the final two steps of purine degradation in humans: the oxidative hydroxylation of hypoxanthine to xanthine, and subsequently, xanthine to uric acid. During these reactions, molecular O_2 acts as the terminal electron acceptor and is reduced to form both $O_2^{\bullet-}$ and H_2O_2 .¹¹⁴

Other enzymatic systems also participate in ROS formation. Uncoupled endothelial nitric oxide synthase (eNOS), localized in the cytosol and plasma membrane caveolae, generates $O_2^{\bullet-}$ instead of nitric oxide (NO) when essential cofactors such as tetrahydrobiopterin (BH_4) or the substrate L-arginine are deficient. The $O_2^{\bullet-}$ produced can rapidly react with NO generated by the canonical coupled eNOS pathway, forming peroxynitrite ($ONOO^-$), a short-lived but highly reactive oxidant that promotes oxidation and nitration of biomolecules including lipids, proteins, and DNA.¹¹⁵ Hypoxia alone and hypoxia-reoxygenation, to favor BH_4 depletion, thus favor eNOS uncoupling and further increasing ROS generation.^{116,117}

The ER is another important site of ROS generation, primarily producing H_2O_2 through ER oxidoreductin-1 (ERO1) during the formation of disulfide bonds essential for protein folding. While moderate ROS levels are required for maintaining redox homeostasis, ER stress or dysfunction leads to protein misfolding and activation of the unfolded protein response (UPR). Among UPR components, inositol-requiring kinase 1 (IRE1) activates inflammatory signaling pathways such as nuclear factor kappa-light-chain (NF κ B), c-Jun N-terminal kinase (JNK), and the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome. Additionally, oxidation of thiol groups in the ryanodine receptor (RyR) triggers calcium (II) cation (Ca^{2+}) release from the ER, amplifying ROS production through mitochondrial dysfunction.¹¹⁸

Further cytochrome P450 (CYP) enzymes, located primarily in the smooth ER and also in mitochondria, also generate ROS in moderate amounts in monooxygenation reactions.

CYPs enzymes activate molecular O_2 to form an oxy-complex, which is reduced via electron transfer from NADPH to form a peroxo-intermediate. Protonation of this intermediate releases Compound I ($Fe^{4+}=O$ porphyrin π -cation radical), a highly reactive species that abstracts hydrogens from the substrates, generating substrate radicals and contributing to ROS formation, including $O_2^{\bullet-}$, H_2O_2 , and singlet oxygen (1O_2).¹¹⁹

Additionally, minor ROS-producing enzymes include haloperoxidases, such as, myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), present in neutrophils, eosinophils, and secretory glands. Their function is to convert H_2O_2 into reactive halogen species (RHS), such as, hypochlorous acid (HOCl) for antimicrobial defense, although persistent activation can lead to chronic oxidative stress. Similarly, cyclooxygenases (COX)1-2 and lipoxygenases (LOX), located in the ER, cytosol, and nuclear envelope, oxidize arachidonic acid, generating prostaglandins, leukotrienes, and hydroperoxides that participate in inflammation, vascular damage, and lipid peroxidation⁹⁰

Collectively, these diverse ROS sources form an intricate and interconnected network, where any imbalance between ROS production and antioxidant defenses results in oxidative stress, a hallmark of inflammation, vascular dysfunction, and aging.^{101,102}

1.1.8. Generation and transport of mitochondrial ROS: From electron transport chain to cellular signaling

Mitochondria consume more than 90% of cellular O_2 to generate ATP. Approximately 1-2% of this O_2 reacts with electrons leaked from Complexes I and III during oxidative phosphorylation (OXPHOS), leading to partial reduction of O_2 and generation of $O_2^{\bullet-}$.¹⁰² Topographically, mitochondria generates mtROS at several sites within the electron transport chain. The most important are those in Complex I, which produces $O_2^{\bullet-}$ at two matrix-facing sites: the FMN/flavin site (the major source) and the ubiquinone-binding Q-site. In contrast, Complex III (Qo site) can generate $O_2^{\bullet-}$ toward both the matrix and the intermembrane space (IMS), making these complexes the primary contributors to

mitochondrial ROS.^{102,120–124} Because $O_2^{\bullet-}$ is membrane-impermeant and extremely short-lived, it acts locally; the propagating species is H_2O_2 , and SODs are in charge of catalyzing the dismutation of $O_2^{\bullet-}$ into H_2O_2 . As an example, each mitochondria site is capable of generating more than $1 \text{ nmol } H_2O_2 \text{ min}^{-1} \text{ mg}^{-1}$ mitochondrial protein under $V_{\max}$ conditions *in vitro* skeletal muscle cells. Of course, the rates of mitochondrial ROS production from a given site are tissue specific¹²⁵. Moreover, $O_2^{\bullet-}$ from the matrix is converted to H_2O_2 by SOD2 and, in the IMS, by SOD1.^{102,120,126,127}

Matrix H_2O_2 exits mitochondria by first crossing the inner membrane, predominantly and more efficiently via peroxiporin aquaporins (AQP), notably AQP8, though it can also cross very slowly by simple diffusion. After that, H_2O_2 traverses the outer membrane through VDAC/porins to enter the cytosol. On the IMS side, H_2O_2 has a short path; it only needs to cross the outer membrane via VDAC/porins to reach the cytosol.¹²⁸ Additionally, mitochondria have their own local ROS sinks, mainly peroxiredoxins (Prx3 and Prx5) and GPx1 and GPx4 in the matrix, and little CAT, that consume H_2O_2 when it is in excess^{102,129,130}. When these antioxidant systems are saturated or inactivated, more mitochondrial H_2O_2 escapes and accumulates in the cytosol, where it can spread to nearby compartments via peroxiporins, AQP3 into endosomes¹³¹ and AQP1,3,5,8 and 9 at the plasma membrane¹³². Furthermore, $\bullet OH$ generated *in situ* from H_2O_2 via Fenton chemistry is ultra-short-lived and does not diffuse appreciably, so its damage is confined to its site of formation. $ONOO^-$, produced where $O_2^{\bullet-}$ meets NO (in the matrix or IMS), also reacts rapidly nearby; its protonated form, $ONOOH$, has limited membrane permeability and short range. Thus, long-range redox signaling and ROS propagation occur predominantly in the form of H_2O_2 , which can diffuse and subsequently generate $\bullet OH$ and derived species in compartments such as the cytosol, endosomes, and endoplasmic reticulum.

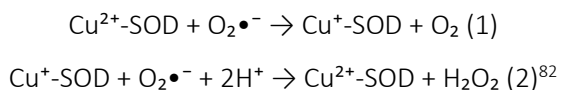
As mentioned previously, beyond mitochondria, NOX provide dedicated O_2 acceptors on membranes. NOX2 (plasma membrane/endosomes/phagosomes) and NOX4 (ER, nucleus, mitochondria-associated membranes) also, generate $O_2^{\bullet-}$ directly into the extracellular

space, cytosol or endosomal lumen, which is locally dismutated to H_2O_2 and then diffuses within the cytosol and into organelles via peroxiporins. Additionally, other mechanisms previously mentioned, such as uncoupled eNOS, H_2O_2 production in the ER, XOs, CYPs, COX, and LOX, follow the same operational rule. $\text{O}_2\bullet^-$ acts locally and is converted to H_2O_2 . Subsequently, H_2O_2 , whose levels are regulated by peroxiporins and antioxidant sinks, serves as the principal mobile redox signal that reaches the cytosol, endosomes, and other organelles.¹³³

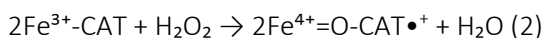
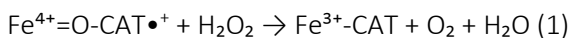
1.1.9 Endogenous antioxidant enzymes against ROS

The human antioxidant defense network comprises both enzymatic and non-enzymatic components that act synergistically to preserve redox homeostasis and protect against damage induced by ROS. The enzymatic antioxidants constitute the primary and most efficient line of defense against ROS. This group includes SOD1-3, CAT, glutathione GPx1-8, and Prx1-6, which function in a coordinated cascade distributed across specific subcellular compartments. These enzymes convert reactive oxygen intermediates into less harmful molecules, maintaining physiological ROS levels required for signaling while preventing oxidative damage.⁸¹

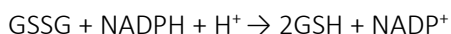
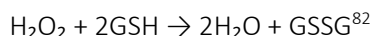
SODs catalyze the dismutation of the $\text{O}_2\bullet^-$ into H_2O_2 and molecular O_2 (**Figure 1.6**). Three isoforms have been identified: SOD1 (copper (Cu)/zinc (Zn)-SOD), located in the cytosol, mitochondrial intermembrane space, and nucleus; SOD2 (manganese (Mn)-SOD), localized in the mitochondrial matrix; and SOD3 (Cu/Zn-SOD), found in the extracellular space. The SOD catalytic mechanism involves a metal-centered redox cycle, which resembles the CeO_2 NPs (explained in the following part). In Cu/Zn-SODs, the copper cation alternates between Cu^{2+} (oxidized) and Cu^+ (reduced) states through two sequential one-electron transfers and subsequently catalyzing the $\text{O}_2\bullet^-$ dismutation:¹²⁶



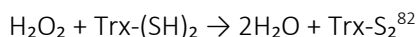
Here, Zn^{2+} stabilizes the enzyme structure, while Cu^{2+} participates directly in electron transfer. In Mn-SOD, manganese cycles between Mn^{3+} and Mn^{2+} cations. Then, The H_2O_2 formed by SOD is detoxified by catalase, GPx, and Prx. Catalase (CAT), primarily localized in peroxisomes, acts efficiently when H_2O_2 concentrations are high, providing rapid detoxification. It decomposes H_2O_2 into H_2O and molecular O_2 through the following stoichiometrically reaction (**Figure 1.6**).^{134,135}



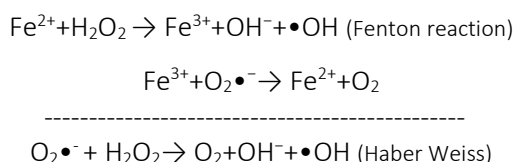
CAT contains a heme prosthetic group at its active site, where the iron atom undergoes a reversible redox cycle, similar to the cerium atom in CeO_2 NPs, but between Fe^{3+} and $\text{Fe}^{4+}=\text{O}$. Further, GPx1-8, distributed in the cytosol, mitochondria, and ER, remove both H_2O_2 and organic peroxides (ROOH) using reduced glutathione (GSH) as an electron donor (**Figure 1.6**):



During this process, GSH is oxidized to glutathione disulfide (GSSG). To sustain antioxidant capacity, glutathione reductase (GR) catalyzes the regeneration of GSH from GSSG using NADPH as the electron donor. Creating then NADPH-dependent glutathione cycle that ensures continuous ROS detoxification and maintenance of intracellular redox balance^{81,90} Additionally, Prx1-6 represent another critical peroxidase family found in the cytosol, mitochondria, peroxisomes, and ER (**Figure 1.6**). They reduce H_2O_2 , ROOH, ONOO^- via the thioredoxin (Trx) system:



The oxidized Trx-S₂ is then restored to its reduced form Trx-(SH)₂ by thioredoxin reductase (TrxR) in an NADPH-dependent reaction. This Trx/Prx redox cycle functions analogously to the GSH/GPx system, both relying on NADPH to sustain their antioxidant activity^{81,90}. Moreover, transition metals such as iron (Fe) or Cu that are present as free ions in solution and can participate in redox cycling, giving rise to the Fenton and Haber-Weiss reaction (Figure 1.6), two interconnected processes responsible for generating the highly reactive •OH. In the Fenton reaction, ferrous iron (Fe²⁺) reacts with H₂O₂, producing ferric iron (Fe³⁺), hydroxide ions (OH⁻), and •OH. Subsequently, O₂•⁻ can reduce Fe³⁺ back to Fe²⁺, thus sustaining the redox cycle and linking this process to the Haber-Weiss reaction:⁷⁸



See a whole schema of the reactions (Figure 1.6):

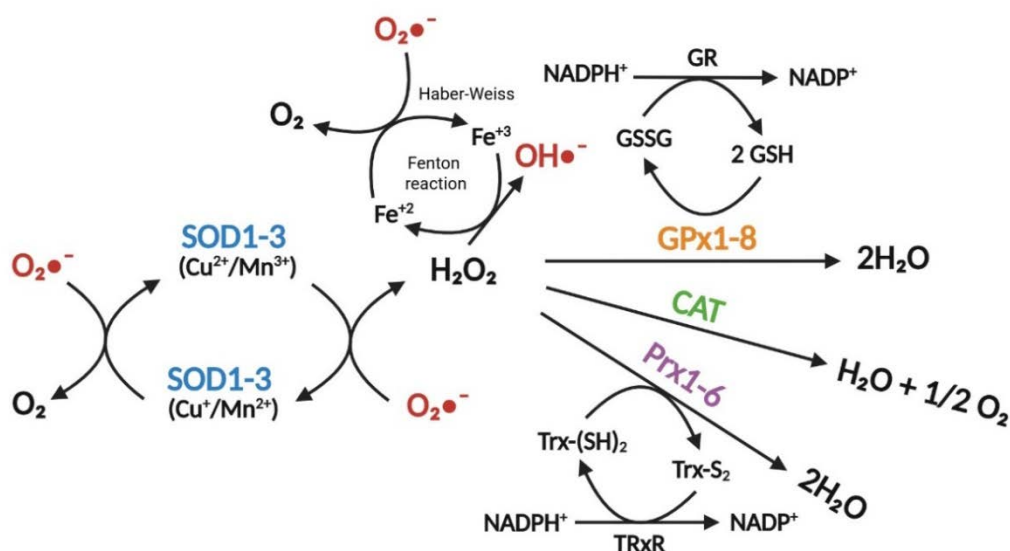


Figure 1.6 Enzymatic antioxidant defense network in human cells, showing the coordinated action of SOD, CAT, GPx, and Prx systems in detoxifying ROS, alongside non-enzymatic Fenton and Haber-Weiss reactions that generate highly reactive •OH.

Complementing the enzymatic defenses, non-enzymatic antioxidants form the secondary defense line, scavenging residual radicals, protecting biomembranes, and regenerating oxidized cofactors. Key examples include vitamin C (ascorbic acid), vitamin E (α -tocopherol and tocotrienols), β -carotenoids (provitamin A), flavonoids, and uric acid. Vitamin C is a water-soluble antioxidant that directly neutralizes $O_2^{\bullet-}$, $\bullet OH$, and $ROO\bullet$, while regenerating oxidized vitamin E and GSH. Vitamin E, a lipid-soluble antioxidant, protects membranes by intercepting lipid peroxyl radicals and terminating lipid peroxidation chain reactions. The uric acid, the final product of purine metabolism, acts as a major antioxidant in plasma, scavenging 1O_2 , $ROO\bullet$, and $ONOO^-$. In parallel, flavonoids and β -carotenoids contribute by chelating transition metals and breaking free-radical propagation chains⁸².

Collectively, the enzymatic and non-enzymatic systems form a dynamic and adaptive antioxidant network that maintains redox balance, prevents oxidative stress, and preserves cellular homeostasis. However, in pathological settings such as PE, the endogenous antioxidant network may become overwhelmed by sustained mitochondrial and extracellular ROS production,^{87,136,137} prompting interest in exogenous strategies that can reinforce or mimic natural antioxidant defenses. In this context, CeO_2 NPs have emerged as promising ROS scavengers that functionally resemble key enzymatic antioxidants, particularly SOD and CAT,^{78–80,82} and may provide targeted protection against oxidative stress.

1.1.10 Why CeO_2 NPs? Integrating cerium oxide nanoparticles into endogenous ROS defenses

From a redox perspective, CeO_2 NPs can be viewed as exogenous enzyme-mimetic antioxidants strategically inserted into the existing antioxidant network. Their surface cerium atoms cycle between Ce^{3+} and Ce^{4+} states, allowing them to disproportionate superoxide and decompose hydrogen peroxide in a manner analogous to SOD and CAT, respectively.^{78–80,82} By integrating into ROS-rich microenvironments, CeO_2 NPs do not replace but rather complement endogenous SOD/CAT/GPx/Prx systems, potentially

restoring redox balance where physiological defenses are insufficient, as occurs in the preeclampsia. The detailed mechanistic basis of the antioxidant and redox activity of CeO₂ NPs will be discussed in **Chapter 2, Section 2.2.2.6**.

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2.1 Introduction

CeO₂ NPs are compelling nanomaterials due to their well-known reversible Ce³⁺/Ce⁴⁺ redox cycling.¹ Defects on the crystalline network arising from the inherent surface tension at the nanoscale favor the presence of oxygen vacancies (Ovs) in the crystal structure and, therefore, unpaired available electrons that impart distinctive surface reactivity.² Moreover, as a lanthanide, cerium features 4f¹ states that can be populated or depopulated during Ce³⁺/Ce⁴⁺ interconversion, providing an extra electronic reservoir that supports this redox flexibility.^{2,3} Consequently, in biological environments, this redox plasticity enables multi-enzymatic activity, including SOD-like, catalase-like, and peroxidase-like functions,^{4,5} positioning CeO₂ NPs as strong candidates for oxidative-stress modulation.^{6,7} However, to have a competitive product requires rigorous control of synthesis and post-processing to ensure reproducibility, stability, and biocompatibility.⁸ In this context, the aim of this chapter has been to control and understand the product, namely which alterations can perturb it and what effects they may have not only at the nanotechnology level but also at the biomedical and translational level. Ultimately, to develop a product useful for treating preeclampsia that has real traceability. Therefore, ensuring biocompatibility and robustness is the starting point for any clinically or industrially relevant NP formulation. Biocompatibility goes beyond cytocompatibility: it requires (i) tight size control and monodispersity to ensure reproducibility between samples and consistent biological effects; (ii) stability at physiological pH and osmolarity; (iii) colloidal stability at working conditions to avoid undesired aggregation;^{7,9} (iv) high chemical purity, because trace reagents can dominate toxicity; and (v) a stable defect profile so that redox/antioxidant capacity remains predictable over time.⁸

Specifically, here a wet-chemistry route is employed that yields approximately monodisperse 3 nm CeO₂ NPs with a stable fluorite phase and narrow polydispersity. In particular, the importance of the ultrasmall size lies in that (i) it prevents toxic aggregation observed in previous cytotoxicity studies of CeO₂ NPs, (ii) it maximizes antioxidant activity

through the optimal $\text{Ce}^{3+}/\text{Ce}^{4+}$ ratio and greater surface vacancies, and (iii) it increases the possibilities of selective arrival at the placenta.^{1,10,11} Building on this, in our 3 nm CeO_2 NP synthesis, we identified different critical steps. The principal ones are: (i) CeO_2 NPs nucleation and growth, (ii) stabilization, (iii) thermal treatment, and (iv) purification/buffer exchange. If not tightly controlled, these stages can spoil size, surface properties, or stability. Accordingly, analysis of the synthesis steps relies on a multi-modal characterization suite, which enables both quality control and mechanistic interpretation.^{7,12} For instance, UV-visible (UV-Vis) spectroscopy verifies NPs formation through the characteristic 283 nm band for 3 nm CeO_2 NPs, checks for the absence of aggregation via a zero signal longer-wavelength region (>400 nm), and probes the electronic structure, with the band gap explored using Tauc analysis.² Similarly, dynamic light scattering (DLS) and zeta potential together quantify hydrodynamic size distributions and colloidal stability/electrostatic stabilization. Furthermore, electron microscopy (transmission electron microscopy (TEM), high-resolution TEM (HR-TEM), and high-angle annular dark-field scanning TEM (HAADF-STEM)) resolves core size, morphology, and lattice fringes, while particle-size histograms provide statistical confirmation of monodispersity. Finally, X-ray diffraction (XRD) further confirms the single-phase fluorite ($\text{Fm}\bar{3}\text{m}$) structure and supports crystallite-size and lattice-parameter assessment.^{2,7}

To complete the validation, functional assays are included in the following compendium: the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) decolorization assay to assess antioxidant behavior of the CeO_2 NPs, and H_2O_2 decomposition to demonstrate redox cycling and catalase-like activity of the CeO_2 NPs.^{4,5} The cytoprotective effects and biological impact of the CeO_2 NPs will not be addressed in this chapter, but in the following ones.

2.2 Results and discussion

2.2.1 CeO₂ NPs synthesis process

The synthesis of CeO₂ NPs begins by adding a cerium (III) chloride heptahydrate (CeCl₃·7H₂O, 10 mM) into a sodium citrate (SC, 20 mM), maintaining a 1:2 molar ratio under vigorous stirring (**Figure 2.1**). The role of citrate in moderating the reactivity of the solution and preventing NP aggregation will be discussed in detail (**Section 2.2.2.2**). After few minutes, tetramethylammonium hydroxide (TMA-OH, 27 mM) is added to the solution in a 1:2.7 molar ratio respect to CeCl₃. TMA-OH induces a pH solution increment, thus promoting the basic precipitation process where Ce³⁺ begins to form solid hydroxylated intermediates. Under an O₂-rich environment, these intermediates progressively oxidize, leading to the conversion of Ce³⁺ to Ce⁴⁺ (in our case, samples were left under stirring overnight (O/N) for practicality, although oxidation is essentially complete after ~4h). The resulting Ce⁴⁺ hydroxo species further transforms into CeO₂ NPs via dehydration. Importantly, hydroxylation, precipitation, oxidation, and dehydration occur simultaneously rather than as strictly separate steps.

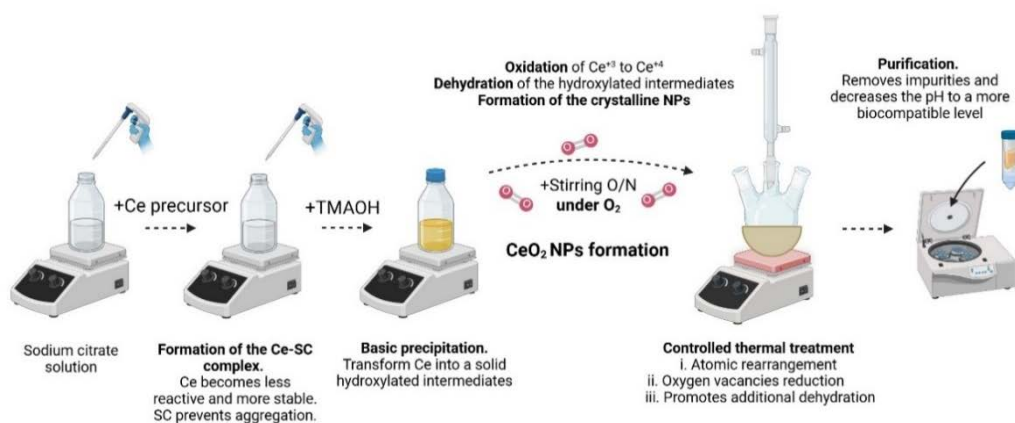


Figure 2.1. Step-by-step synthesis of CeO₂ NPs highlighting the key stages governing crystallinity and stability.

The final step in the CeO₂ NPs synthesis is the controlled thermal treatment, which in our protocol is performed at 100 °C during 4h. This step is crucial to (i) promote atomic rearrangement, and (ii) improve the stability and homogeneity of the NPs. Atomic

rearrangement reduces the number of Ovs, yielding more stable and homogeneous crystals. Although a slight loss of catalytic activity occurs, this step significantly increases stability, a key requirement to have a final biocompatible product. This results in 3 nm highly monodisperse NPs. In addition, the thermal treatment facilitates dehydration, as residual $\text{Ce}(\text{OH})_4$ is converted into CeO_2 through water loss. Thus, the thermal treatment yields a final product with enhanced crystallinity, improved monodispersity, and increased structural stability. These features are particularly critical for nanoparticles intended for biomedical applications, where stability and uniformity are essential to ensure safety, efficacy, and reproducibility.

Following thermal treatment, the colloidal suspension exhibits a pH around 9.4 due to the excess TMA-OH. To achieve a biocompatible product suitable for future GMP production, CeO_2 NPs are purified by three centrifugation cycles using Centricon® devices with a molecular weight cut-off of 10 kDa, corresponding a pore of 1.5 nm approximately. After each cycle, the pellet is resuspended in a diluted SC solution (2.2. mM). As a result, the NPs solution is purified approximately 10.000-fold. This procedure, also, enhances biological compatibility by neutralizing the suspension to a pH similar of 7.9, closer to physiological pH (7.4) and removing residual impurities, including (i) excess citrate, (ii) free ions (TMA^+ , OH^- , Na^+ , Cl^-), and (iii) oxidation by-products. Finally, the solution is filtered through a 0.22 μm hydrophilic membrane to ensure sterility and is evaluated using the Limulus Amebocyte Lysate (LAL) assay to confirm the absence of bacterial endotoxins, which can induce septic shock or death in living organisms.

2.2.2 Thermodynamic pathways of CeO_2 NPs: insights from Pourbaix diagrams

To understand the thermodynamics and the different steps involved in the synthesis of CeO_2 NPs, it is useful to understand the cerium Pourbaix diagram in water. A Pourbaix diagram is a thermodynamic representation of the equilibrium of chemical species in an aqueous system, as a function of pH and electrochemical potential (E). It is also known as a potential-pH diagram. In the Pourbaix diagram, we can observe (i) the stability regions of

different forms of an element (ions, oxides and hydroxides) in aqueous solution and (ii) the thermodynamics, that is the energy equilibrium of redox and dissolution/precipitation reactions of a chemical compound. It is important to know that Pourbaix diagram does not consider kinetics, but only whether a transformation is thermodynamically possible from an equilibrium point of view.³ Since the CeO₂ NPs synthesis is performed in an aqueous medium without the application of external electrical field, the formation pathway can be followed step by step using this diagram around the 0 eV value (**Figure 2.2**). Initially, cerium cations form complexes with SC, which plays a crucial role in (i) controlling nanoparticle stabilization, (ii) preventing aggregation, and (iii) regulating growth. This complexation step, however, is not represented in the Pourbaix diagram. Next, TMA-OH is added to the fully solvated Ce-SC complex solution. As a strong organic base, TMA-OH dissociates completely in aqueous media, releasing OH⁻ and raising the pH of the reaction from approximately 5 to 11.45. This increase in pH and specially of free OH⁻ induces the hydroxylation of Ce³⁺ cations, resulting in the formation of intermediate hydroxo complexes. Specifically, Ce(OH)²⁺ forms between pH 8.4 and 9.2, and Ce(OH)₂⁺ forms between pH 9.2 and 9.8. Upon further increase of the pH above 9.8, cerium hydroxide precipitates as Ce(OH)₃, which is a solid hydroxide precursor, and indicated as a solid phase (s) in the Pourbaix diagram. This step of base precipitation reaches a maximum near 11.45. (from left to right, **Figure 2.2**)

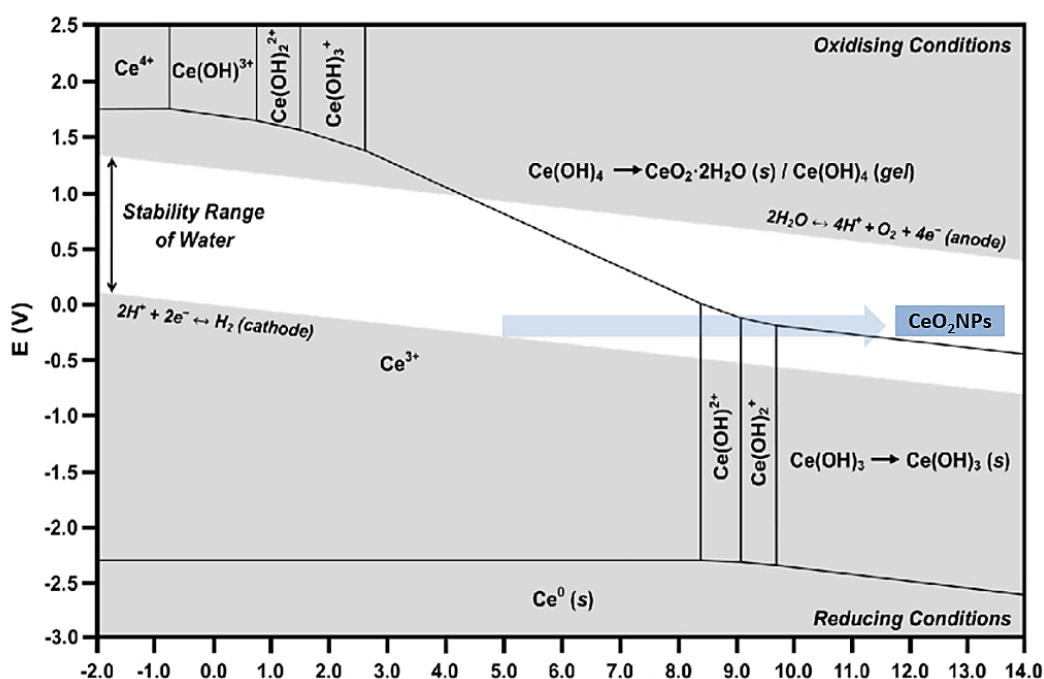


Figure 2.2. Calculated Pourbaix (E-pH) diagram for the Ce-H₂O system. Adapted from: ¹³.

In parallel with the hydroxylation of Ce^{3+} , these hydroxo complexes undergo oxidation to Ce^{4+} , leading to the formation of $\text{Ce}(\text{OH})_4$ as an intermediate species. This intermediate subsequently transforms into CeO_2 NPs through a dehydration process, which is thermodynamically favored due to the higher stability of the Ce–O network in crystalline CeO_2 . This transformation is depicted by the blue arrow in **Figure 2.2**. Although the Pourbaix diagram may imply that hydroxylation and oxidation are distinct steps, both processes occur simultaneously, resulting in the conversion of the solid hydroxide precursor into CeO_2 NPs that stabilize in the characteristic face-centered cubic (fcc) fluorite-type crystal structure. This controlled transformation plays a key role in determining the final physicochemical properties of the NPs, including particle size, crystallinity, and surface characteristics.

2.2.3 Physicochemical characterization of 3 nm monodisperse CeO₂ NPs

Once the synthesis is completed, the material is extensively characterized using a range of physicochemical techniques. These analyses confirm the reproducibility of the synthesis, which is a mandatory requirement for materials intended for biomedical applications, and establish the key physicochemical properties of the 3 nm monodisperse CeO₂ NPs. Detailed descriptions of the experimental methods are provided in **Chapter 5**.

2.2.3.1 UV-Visible spectroscopy

The first technique applied for characterization is UV-visible spectroscopy (**Figure 2.3**), a routine tool in any nanoparticle synthesis laboratory. The absorption spectrum displays a distinct and asymmetric absorption band at 283 nm and no extinction band (absorbance + scattering) above 400 nm, confirming the absence of aggregates (**Figure 2.3A**). Using the Beer-Lambert law, the colloidal nanoparticle concentration can indirectly be calculated from the absorption spectrum (see **Chapter 5**). For NPs smaller than 10 nm, scattering is negligible, so the measured absorbance originates almost exclusively from absorption and is linearly proportional to nanoparticle concentration.

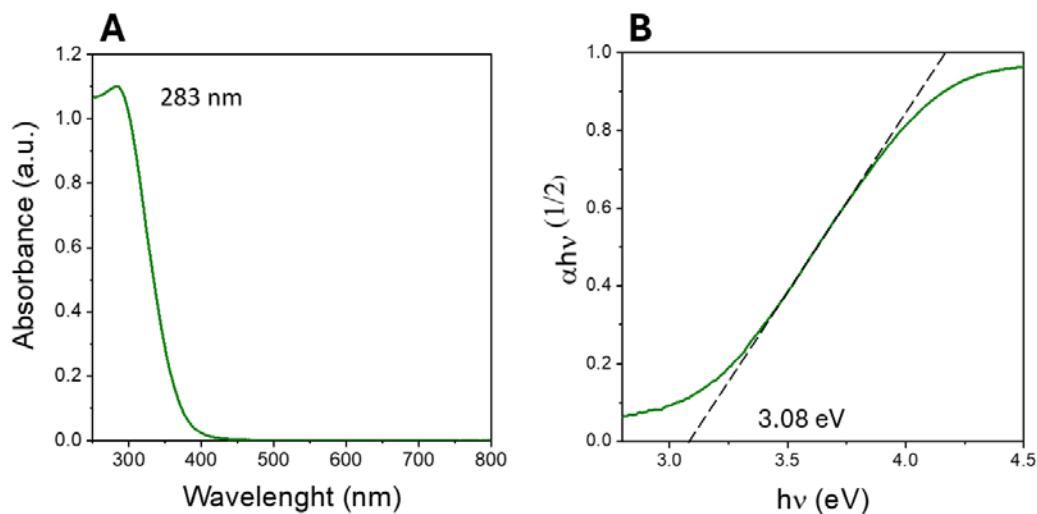


Figure 2.3. UV-vis absorption spectrum of CeO₂ NPs and Tauc Plot. **(A)** UV-vis spectrum shows the characteristic absorption band at 283 nm and the absence of aggregation-related features above 400 nm. **(B)** Tauc plot used for the determination of the optical band gap, calculated to be 3.08 eV.

The optical band gap, estimated from the Tauc plot, is calculated to be 3.08 eV (**Figure 2.3B**), whereas the bulk band gap of CeO₂ is reported to be 3.15-3.20 eV.¹⁴⁻¹⁶ Semiconductor band gaps typically increase as NP size decreases below the Bohr radius (7 nm for CeO₂) due to quantum confinement.¹⁷ However, in the present 3 nm CeO₂ NPs, the presence of Ovs introduces Ce³⁺ ions with an extra electron in the 4f¹ orbital, compensating for this effect. As a result, the NP band gap is slightly smaller than the bulk value.⁷

2.2.3.2 Dynamic light scattering and zeta potential

DLS is employed to determine the hydrodynamic diameter and size distribution of the NPs (**Figure 2.4A-C**). Measurements confirm a uniform distribution with an average hydrodynamic diameter of 4.5 nm by intensity (**Figure 2.4A**), and 3.4 by number (**Figure 2.4B**), respectively. The intensity distribution reflects how strongly each nanoparticle population contributes to the total scattered light and therefore over-represents larger particles. In contrast, the number distribution is a recalculated size distribution obtained by converting the intensity data using an optical scattering model (in our case, Mie theory) and assumed values for the refractive index and particle shape. As such, it provides an estimate of the relative particle count within each size class. It is important to highlight that the average hydrodynamic diameter obtained by DLS is slightly larger than the size determined by TEM, (**Figure 2.5**), as it also includes contributions from associated surface molecules (i.e., SC) and solvation shells (water molecules and counterions). Moreover, the peaks observed above 100 nm in the intensity distribution are considered artefacts as described in Krug et al.⁹ Moreover, the sharp, single peak observed in the size distribution indicates that the NPs are highly monodisperse. This is further supported by the low polydispersity index (PDI) of 0.3. Notably, this dispersity compares favorably with many

reports in the field, where broader DLS profiles and higher PDI values are frequently observed.^{18,19}

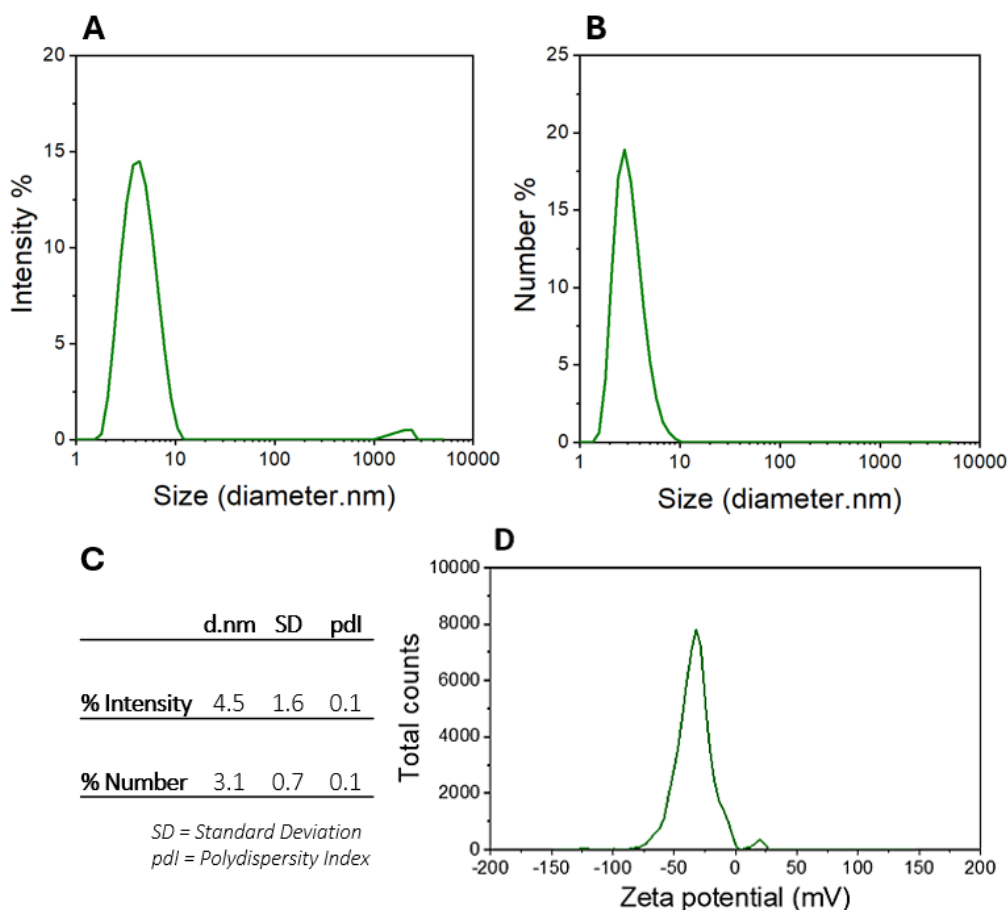


Figure 2.4. DLS spectra of scattered light by CeO₂ NPs and zeta potential. **(A).** DLS spectra of CeO₂ NPs by intensity distribution (logarithmic scale). **(B).** DLS spectra of CeO₂ NPs by number distribution, showing a narrow unimodal population at 3.4 nm. **(C)** Summarizing table of DLS CeO₂ NPs size distribution by number, volume and intensity with its standard deviation (SD) and polydispersity index. **(D)** Zeta potential distribution centered at -33 mV, indicating strongly negative surface charge and high colloidal stability.

Additionally, zeta potential analysis (**Figure 2.4D**) provides information on surface charge and colloidal stability. The NPs exhibit a zeta potential of -33.8 mV, indicating strong negative surface charge and high colloidal stability. Absolute values greater than ± 30 mV are generally associated with stable dispersions due to effective electrostatic repulsion. Stability is a critical parameter for long-term storage and biomedical applications, where uncontrolled aggregation can impair reproducibility and biocompatibility.

2.2.3.3 Transmission electron microscopy

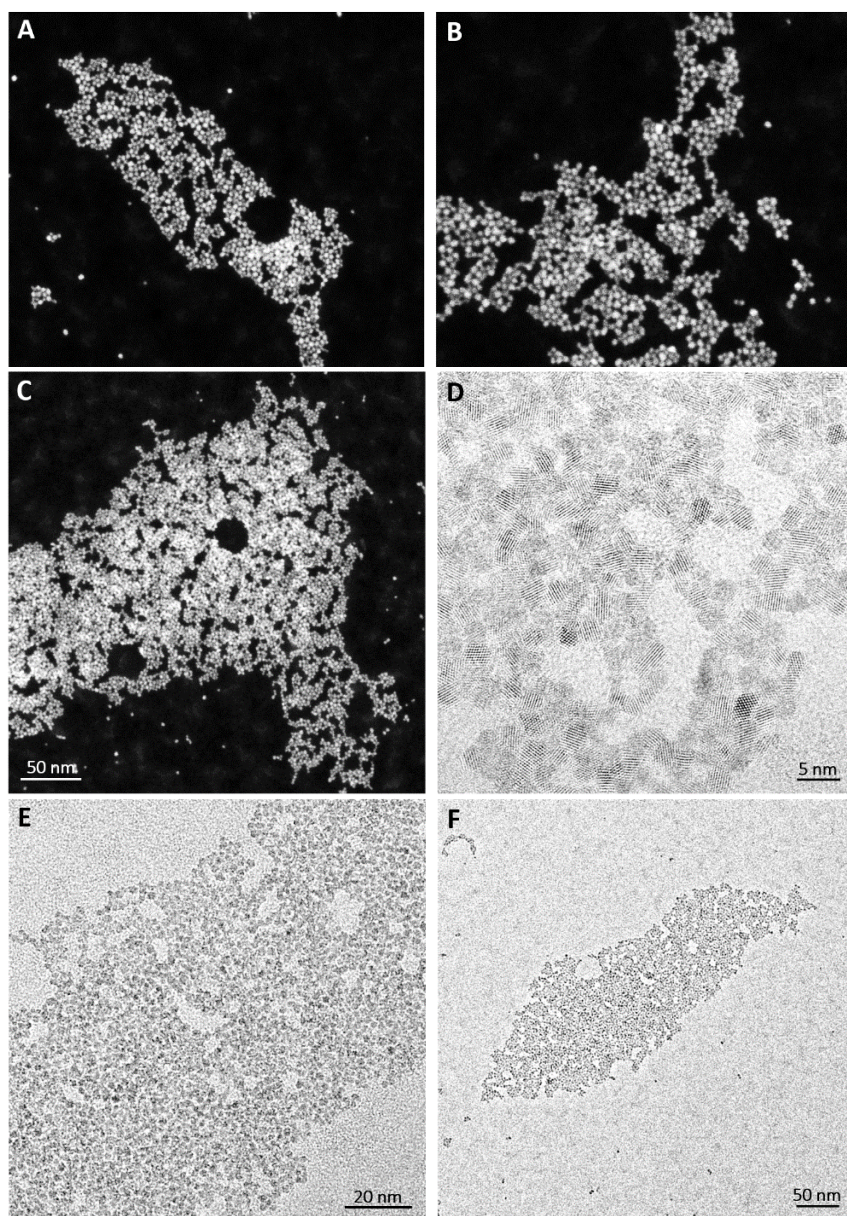


Figure 2.5. Characterization of ~ 3 nm CeO_2 NPs using a Tecnai F20 S/TEM operated in HAADF-STEM, HR-TEM, and TEM modes. (A-C) HAADF-STEM images at magnifications of 450.000 $\times$, 640.000 $\times$, and 320.000 $\times$, respectively. (D) HR-TEM image acquired at 400.000 $\times$, showing clearly resolved atomic lattice planes of the NPs. (E-F) Bright-field TEM images at magnifications of 145.000 $\times$ and 43.000 $\times$, respectively.

HAADF-STEM (Figure 2.5A-C) and TEM (Figure 2.5E-F) images are used to determine nanoparticle size and morphology, while HR-TEM provides additional insight into the crystalline nature of the nanoparticles (Figure 2.5D). These techniques reveal highly uniform, spherical NPs with an average diameter of ~2.9 nm. HR-TEM images show clearly resolved lattice fringes corresponding to atomic planes, confirming the single-crystalline nature and high crystallinity of the nanoparticles at the nanoscale (Figure 2.5D).

Image analysis of particle size distribution, represented with a histogram (Figure 2.6), yields an average diameter of ~2.9 nm with narrow standard deviation, further supporting the size and monodispersity indicated by DLS results.

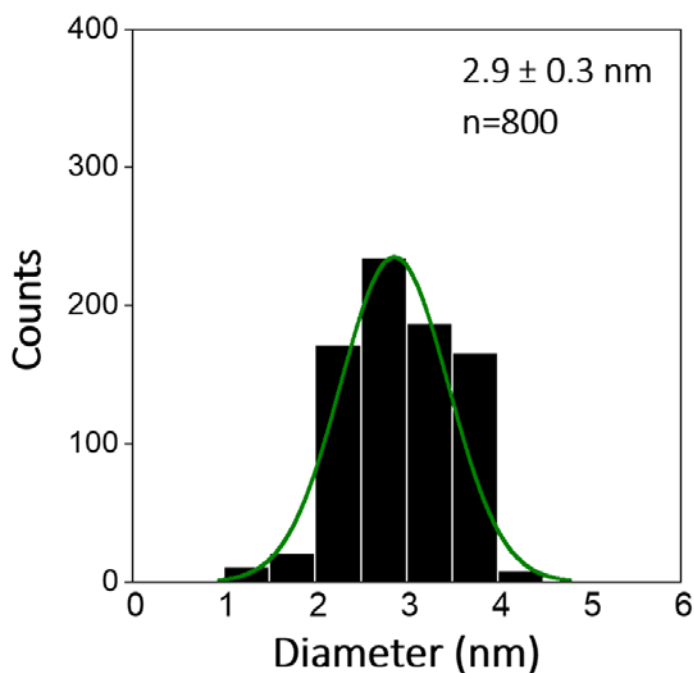


Figure 2.6. Histogram of nanoparticle diameters fitted with a Gaussian curve (green line). The average diameter is 2.9 ± 0.3 nm; $n=800$.

2.2.3.4 X-ray diffraction

The XRD pattern displays characteristic diffraction peaks at 2θ values corresponding to the crystallographic planes (111), (200), (220), (311), (222), (400), (331), (420), (422), and (511) of the standard CeO_2 crystal lattice, in agreement with the reference ICDD card No. 34-0394 (**Figure 2.7A**).^{7,20} This confirms that the NPs possess a single-phase *fcc* fluorite-type structure (space group $Fm\bar{3}m$).²¹

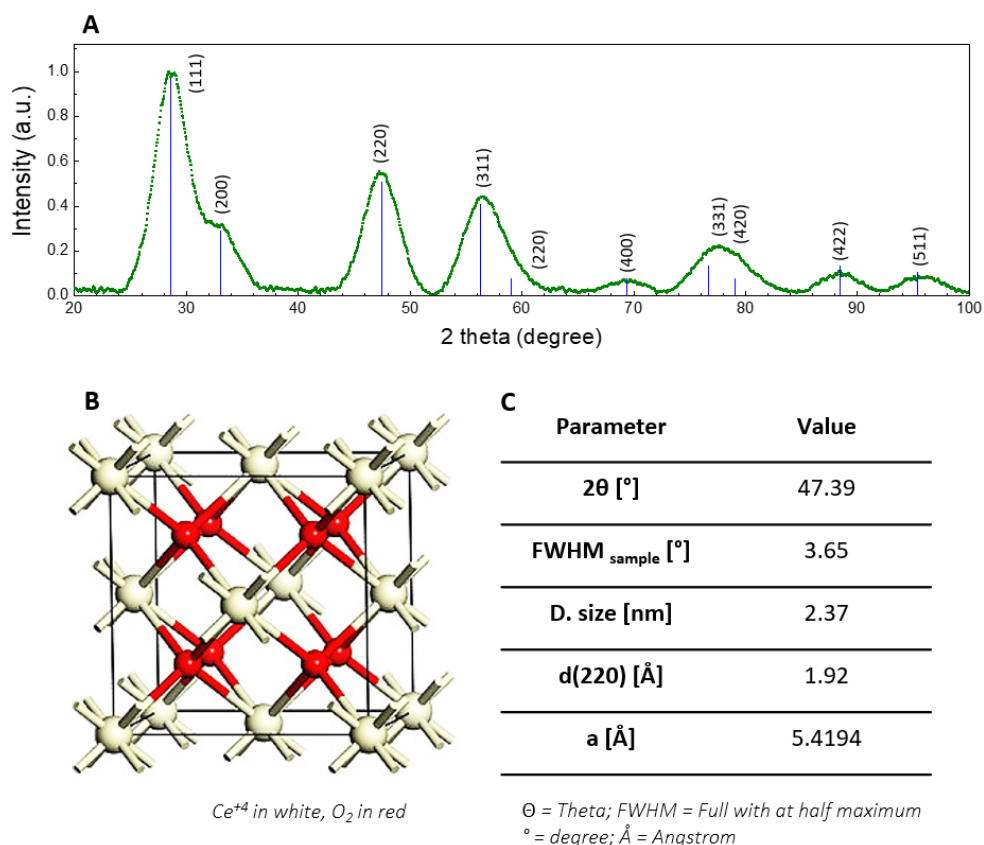


Figure 2.7. Structural characterization of CeO_2 NPs. (A) X-ray diffraction (XRD) pattern of the ~ 3 nm CeO_2 NPs (green line), matching the reference pattern of CeO_2 (ICDD no. 34-0394, blue bars). (B) Schematic representation of the cubic fluorite crystal structure of CeO_2 NPs (Ce^{4+} cations in white and O^{2-} ions in red) from ²² (C) Summary table of structural parameters calculated from the (220) crystallite plane, including 2θ of (220), full width at half maximum (FWHM), crystallite size, interplanar spacing d_{220} , and lattice parameter.

In this structure, Ce⁴⁺ ions occupy the corners and face centers of the cubic unit cell, while O²⁻ ions occupy the tetrahedral interstitial sites, resulting in each Ce⁴⁺ ion being coordinated by eight O²⁻ ions, and each O²⁻ ion by four Ce⁴⁺ ions (**Figure 2.7B**).^{21,22}

The broad diffraction peaks indicate the nanocrystalline nature of the sample. Thus, applying the Scherrer equation, in that case to the (220) peak, it yields a crystallite size of 2.37 nm (**Figure 2.7C**), in agreement with the TEM and HR-TEM observations (**Figure 2.5** and **Figure 2.6**). The lattice parameter calculated from the (220) peak is 5.4194 Å (**Figure 2.7C**), slightly larger than the canonical bulk value for CeO₂ ($\approx$ 5.411 Å, ICDD no. 34-0394). This small expansion is commonly observed in nanostructured ceria and can be attributed to a high concentration of Ovs, since the removal of oxygen ions increases the electrostatic repulsion between adjacent cerium cations, which in turn induces a slight expansion of the crystal lattice.^{2,7}

2.2.3.5 Inductively coupled plasma mass spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) is used to quantify the cerium content in the purified sample and supernatant, allowing calculation of reaction yield. The technique yields a Ce concentration of 1.39 mg/mL, corresponding to a synthesis yield of 99.6%. This translates to a CeO₂ NP concentration of 1.71 mg/mL (9.94 mM), corresponding to approximately 1.67×10^{16} NPs/mL with a total surface area of 4.73×10^{17} nm²/mL.

2.2.4 Critical steps in the 3 nm CeO₂ NPs synthesis

As mentioned above in the following sections some of the critical steps of the synthesis studied on the framework of this thesis will be discussed. Importantly, the detailed study of these steps contributed to the optimization of CeO₂ NPs synthesis protocol developed by our group.

2.2.4.1 Synthesis precursor: $\text{Ce}(\text{NO}_3)_3$ vs. CeCl_3

The UV-vis spectra show that cerium (III) nitrate ($\text{Ce}(\text{NO}_3)_3$) exhibits stronger absorption in the 240 nm region compared with CeCl_3 . This indicates that, prior to reaction, the two precursors display distinct optical profiles. However, after synthesis, the resulting CeO_2 NPs present identical properties: their UV-vis absorption spectra overlap and their DLS size distributions are very similar. These results confirm that, under our synthesis conditions, both $\text{Ce}(\text{NO}_3)_3$ and CeCl_3 are suitable precursors for producing CeO_2 NPs without significant influence on physicochemical characteristics, optical absorption, or size distribution (Figure 2.8).

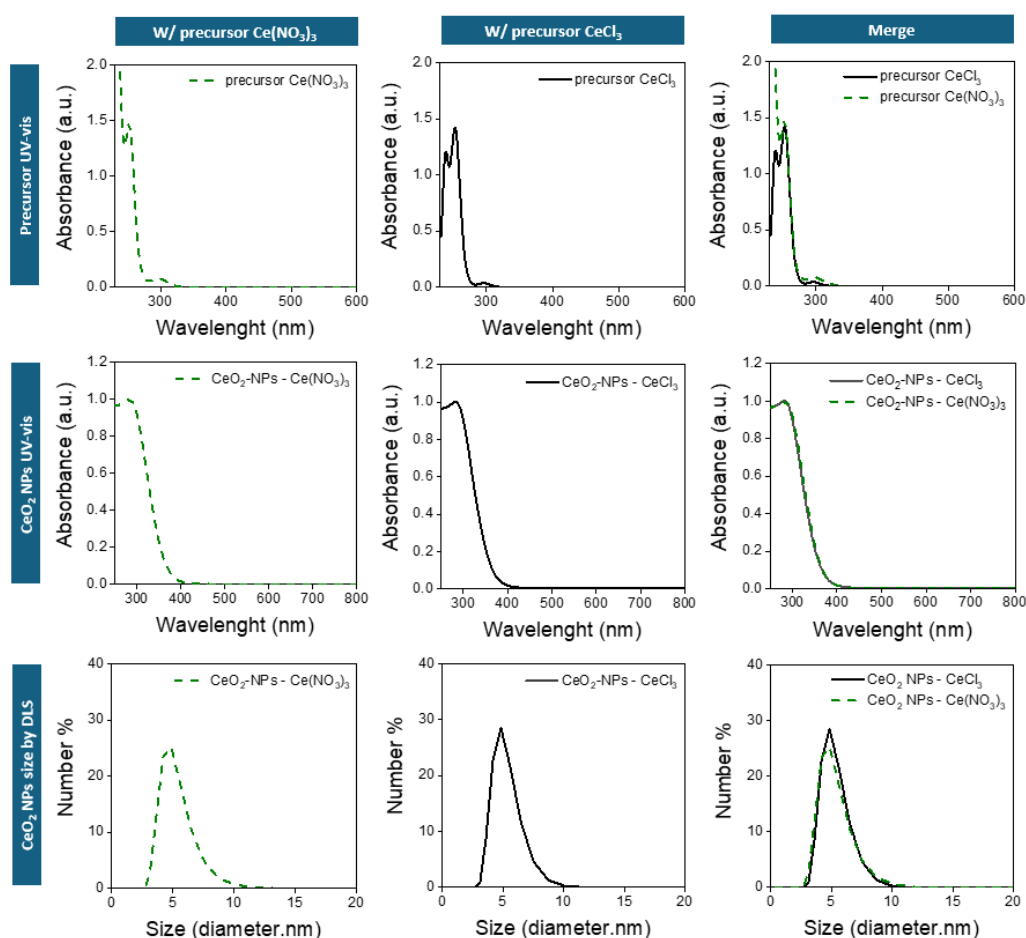


Figure 2.8. Characterization by UV-vis and DLS of CeO_2 NPs synthesized from different precursors $\text{Ce}(\text{NO}_3)_3$ and CeCl_3 .

Despite this equivalence, CeCl₃ is generally considered the more environmentally sustainable option. This is because its use avoids the release of nitrate ions potentially forming nitrogen oxides (NO_x), both of which present ecological and health risks. Nitrate ions contribute to the eutrophication of water, while NO_x gases released upon heating are toxic, irritant, and in some cases carcinogenic.²³ In addition, residual nitrate salts are more chemically reactive and oxidizing than chlorides, which makes them less safe and, under certain conditions, capable of promoting combustion.

Another possible concern in nitrate-based syntheses is the potential formation of nitrosamines, compounds characterized by the -N-N=O functional group, which are typically derived from secondary amines. These are toxic and have been linked to carcinogenic effects during long-term exposure. In the present case, however, the likelihood of their formation is minimal.²⁴ The most plausible route would involve dimethylamine (DMA), a trace byproduct of TMA-OH, reacting with nitrate species under mildly acidic conditions. Since TMA-OH provides a strongly basic environment, the reaction medium remains basic to near-neutral throughout the synthesis, preventing the formation of nitrosamines.

In contrast, chloride by-products are well-known to be less harmful and persistent than other counterions, and can be easily neutralized in aqueous media. For this reason, CeCl₃ represents a greener precursor choice for the synthesis of CeO₂ NPs, offering environmental and safety advantages while maintaining comparable NP quality and higher biocompatibility.

2.2.4.2 Sodium citrate as a stabilizing agent

(C₆H₅O₇³⁻), a polydentate ligand that contains three carboxylate groups (-COO⁻) and one -OH on the central carbon. Each carboxylate group can donate electron density to metal cations. Ce cations have a high affinity for oxygen donors, and citrate can form chelated complexes by binding through two or three donor groups, giving rise predominantly to

bidentate or tridentate interactions in most of the cases. These interactions generate Ce–O–C bonds, stabilizing complexes depending on the number of -COO^- and -OH groups involved. The role of pH is critical in this process because the protonation state of citrate determines the availability of -COO^- groups for coordination. Citrate has three pK_a values (2.9, 4.4, 5.7, **Figure 2.9**). At low pH (<2.9), citrate is largely protonated and exists mainly in the carboxyl (-COOH) form, which is unable to coordinate effectively with Ce^{3+} . As the pH increases, successive deprotonation occurs: near pH 2.9, the first -COOH group deprotonates to -COO^- ; around pH 4.4, a second deprotonates; and by pH 6.40, citrate is fully deprotonated (Cit^{3-}), providing three -COO^- groups for complexation. Moreover, the -OH group deprotonates only at very high pH ($\text{pK}_a = 13.5$), such conditions are not reached during Ce^{3+} complexation with SC. Consequently, the hydroxyl group does not participate directly in coordination; instead, it contributes indirectly by influencing the chelating architecture of SC.²⁵

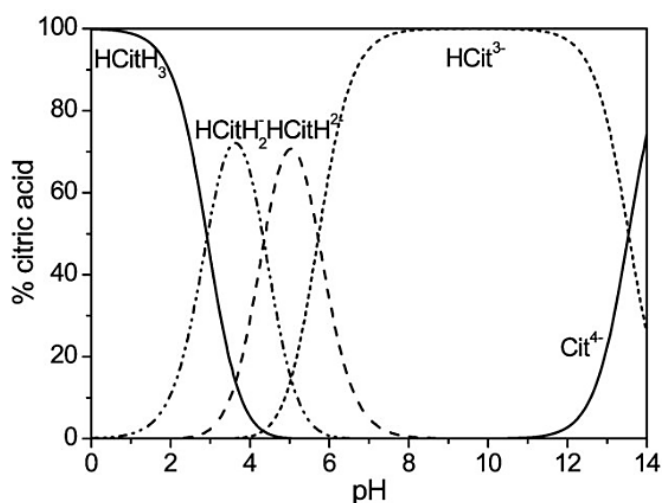


Figure 2.9. Speciation diagram of 0.1 M citric acid in aqueous solution at ionic strength $I = 0.1$ M and RT showing the distribution of H_3Cit , H_2Cit^- , HCit^{2-} , and Cit^{3-} as a function of pH. Obtained from:²⁵

In our synthesis, citrate coordination with Ce^{3+} occurs at approximately pH 5, where citrate is partially to fully deprotonated, specifically in the forms HCit^{2-} and Cit^{3-} . At this pH, Ce^{3+} remains soluble and begins to form stable Ce-citrate complexes at a 1:2 Ce: Cit ratio (pH 3-5 range) and a 1:1 ratio (pH 5-6.5 range), thus forming primarily $[\text{Ce}(\text{citrate})]$ and

[Ce(citrate)₂]³⁻ complexes. These provide (i) electrostatic stabilization and (ii) prevention of premature hydrolysis. The electrostatic stabilization provided by Cit⁻ prevents agglomeration and slows down Ce³⁺ hydrolysis. During nanoparticle growth, citrate continues to adsorb, blocking uncontrolled growth and resulting in monodisperse CeO₂ NPs. This coordination step is fundamental to achieving monodisperse and unimodal CeO₂ NPs. The behavior of sodium citrate is similar to that of other carboxylic stabilizers, such as malic acid (C₄H₆O₅), which also contains two -COO⁻ groups capable of coordinating to Ce cation.²⁶ Besides, SC not only provides colloidal stability by preventing NP aggregation but also enhances biocompatibility due to its biodegradable and non-toxic nature. These features make SC an effective stabilizer and capping agent in the synthesis of CeO₂ NPs.

2.2.4.2.1 Formation of the Ce-SC complex

UV-Vis spectroscopy was employed to evaluate the formation of the Ce-SC complex and its influence on the synthesis of CeO₂ NPs. With only 1 minute of complexation, the Ce-SC precursor is already formed and is sufficient to carry out the synthesis and obtain CeO₂ nanoparticles. The UV-Vis spectra of the Ce-SC complex show that coordination between Ce³⁺ and citrate occurs very rapidly, as the characteristic double-peak profile is already present after the first minute (**Figure 2.10A**). When the complexation time is extended to 2h, only minor changes in the spectral features are observed, which can be attributed to a slight reorganization of the coordination environment, without any noticeable impact on CeO₂ NP formation, indicating that this step is fast and robust.

After completion of the synthesis, the nanoparticles obtained under both conditions exhibit indistinguishable optical behavior. The UV-Vis spectra and Tauc analysis yield the same band gap, around 3.07 eV, in agreement with reported values for nanocrystalline CeO₂ (**Figure 2.10B**).⁷ Consistently, DLS analysis confirms nanoparticle formation in both cases, with narrow size distributions centered around 4 nm (**Figure 2.10C**), as discussed in **Section 2.2.3.2**.

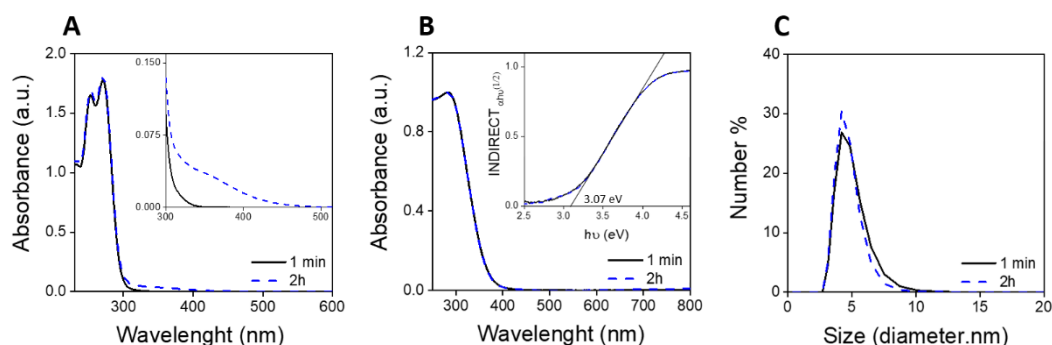


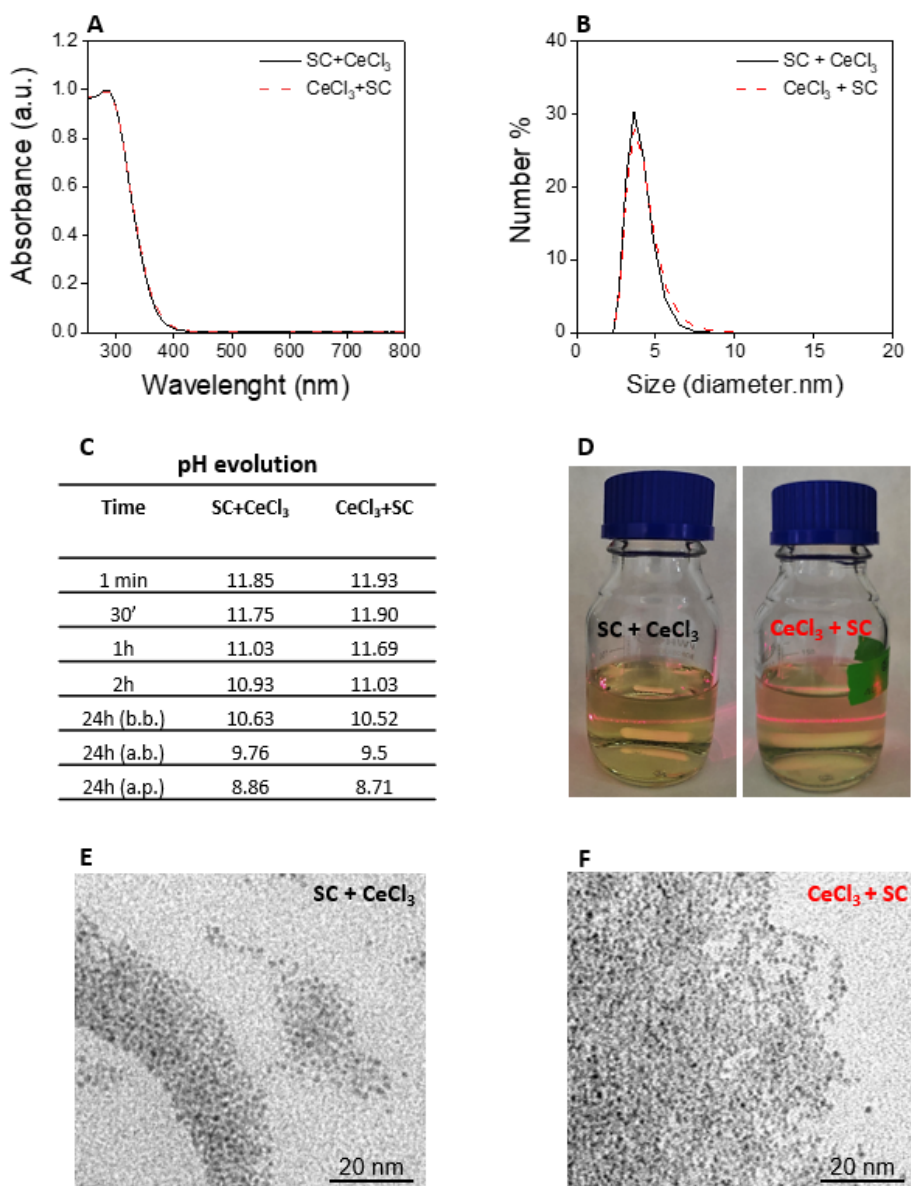
Figure 2.10. Time-dependent complexation of SC-Ce and its impact on CeO_2 NPs formation. **(A)** Ce-SC complexes at 1 min and 2h. **(B)** UV-Vis spectra and Tauc plot of synthesized CeO_2 NPs; Ce-SC complexes aged for 1 min and 2h **(C)** DLS size distribution of CeO_2 NPs synthesized with Ce-SC complexes aged for 1 min and 2h.

2.2.4.2.2 The order between SC and the Ce precursor

Another aspect addressed in this thesis is the order of reagent addition. The robustness of the process was evaluated by varying the sequence in which the reagents forming the Ce–SC complex were added. The UV-Vis spectra show that both syntheses yield overlapping curves (**Figure 2.11A**), indicating that the mixing order does not significantly affect the formation of NPs. The NP size distributions obtained by DLS further confirm this trend, as both syntheses display almost identical profiles with narrow peaks at approximately 3.5-5 nm, and reassuring the formation of monodisperse NPs for both synthetic routes (**Figure 2.11B**). Similarly, the monitoring of pH over time reveals very close values for both cases, with slight variations attributable to natural experimental bias, but without substantial differences between the syntheses results (**Figure 2.11C**).

Eventually, we show photographs of the colloidal suspensions exhibit the classical Tyndall effect in both cases (**Figure 2.11D**). The Tyndall effect is a well-known physical phenomenon observed when a beam of light passes through a colloidal dispersion and is scattered by the particles present in it. This scattering makes the light path visible, in contrast to a true solution where the solute is fully dissolved at the molecular level and no light scattering

occurs. The presence of this phenomenon confirms the formation of NPs in both suspensions, regardless of the reagent addition sequence.



b.b. = before boiling, *a.b.* = after boiling, *a.p.* = after purification

Figure 2.11. Influence of mixing order (SC + CeCl₃ vs. CeCl₃ + SC) on physicochemical properties and NPs formation. **(A)** UV-Vis spectra comparison of two syntheses: SC + CeCl₃ and CeCl₃ + SC mixtures. **(B)** pH evolution over the full synthesis time for both mixing orders of the complexes. **(C)** DLS size distribution of as-synthesized CeO₂ NPs obtained from both mixing orders. **(D)** Photographs of the two CeO₂ NP syntheses showing the Tyndall effect, **(left)** synthesized with the SC + CeCl₃ complex and **(right)** with the CeCl₃ + SC complex. **(E-F)** TEM images at 200.000× of CeO₂ NPs synthesized from both mixing orders of the complexes.

Finally, TEM images demonstrate the successful formation of CeO₂ NPs in both synthesis routes (**Figures 2.11E-F**). The morphology and distribution of the NPs show no significant differences, reinforcing the conclusion that the mixing order of SC and CeCl₃ does not critically influence the physicochemical properties of the NPs.

2.2.4.2.3 Effect of the SC in the NPs colloidal stability

The role of SC as a stabilizing agent in the colloidal stability of CeO₂ NPs has been assessed by comparing the synthesis. As shown in the UV-Vis absorbance spectra (**Figure 2.12A**), the sample stabilized with SC displays a sharp absorption peak at 283 nm, a feature typically associated with small and well-dispersed CeO₂ NPs. This spectral behavior contrasts strongly with that of CeO₂ NPs synthesized without SC, where a noticeable redshift and broadening of the absorbance curve indicate the presence of NPs aggregates, leading to increased light-scattering effects. The third condition, in which SC was added four hours after the synthesis began, results in an intermediate spectrum. While the spectral edge is shifted relative to the SC-stabilized sample, it shows slightly improved monodispersity compared to the TMA-OH-only system, suggesting partial re-dispersion of aggregated particles upon SC addition.

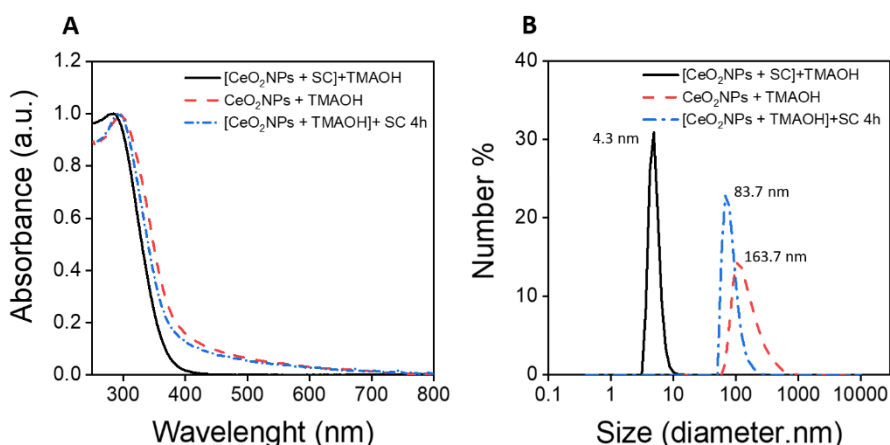


Figure 2.12. SC mediates stabilization of CeO₂ NPs. (A) UV-Vis absorbance spectra of CeO₂ NPs synthesized with SC, without SC, and with delayed SC addition (4h). **(B)** DLS size distribution profiles showing a narrow population at 4.3 nm for SC-stabilized CeO₂ NPs, a broad distribution centered at 163.7 nm for the TMA-OH-only system, and an intermediate distribution around 83.7 nm when SC is added after 4 h.

Complementary to the absorbance measurements, the DLS results (**Figure 2.12B**) further confirm the role of SC as a stabilizing agent on CeO₂ NP size distribution. The SC-coated sample is characterized by an extremely narrow size distribution with a hydrodynamic diameter around 4.3 nm, consistent with monodisperse NPs. In comparison, CeO₂ NPs synthesized only in TMA-OH exhibit a much broader distribution, with a dominant population centered at 163.7 nm, reflecting extensive aggregation and the formation of larger colloidal clusters. Furthermore, the delayed addition of SC reduces the average particle size to approximately 83.7 nm, suggesting that SC can partially break down or prevent further growth of aggregates, but cannot fully reverse the NPs aggregation. These observations demonstrate that SC plays a critical role in maintaining the stability, small size, and monodispersity of CeO₂ NPs, likely by providing electrostatic and steric stabilization that counteracts the aggregation tendency of the NPs synthesized only with TMA-OH.

Controlling NPs stability and size is particularly important for biomedical applications, where robust and reproducible formulations are required. Well-dispersed small NPs can circulate in the bloodstream, cross biological barriers, and accumulate at target tissues, whereas aggregation not only diminishes surface activity and cellular interactions but also increases the risk of embolism, mechanical stress, and undesired immune responses. Thus, the stabilizing and coating effect of SC is essential to ensure the safe and effective use of CeO₂ NPs in biomedical contexts²⁷.

2.2.4.3 The role of oxygen in CeO₂ NPs formation

Molecular oxygen plays a key role in promoting the oxidation of Ce³⁺ in Ce(OH)₃ to Ce⁴⁺, resulting in the formation of Ce(OH)₄. Indeed, in the absence of atmospheric oxygen to refill the dissolved oxygen employed to the oxidation of Ce³⁺, NPs are not properly formed (see **Figure 2.13**). The oxidation process involves the transient formation of a hydroxo complex, Ce(OH)₄. Since Ce⁴⁺ species are insoluble in water, they precipitate under these conditions as CeO₂ NPs, and this transformation proceeds through dehydration and structural atomic rearrangement. More in detail, after initial formation of different

hydroxides, Ce ions mineralize in the form of $\text{Ce}(\text{OH})_3$ hydroxide which then follows oxidation and dehydration. The chemical pathway for the precipitated species can be summarized as:

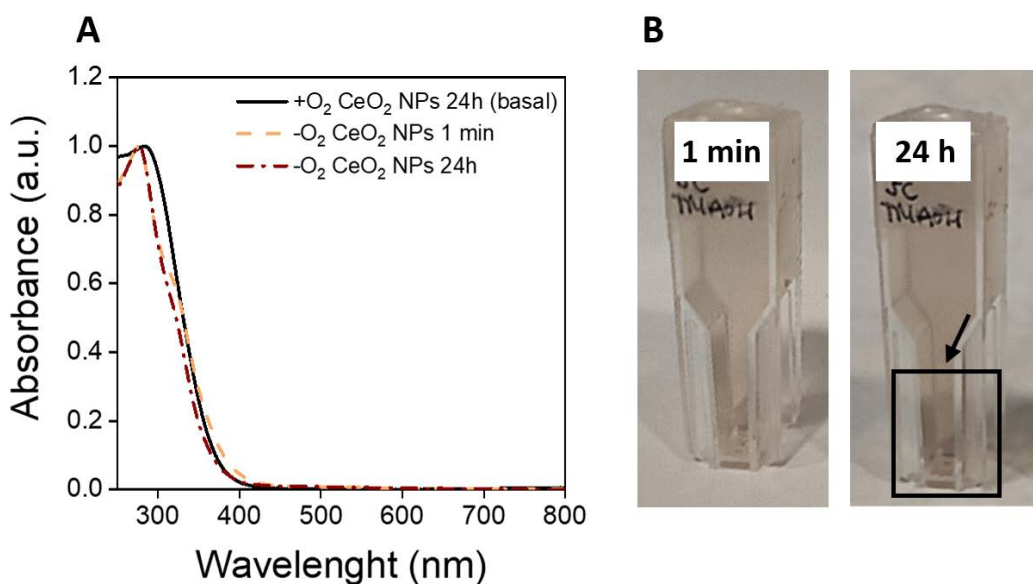
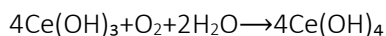


Figure 2.13. Oxygen as a key factor in CeO_2 NPs formation. (A) UV-Vis absorbance spectra of CeO_2 NPs synthesized under atmospheric oxygen conditions (+O₂) and of synthesis precursors incubated under oxygen depletion (-O₂) for 1 min and 24 h. (B) Images of precursor suspensions incubated under -O₂ conditions for 1 min and 24 h, with visible aggregates observed after 24 h.

Accordingly, when analyzed by UV-vis a sample exposed to O₂, the suspension exhibits the typical asymmetric peak of CeO_2 NPs at 283 nm (Figure 2.13A). In contrast, when the synthesis was performed in a cuvette without headspace (under oxygen-depleted conditions) this transformation was compromised, leading to a UV-vis spectra inconsistent with the formation of CeO_2 NPs. Further, by simple visual inspection one could see big aggregates at 24h (Figure 2.13B).

As shown in **Figure 2.13**, oxygen depletion compromises nanoparticle transformation, leading to particles instability, aggregation, and eventual precipitation, as observed experimentally. These results highlight oxygen as one of the key factors governing the formation of CeO₂ NPs.

2.2.4.4 Enhancing the stability of CeO₂ NPs via thermal treatment

As described in **Section 2.2.1**, the final step of the synthesis involves boiling the CeO₂ NPs at 100 °C for up to 4h. In the context of nanomaterials, this step can be regarded as a mild thermal treatment, consisting of heating the material at a defined temperature for a fixed period in order to modulate its structural and physicochemical properties. Here, the impact of this treatment on the optical and structural characteristics of CeO₂ NPs was evaluated by comparing samples kept at room temperature (RT) with those subjected to boiling for 2h and 4h (**Figure 2.14**). As shown in **Figure 2.14A**, all samples display the characteristic asymmetric UV-Vis absorption band of CeO₂ NPs centered at approximately 283 nm, indicating that the overall electronic structure of ceria is preserved regardless of the thermal treatment. However, inspection of the absorption tail in the 350-450 nm region reveals a blue shift for the 2h- and 4h-boiled samples compared to the RT sample. These spectral changes are consistent with modifications in the Ce³⁺/Ce⁴⁺ balance and, consequently, in the concentration of Ovs. In particular, the results suggest a progressive oxidation of residual Ce³⁺ to Ce⁴⁺ during boiling, in agreement with the oxidation mechanism discussed in **Section 2.2.2.3**. Overall, boiling at 100 °C appears to fine-tune the electronic defect landscape of CeO₂ NPs while preserving their colloidal integrity. This is further supported by the absence of light scattering above 400 nm and by the negligible variations in pH, which remains close to ~9 for all samples (**Figure 2.14B**).

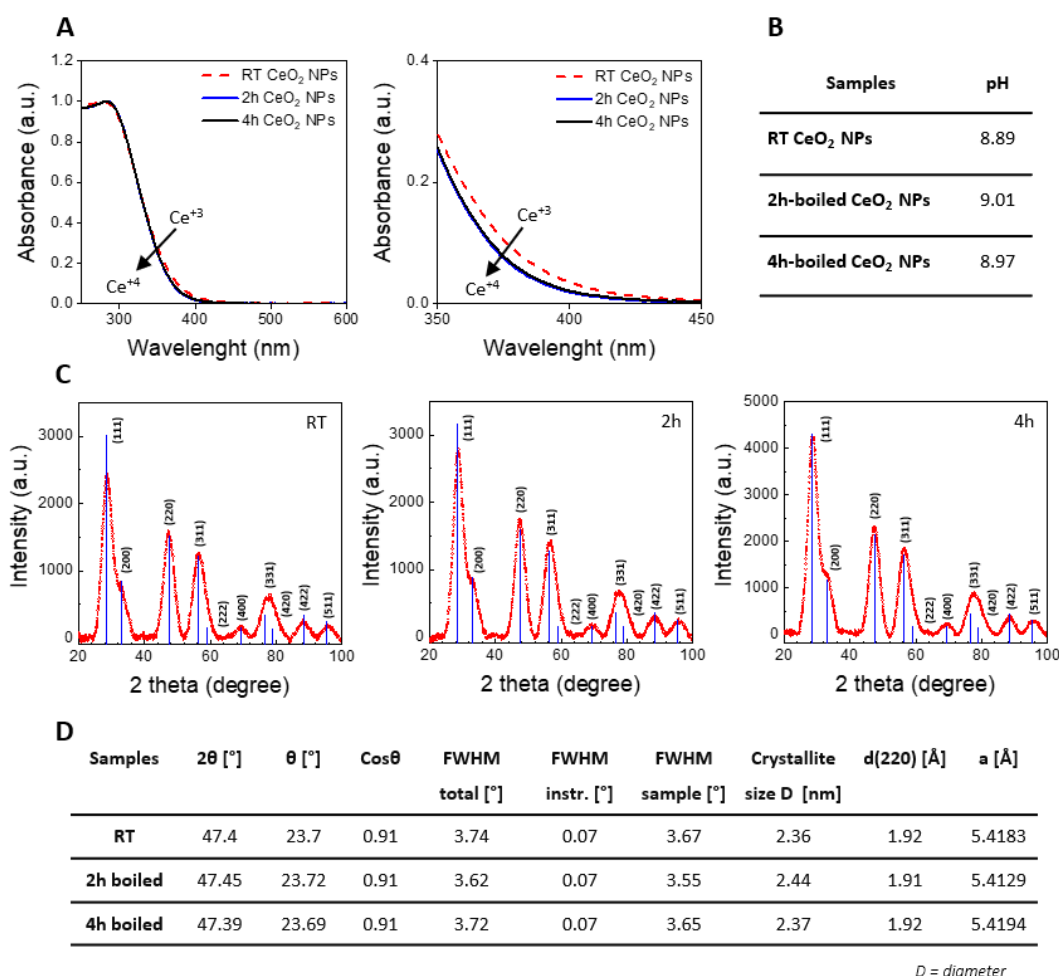


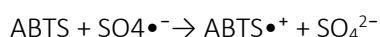
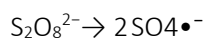
Figure 2.14. Characterization RT, 2h and 4h boiled CeO₂ NPs. (A) UV-Vis absorption. (B) Measured pH values. (C) XRD patterns (red lines) matching the reference pattern of CeO₂ (ICDD no. 34-0394, blue bars). (D) XRD-derived structural parameters (2 θ , θ , FWHM, crystallite size, d-spacing, and lattice parameter).

Furthermore, the XRD patterns confirm that all samples adopt a single-phase cubic fluorite structure (space group $Fm\bar{3}m$), in agreement with the CeO₂ reference pattern (ICDD no. 34-0394), represented by the blue bars in **Figure 2.14C**. Closer inspection of the diffraction profiles reveals deviations from the reference in the relative intensity of the (111) reflection. The RT and 2h-boiled samples exhibit a reduced intensity of this peak, with the effect being more pronounced in the RT sample. A decrease in peak intensity, without significant peak broadening, is consistent with the presence of oxygen-related defects, including Ovs or local lattice distortions in CeO₂, although experimental factors such as

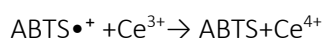
sample packing, amount of material, or preferred orientation may also contribute to intensity variations. When considered together with the UV-Vis results, which indicate changes in defect-related electronic states, these observations are consistent with a relatively increased concentration of Ovs in the non-boiled samples. This interpretation is supported by XPS analysis performed in our group on the RT and 4 h-boiled samples, which reveals a decrease in the Ce³⁺ fraction from ~40% in the RT sample to ~30% after 4h of boiling. Since the presence of Ce³⁺ is commonly associated with Ovs formation for charge compensation. By suppressing Ovs and with them the associated surface redox activity, boiling stabilizes the NPs, moderates their catalytic reactivity but yields a more stable product. In contrast, it has been observed (data not shown) that samples prepared without boiling tend to keep evolving, undergoing progressive mild oxidation and optical signatures drift, which prevents the tight control that is indispensable for reproducible biomedical applications.

2.2.2.5 Antioxidant activity of CeO₂ NPs measured by ABTS decolorization assay

The antioxidant capacity of CeO₂ NPs was further quantified using the ABTS decolorization assay, a single-electron transfer method to measure antioxidant capacity of a substance. The ABTS radical cation (ABTS•⁺) is generated by oxidizing ABTS with potassium persulfate (K₂S₂O₈) over night in the dark. During this step, colorless/pale ABTS is converted into the characteristic dark-green ABTS•⁺ solution according to:



Subsequently, upon addition of CeO₂ NPs, the particles act as heterogeneous electron donors via the Ce³⁺/Ce⁴⁺ redox couple at the NP surface and the associated Ovs.



The reaction probed in the following experiments is the reduction of $\text{ABTS}^{\bullet+}$ by surface Ce^{3+} , which produces decolorization, turning the solution from dark green toward transparent and a concomitant decrease in absorbance. This process is monitored by UV-Vis spectrophotometry at 734 nm (Figure 2.15).

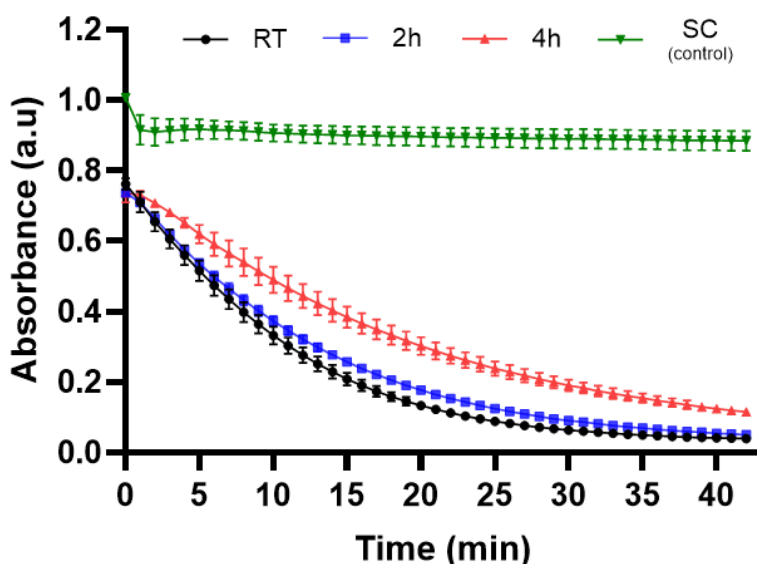


Figure 2.15. ABTS decolorization kinetics at 734 nm for CeO_2 NPs measured at RT (black ●), after 2 h boiling (blue ■), after 4 h boiling (red ▲), and for the SC, vehicle of the NPs and control (green ▼).

In the graph, RT and 2 h boiled CeO_2 NPs samples rapidly decolorize $\text{ABTS}^{\bullet+}$, while the 4 h boiled CeO_2 NPs sample shows a modestly slower decay of absorbance. As discussed previously in **Section 2.2.2.4**, it evidences a slight loss of catalytic activity that is consistent with a small reduction in Ovs content and thus, in surface Ce^{3+} . However, also as mentioned previously, despite the higher capacity of non-boiled samples, the boiling treatment stabilizes the NPs' surface chemistry and prevents further time-dependent evolution, which is crucial for reliable biomedical use. Note that the SC sample shows essentially no change over time, indicating no detectable antioxidant activity under these conditions. Thus, the antioxidant effect depends totally on the CeO_2 NPs.

2.2.2.6 Insights into the redox mechanism of CeO₂ NPs

CeO₂ NPs has emerged as a unique nanomaterial with exceptional antioxidant properties. As mentioned, CeO₂ NPs can cycle between the Ce³⁺ and Ce⁴⁺ oxidation states, allowing them to repeatedly scavenge ROS. This redox-switching ability, coupled with the presence of Ovs on their surface, enables ceria to mimic the activity of key antioxidant enzymes such as SOD, CAT, GPx and Prx. As a result, CeO₂ NPs exhibits significant potential in mitigating oxidative stress, a process closely linked to inflammation, endothelial damage, aging, cardiovascular diseases, cancer and neurodegenerative disorders.⁵

2.2.2.6.1 H₂O₂ decomposition and catalase-like redox cycling of CeO₂ NPs

The redox cycling between Ce³⁺ and Ce⁴⁺ surface states enable CeO₂ NPs to fully mimic CAT-like activity. In this process, both oxidation states of cerium participate in the decomposition of H₂O₂ into H₂O and O₂, analogously to the heme-centered catalytic mechanism of natural catalase (**Figure 2.16**).⁴

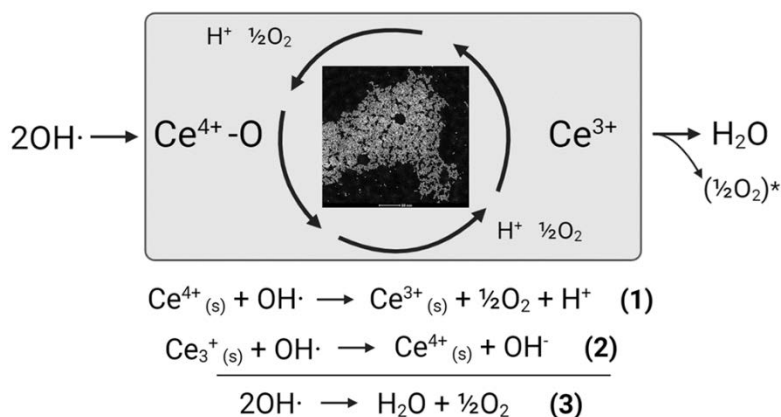
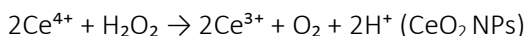


Figure 2.16. CAT-like activity of CeO₂ NPs in the presence of H₂O₂. Adapted from:¹

In the Ce⁴⁺-driven step, Ce⁴⁺ acts as an oxidizing agent and accepts electrons from another H₂O₂ molecules becoming reduced Ce³⁺, with more Ovs than before. This step is similar to the second half of the catalase reaction, in which Fe⁴⁺=O is reduced back to Fe³⁺ by a second H₂O₂ molecule, releasing molecular O₂:^{1,6}



Conversely, in the Ce^{3+} -driven step, Ce^{3+} acts as a reducing agent donating electrons to H_2O_2 , reducing it to water while being oxidized back to Ce^{4+} . This reaction parallels the first half of the CAT catalytic cycle, where the heme- Fe^{3+} center is reduced by H_2O_2 to form the intermediate $\text{Fe}^{4+}=\text{O}$, with concurrent production of water:^{1,6}:



Additionally, other endogenous enzymes capable of decomposing H_2O_2 through a similar redox mechanism to Ce^{4+} are GPx and Prx. The biggest difference is that these enzymes, however, require co-factors, GSH for GPx and Trx for Prx. Moreover, it is important to mention that CeO_2 NPs can also exhibit SOD-like activity under biological conditions, although this is not the case in this experiment. In the SOD-like mechanism, both Ce^{3+} and Ce^{4+} catalyze the dismutation of $\text{O}_2\bullet^-$.²⁸

CeO_2 NPs were exposed to H_2O_2 at a 1:1 molar ratio (10 mM CeO_2 NPs and 10 mM H_2O_2), and the redox cycling was monitored over time by UV-Vis spectroscopy (**Figure 2.17A**). The resulting spectral changes were analyzed, noting that they partially overlap with bands from intermediate Ce-peroxo complexes formed upon H_2O_2 addition (non-deconvoluted spectra are shown here for simplicity). Immediately after H_2O_2 addition, an increase in the low-energy absorption band ($\approx 350\text{-}500$ nm) was observed. This feature is consistent with an increased population of $4f^1$ states associated with Ce^{3+} . In parallel, H_2O_2 decomposed into H_2O and O_2 , and oxygen bubble formation was observed (**Figure 2.17B**), consistent with the Ce^{4+} -driven step of the CAT-like mechanism described previously.

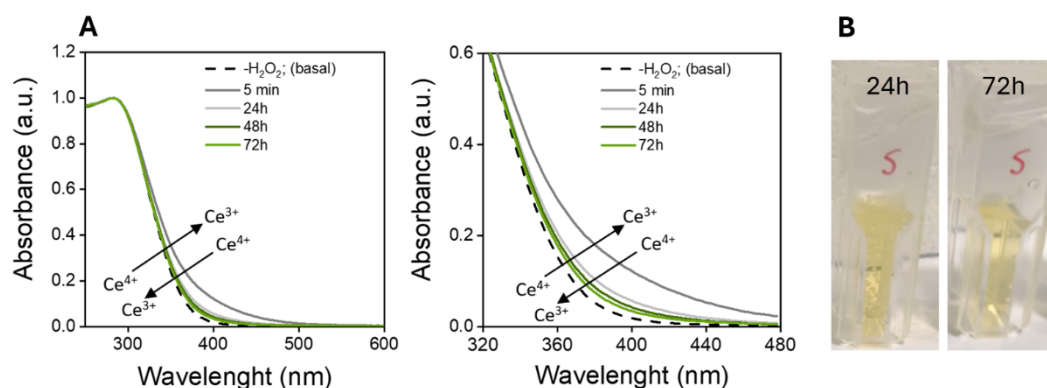


Figure 2.17. Redox cycling behavior of CeO₂ NPs in the presence of H₂O₂ (1:1 molar ratio). (A) (left) UV-Vis absorption spectra of CeO₂ NPs recorded at different times (basal-72h) exposure to a 1:1 molar ratio of H₂O₂. (right) Zoom image of the red and blue shift. (B) Optical appearance of CeO₂ NP suspensions at 24 h and 72h, showing bubbles generated by the O₂ at 24h and no bubbles at 72h.

As the reaction proceeds, the low-energy absorption band (≈ 350 -500 nm) gradually disappears, indicating the re-oxidation of Ce³⁺ to Ce⁴⁺. After approximately 72h, the UV-Vis spectrum nearly returns to the baseline of the untreated sample, suggesting that the reaction has reached completion. This final stage corresponds to the Ce³⁺-driven step, in which Ce³⁺ reduces H₂O₂ to H₂O. Overall, this cyclic spectral transition between Ce³⁺ and Ce⁴⁺ absorbance bands confirm the bidirectional redox mechanism of CeO₂ NPs, closely analogous to the two-step catalytic cycle of natural catalase.

Additionally, the influence of a mild thermal treatment at 100 °C on the optical properties of CeO₂ NPs exposed to H₂O₂ was examined by comparing samples synthesized at RT and after boiling for 2 h and 4 h. The evolution of the optical band gap with boiling time was analyzed using Tauc plots for indirect transitions (**Figure 2.18**). Consider that, as mentioned previously, the band partially overlap with the intermediate Ce-peroxo complexes formed. The apparent indirect band gap energy of the RT and 2 h-boiled samples was slightly lower, 2.99 eV and 2.93 eV, respectively, compared to the 3.08 eV obtained for the 4 h-boiled sample. A similar trend was observed for the samples treated with a 1:1 molar ratio of H₂O₂, where the apparent band gap initially narrowed after 5 minutes of exposure under all conditions and then gradually recovered up to 72h. Interestingly, the RT and 2 h-boiled

samples at 72h displayed higher apparent band gap values than their initial counterparts, suggesting that the H_2O_2 treatment induces a comparable effect on CeO_2 NPs to that of the 100 °C thermal treatment.

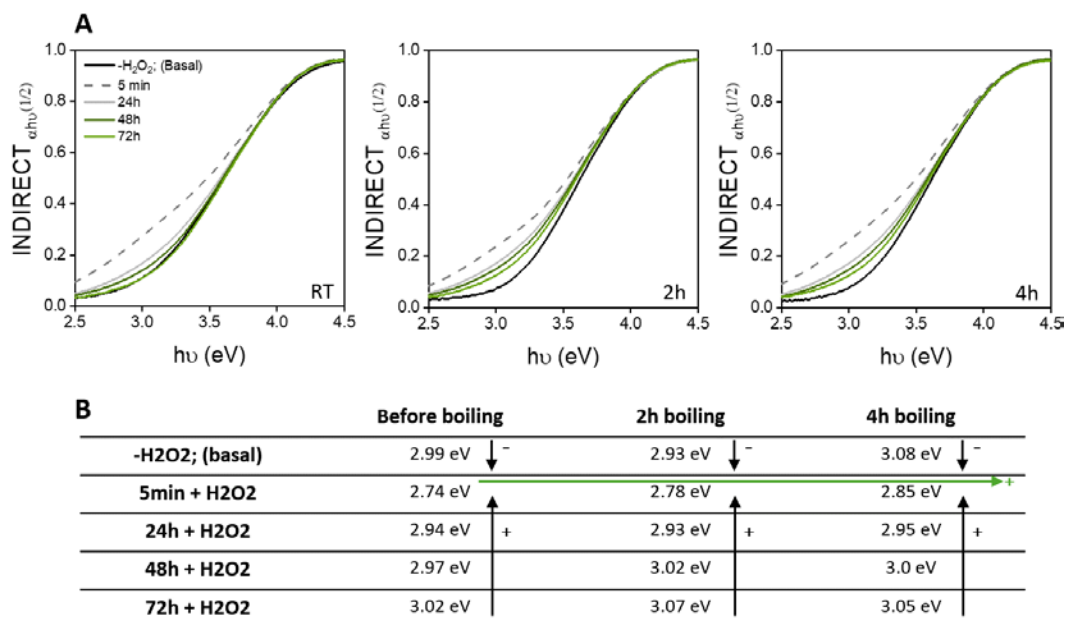


Figure 2.18. Influence of H_2O_2 exposure and thermal treatment on the optical band gap of CeO_2 NPs synthesized at RT and after boiling for 2 h and 4 h. (A) Indirect transitions Tauc plots of CeO_2 NPs synthesized at RT and after boiling for 2 h and 4 h, showing band gap variations with different H_2O_2 treatment times. (B) Extracted indirect band gap values illustrating that both thermal treatment and prolonged H_2O_2 exposure promote Ce^{3+} oxidation and oxygen vacancy healing, leading to band gap widening.

Summarizing, this behavior can be interpreted in terms of the dynamic balance between $\text{Ce}^{3+}/\text{Ce}^{4+}$ species and the associated Ovs. The initial narrowing of the band gap upon short H_2O_2 exposure is attributed to an increase in Ovs concentration, which introduces localized defect levels near the conduction band and facilitates sub-band-gap transitions. From a catalytic perspective, during the initial stage, a lower band gap is associated with a higher concentration of Ovs and, consequently, with an increased presence of Ce^{3+} . This process is typically characterized by the visible formation of O_2 bubbles, indicating the decomposition of H_2O_2 . This observation suggests that Ce^{4+} acts as an oxidizing agent, accepting electrons from H_2O_2 molecules and being reduced to Ce^{3+} . Subsequently, as the

band gap increases again, Ce³⁺ acts as a reducing agent, donating electrons to H₂O₂ and converting it into H₂O while being re-oxidized to Ce⁴⁺. This redox cycling between Ce³⁺ and Ce⁴⁺, as discussed previously, progressively restores the stoichiometry of the CeO₂ NPs and reduces the concentration of Ovs.⁷

Overall, these results demonstrate that mild thermal treatment at 100 °C promotes a reduction of Ovs in CeO₂ NPs, in favor of stability, favoring a more stable oxidation state that is desirable for biological systems, as also discussed in **Section 2.2.2.4**. The observed band gap evolution provides clear evidence that the optical response of CeO₂ is highly sensitive to its Ce³⁺/Ce⁴⁺ chemistry. Furthermore, UV-visible spectroscopy proves to be a valuable technique for monitoring these subtle changes, offering important insights into the relationship between oxidation state dynamics, Ovs concentration, and the resulting optical properties of CeO₂ NPs.

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3.1 Introduction

3.1.1. *In vitro* modeling of PE by exposing HTR-8/SVneo trophoblasts to 0.5% oxygen

To provide context, during early placental development trophoblasts differentiate into distinct subtypes with specialized functions. Cytotrophoblasts (CT_s) form a proliferative progenitor pool that can fuse to generate the syncytiotrophoblast (STB), which primarily mediates maternal-fetal exchange and endocrine activity. In parallel, a subset of CT_s differentiates into EVT_s, a highly migratory and invasive phenotype that leaves the villous compartment to invade the maternal decidua and myometrium and to remodel the uterine spiral arteries. Because impaired EVT invasion and deficient spiral artery remodeling are central features of placental disorders such as PE, particularly early-onset PE, which is associated with hypoxia, oxidative stress, and inflammation, EVT-based models are especially well suited to investigate mechanisms of placental dysfunction.^{1–3} By contrast, CT-derived choriocarcinoma cell lines such as BeWo are widely used to study trophoblast proliferation, differentiation, and syncytialization, but they do not fully recapitulate the invasive and vascular remodeling behaviors that are characteristic of EVT_s.^{4,5}

Consistent with this rationale, we used HTR-8/SVneo cells (**Figure 3.1**), an EVT-like line derived from primary first-trimester EVT_s that retains many intrinsic properties of early invasive trophoblasts, including anchorage-dependent growth and robust migratory and invasive capacity.^{4,6} HTR-8/SVneo cells can also upregulate key markers implicated in the pathophysiology of preeclampsia. Under inflammatory and oxidative stress conditions, both relevant to preeclampsia, they upregulate pro-inflammatory mediators such as interleukin (IL)-6, IL-1 β , and the CCL2.^{7–9} Under hypoxia and metabolic stress, glucose transporter 1 (GLUT1) can be upregulated, consistent with metabolic adaptations reported in preeclampsia-associated placental dysfunction.^{10,11} In the context of trophoblast invasion and vascular remodeling, these cells also upregulate MMP-2 and MMP-9, while the angiogenic imbalance characteristic of preeclampsia is reflected by altered expression

of VEGF-A and PGF.^{5,12,13} These responses are coordinated by HIF-1 α , a central regulator of cellular adaptation to low-oxygen environments, which has been reported to be robustly inducible in HTR-8/SVneo cells compared with other trophoblast cell models under hypoxic stimulation.^{5,13–15} Together, these characteristics make HTR-8/SVneo cells more physiologically representative and translationally relevant than alternative choriocarcinoma-derived trophoblast models (e.g., BeWo, JEG-3, JAR) for studying cellular processes central to preeclampsia pathogenesis, including impaired trophoblast invasion and vascular remodeling, as well as stress-response signaling.^{4–6} They are also well suited for experiments under controlled oxygen tension, enabling robust comparisons between hypoxic and normoxic conditions.^{5,10} From a practical standpoint, the line is easy to maintain, exhibits stable and reproducible growth under standard serum culture conditions, and avoids the donor-to-donor variability associated with primary trophoblast cultures, advantages that make it a suitable model for exploratory mechanistic studies.^{4,8}

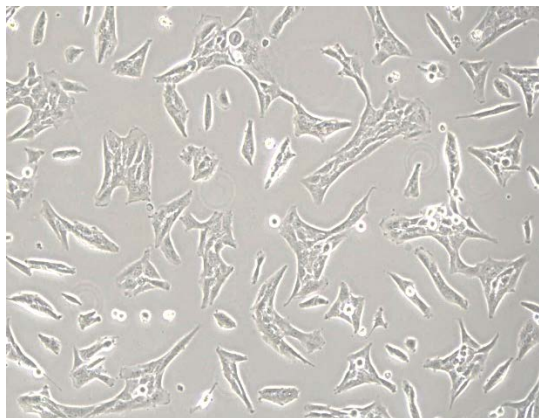


Figure 3.1. Image of HTR-8/SVneo trophoblasts 400 $\times$ take at SCAC-UAB microscope.

To generate hypoxia condition in our experiments, HTR-8/SVneo trophoblasts were cultured in the InvivoO₂[®] 300 hypoxia workstation with the oxygen concentration set to 0.5% for 48h, a condition considered to represent severe hypoxia and widely used as an *in vitro* model relevant to PE.^{5,10} This experimental approach was selected after evaluating alternative models, including hypoxia-reoxygenation, lipopolysaccharide (LPS) stimulation,

and cobalt (II) chloride (CoCl₂) treatment.¹⁰ Our results indicate that this 48-hour sustained hypoxia model elicits more pronounced pathological alterations than hypoxia-reoxygenation paradigms or chemical hypoxia induced by CoCl₂, or LPS, making it more suitable for mimicking PE *in vitro*. (see **Annex** for details).^{10,16–18} Although the use of a hypoxia chamber may complicate handling compared with direct treatment using pro-inflammatory and/or pro-oxidant agents such as LPS or H₂O₂, the model remains highly reproducible and offers an excellent balance between experimental control and physiological relevance.^{8,10} Overall, this approach better reflects the *in vivo* environment observed in PE, more faithfully reproducing the chronic hypoxia and trophoblastic dysfunction characteristic of the disease.^{1,2,5} Thus, it constitutes a robust and physiologically relevant tool for studying the molecular mechanisms underlying PE.

3.1.2 Hypoxia-driven metabolic and signaling reprogramming in trophoblasts

Under conditions of aberrant hypoxia (around 0.5% O₂), as in PE, oxygen availability itself becomes the key regulator of trophoblast metabolism and behavior.^{19,20} At the mitochondrial level, oxygen serves as the final electron acceptor of the electron transport chain, so its scarcity forces mitochondria to shift away from OXPHOS toward metabolic pathways that do not rely on electron transfer to oxygen to support ATP production, namely anaerobic glycolysis, thereby limiting excessive ROS generation that would otherwise arise from electron leakage along the ETC in the absence of an adequate terminal acceptor.^{19,21} However, the ROS that is still produced under these conditions also acts as a signaling molecule, functioning as a secondary messenger that can modulate HIF-1 α stability and downstream target gene expression, thus becoming an additional regulator of trophoblast adaptation to hypoxia. In parallel, oxygen directly regulates HIF-1 α stability, such that low oxygen tensions favor HIF-1 α accumulation and activation of its transcriptional program.^{21,22}

HIF-1 α further reinforces this metabolic reprogramming by binding hypoxia-responsive elements in target genes such as GLUT-1, promoting its overexpression and thus increasing

glucose uptake to sustain ATP production through enhanced anaerobic glycolysis and lactate generation.^{23,24} Under physiological, time-limited hypoxia this response is largely adaptive, but persistent activation of HIF-1 α in an abnormally oxygenated environment, as in established preeclampsia, triggers a maladaptive transcriptional response in extravillous trophoblasts.^{23,25} In this context, trophoblasts maintain hypoxia-dependent pro-inflammatory signaling that initially increases their invasive capacity, but in a disorganized and unsustained manner that ultimately compromises proper spiral artery remodeling.^{25,26} Beyond metabolism, HIF-1 α acts as a key regulatory and immunomodulatory factor in trophoblasts by driving overexpression of cytokines such as macrophage migration inhibitory factor (MIF), IL-1 β , IL-6, and the chemokine CCL-2, thereby creating an active immuno-inflammatory microenvironment at the maternal-fetal interface and stimulating a more aggressive early trophoblast invasion.^{27,28} When hypoxia becomes prolonged or intermittent, as in preeclampsia, trophoblast invasion remains heightened but disorganized, accompanied by increased activity of matrix metalloproteinases MMP-2 and MMP-9 that further disturb controlled matrix remodeling.^{19,25} In parallel, the angiogenic balance is disrupted, with dysfunctional activation and signaling of VEGF-A and PlGF driving aberrant and poorly organized placental angiogenesis, which contributes to the systemic endothelial dysfunction characteristic of the disease.^{26,28}

3.1.2.1 HIF-1 α mechanism in normoxia

To understand how the HIF-1 α mechanism functions, we first describe its operation under normoxic conditions, approximately 21% O₂ for humans and ~18% for standard cell culture (see **Figure 3.2**).^{29,30} Importantly, HIF-1 α is constitutively transcribed and continuously translated, so its cellular levels are primarily controlled post-translationally through oxygen-dependent hydroxylation and proteasomal degradation.^{31,32} This constitutive production, coupled with rapid degradation under normoxia, enables immediate stabilization and a transcriptional response when oxygen availability decreases.^{33,34} Under normoxia, PHD1-3 and FIH, Fe²⁺/ α -ketoglutarate (α KG)-dependent dioxygenases, keep HIF-

1 α repressed.^{31,33} α KG binds to the Fe²⁺ active site, followed by HIF-1 α ; the entry of O₂ generates a Fe(III)-superoxide intermediate that attacks the C2 of α KG, forming a peroxide intermediate that subsequently breaks down to produce succinate, carbon dioxide (CO₂), and the ferryl intermediate (Fe(IV)=O).^{34,35} The Fe(IV)=O then abstracts a hydrogen atom from the proline or asparagine residue, transferring an oxygen atom to form the hydroxylated substrate.³⁵ PHD hydroxylates proline (Pro)402 and Pro564 in the oxygen-dependent degradation domain (ODD) of HIF-1 α , enabling recognition by the E3 ligase von Hippel-Lindau tumor suppressor protein (pVHL), ubiquitination, and proteasomal degradation.^{31,33} In parallel, FIH hydroxylates asparagine (Asn)803 in the C-terminal transactivation domain (CTAD), preventing binding to the coactivators CREB-binding protein (CBP) and E1A-binding protein (p300), which are essential for recruiting RNA polymerase II and initiating gene transcription.^{31,32} Together, prolyl and asparaginyl hydroxylation suppress HIF-1 α stability and transcriptional activity.^{31,32} Once the catalytic cycle is completed, the iron returns to the Fe²⁺ state and succinate is released and HIF-1 α has been degraded.^{33,35}

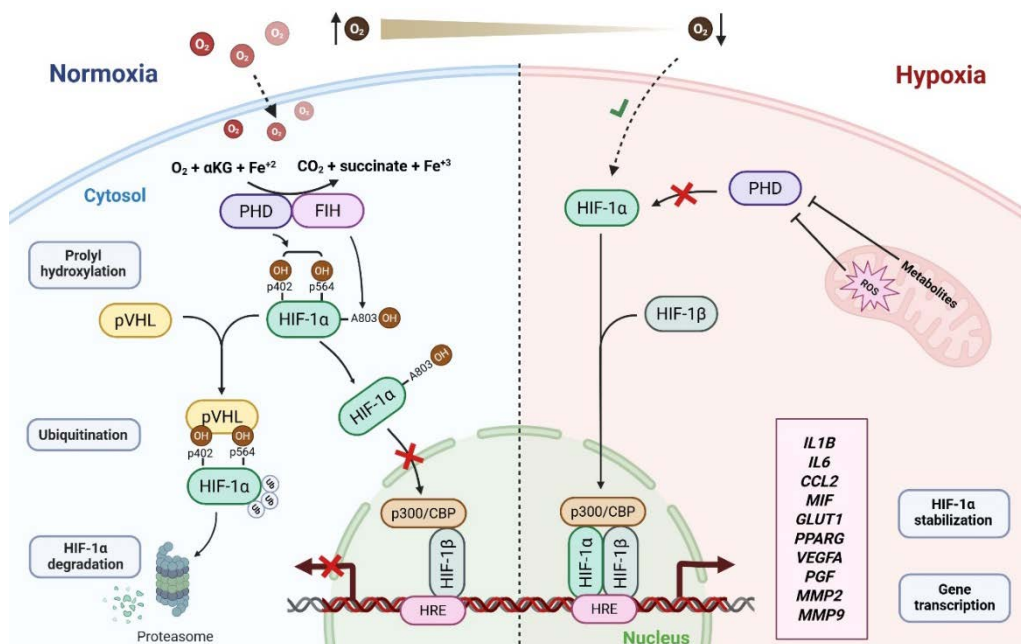


Figure 3.2. Schematic representation of the molecular mechanisms controlling HIF-1 α stability, degradation, and transcriptional activity under normoxic and hypoxic environments.

3.1.2.2 HIF-1 α response in hypoxia: the role of O₂ as sensor and main signal, and the function of ROS

Under hypoxia (see **Figure 3.2**), the limitation of O₂ directly inactivates PHD and FIH. On the contrary, it becomes stabilized, translocates to the nucleus, dimerizes with HIF-1 β , and binds to HREs to activate its gene expression program.^{29,30} Thus, oxygen functions both as a sensor and regulator: its absence activates the transcription of specific target genes, while its presence inhibits the pathway through hydroxylation, ubiquitination, and proteasomal degradation.^{31,32} Moreover, under hypoxic conditions, the production of ROS (mainly from mitochondria) increases. Although ROS are not substrates of PHD/FIH, they modulate their activity. ROS oxidize Fe²⁺ $\rightarrow$ Fe³⁺ at their catalytic center and deplete ascorbate, which is required to maintain iron in the reduced state; as a result, they inhibit PHD and FIH. This effect can even occur in the presence of O₂ in certain situations, generating a state of pseudohypoxia. This mechanism further enhances the stabilization of HIF-1 α and reinforces the progression of the transcriptional program, synchronizing cellular adaptation with the redox state.^{33–35}

Furthermore, additional non-canonical pathways reinforce HIF-1 α activation under hypoxia. The TCA cycle becomes truncated, and several mitochondrial metabolites, including NADH, acetyl-CoA, ATP, and succinate/succinyl-CoA, can inhibit the pyruvate dehydrogenase complex (PDH) directly or activate PDK, which phosphorylates and inactivates PDH. This supports the hypoxic metabolic shift and limits more oxidative TCA cycle activity.^{30,32} As a consequence, reducing equivalents and intermediates such as succinate and fumarate accumulate, competitively inhibiting PHDs, thereby preventing HIF-1 α degradation and further stabilizing the transcriptional response to low oxygen.^{31,33,34}

3.1.3 Assessing intracellular ROS under hypoxia: modulation by CeO₂ NPs and technical challenges

ROS levels were measured as they play a pivotal role in hypoxic conditions. Under hypoxic conditions they are primarily generated by leakage of electrons from the mitochondrial ETC most notably from Complex III of the inner mitochondrial membrane due to the lack of O₂ as the final electron acceptor, as detailed further in **Section 1.1.7.1**. In this context, ROS function dually: as pathogenic mediators exacerbating cellular damage and as key signaling molecules that activate critical inflammatory cascades, including NF-κB, MAPK, and AKT pathways. Most notably, elevated ROS contribute to the reinforced stabilization of HIF-1α by inhibiting PHDs and FIH, thereby promoting its nuclear translocation and target gene expression, as described in **Section 3.1.2.2**. To precisely quantify intracellular ROS accumulation, HTR-8/SVneo trophoblast cells were stained with the CellROX™ Deep Red Reagent, a cell-permeant dye that fluoresces in the presence of ROS, and subjected to flow cytometric analysis following 48h of severe hypoxia (0.5% O₂) exposure (**Figure 3.3**).

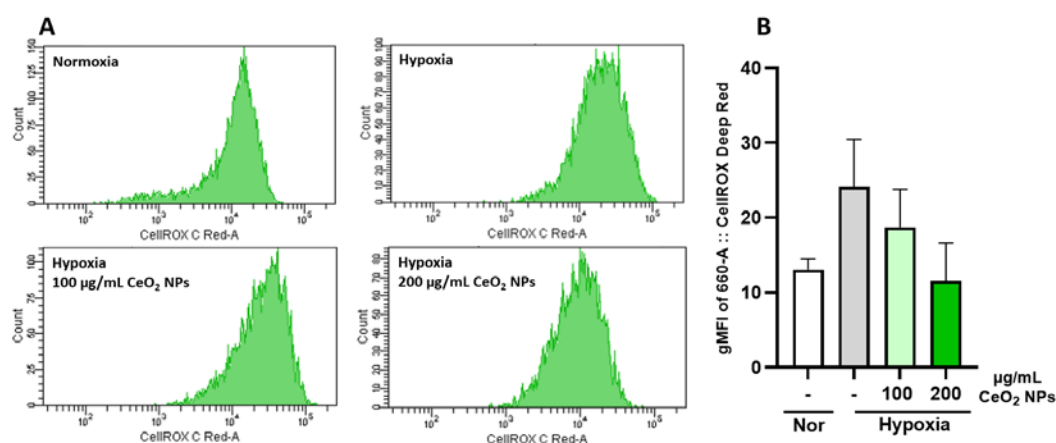


Figure 3.3. Intracellular ROS assessment using CellROX™ Deep Red under normoxic and 0.5% O₂ hypoxic conditions, with or without CeO₂ NPs. **(A)** Representative histograms of CellROX™ Deep Red fluorescence acquired in the R660-A channel, excited with the red laser (640 nm), in trophoblasts exposed for 4h to normoxia (top left), hypoxia (0.5% O₂) (top right), hypoxia + 100 µg/mL CeO₂ NPs (bottom left), and hypoxia + 200 µg/mL CeO₂ NPs (bottom right). **(B)** geometric mean fluorescence intensity (gMFI) of CellROX™ Deep Red measured by flow cytometry in the R660-A channel, excited with the red laser (640 nm), in trophoblasts exposed for 48h to normoxia, hypoxia (0.5% O₂), hypoxia + 100 µg/mL CeO₂ NPs, or hypoxia + 200 µg/mL CeO₂ NPs.

The representative histograms in **Figure 3.3A** clearly demonstrate a substantial rightward shift in CellROX™ Deep Red fluorescence intensity under hypoxic conditions relative to normoxia, reflecting an increase in intracellular ROS following sustained low-oxygen exposure. Remarkably, pretreatment with CeO₂ NPs under hypoxia elicited a dose-dependent attenuation of this shift: 100 µg/mL partially normalized the distribution, while 200 µg/mL restored it closest to normoxic levels, underscoring the CeO₂ NPs antioxidative efficacy. These observations are quantitatively substantiated by the gMFI data in **Figure 3.3B**, which reveal that 48h hypoxia significantly elevated ROS ($p < 0.05$ versus normoxia), whereas CeO₂ NPs counteracted this surge in a concentration-dependent manner (100 µg/mL: partial reduction; 200 µg/mL: maximal suppression, $p < 0.05$ versus hypoxia). Together, these findings confirm that CeO₂ NPs effectively counteract hypoxia-induced oxidative stress by scavenging excess ROS, thereby protecting trophoblasts from dysfunction characteristic of preeclampsia.

3.2 Results and Discussion

3.2.1 Surface chemistry meets biology: how fetal bovine serum and ionic strength control CeO₂ NP aggregation and identity

After synthesis, SC stabilized CeO₂ NPs remain colloidally dispersed due to electrostatic repulsion: SC coordinates with Ce cations, leaving an excess outward-facing negative charge. The resulting electric double layer generates electrostatic repulsion that counteracts ubiquitous van der Waals attraction and prevents NPs agglomeration.^{36,37} Conversely, in biological media, serum acts mostly as a steric stabilizer through rapid protein adsorption: abundant species such as albumin (dominant by mass), immunoglobulins, transferrin, and lipoproteins bind within seconds, forming a protein coating on the NP surface. This layer increases hydrodynamic size and creates entropic/steric repulsion that can prevent salt-induced aggregation.^{38,39}

When NPs enter biological media, such as, cell-culture media, serum, or plasma, their as-synthesized properties no longer define their behavior. Instead, a combination of intrinsic physicochemical traits, such as, (i) size, (ii) morphology, (iii) composition, and (iv) surface chemistry and extrinsic

environmental factors, such as, (i) pH, (ii) ionic strength, (iii) total protein content and composition, (iv) and NPs concentration will govern their colloidal stability, surface identity, and ultimately their pharmacology and toxicity.^{37,40,41} As a result of these processes, several key transformations can occur when CeO₂ NPs are dispersed in biological media.^{36,37,39}

a) Aggregation/agglomeration. Elevated ionic strength (e.g., phosphate-buffered saline (PBS) or cell-culture media) compresses the electric double layer around CeO₂ NPs, weakening electrostatic repulsion and promoting aggregation. Introducing steric protection, commonly via a protein coating (<10 nm), protein corona (≥10 nm) or polymer brushes, can mitigate salt-induced clustering in NPs.^{36,42}

b) Protein coating formation in biological media: In cell culture, fetal bovine serum (FBS) is commonly used as a standard medium supplement. FBS proteins rapidly adsorb onto CeO₂ NP surfaces, forming a dynamic protein coating. This process can be monitored by UV-Vis spectroscopy, DLS, and ζ-potential measurements, which together reveal the swift formation of a protein layer that increases hydrodynamic size, modulates surface charge, and governs colloidal stability in biological conditions.^{38,39,42,43}

c) Corrosion/dissolution. Depending on composition and medium, NPs can undergo chemical transformations, including oxidation, ligand exchange, and dissolution into ionic species, altering their physicochemical properties and biological responses.^{36,37}

Further, a critically, characterization must be performed in the intended physiological media, because NPs stability will compromise their pharmacokinetics, thus their biocompatibility, cytotoxicity and therapeutic efficacy.^{37,39} For the experiments conducted in this chapter, it was therefore essential to first evaluate the stability of CeO₂ NPs. CeO₂ NPs were either pre-incubated with FBS for 24h to obtain conjugated NPs or used directly without pre-incubation (non-conjugated). Experiments were conducted in the working medium, Roswell Park Memorial Institute (RPMI)-1640, either without supplements or supplemented with 10% FBS (see schematic in **Figure 3.4A**; note that serum supplementation is required for normal cell growth). CeO₂ NPs were tested at five concentrations (50, 100, 200, 400, and 800 µg/mL), and their time-dependent behavior was analyzed by DLS at 1 min, 24h, and 48h.

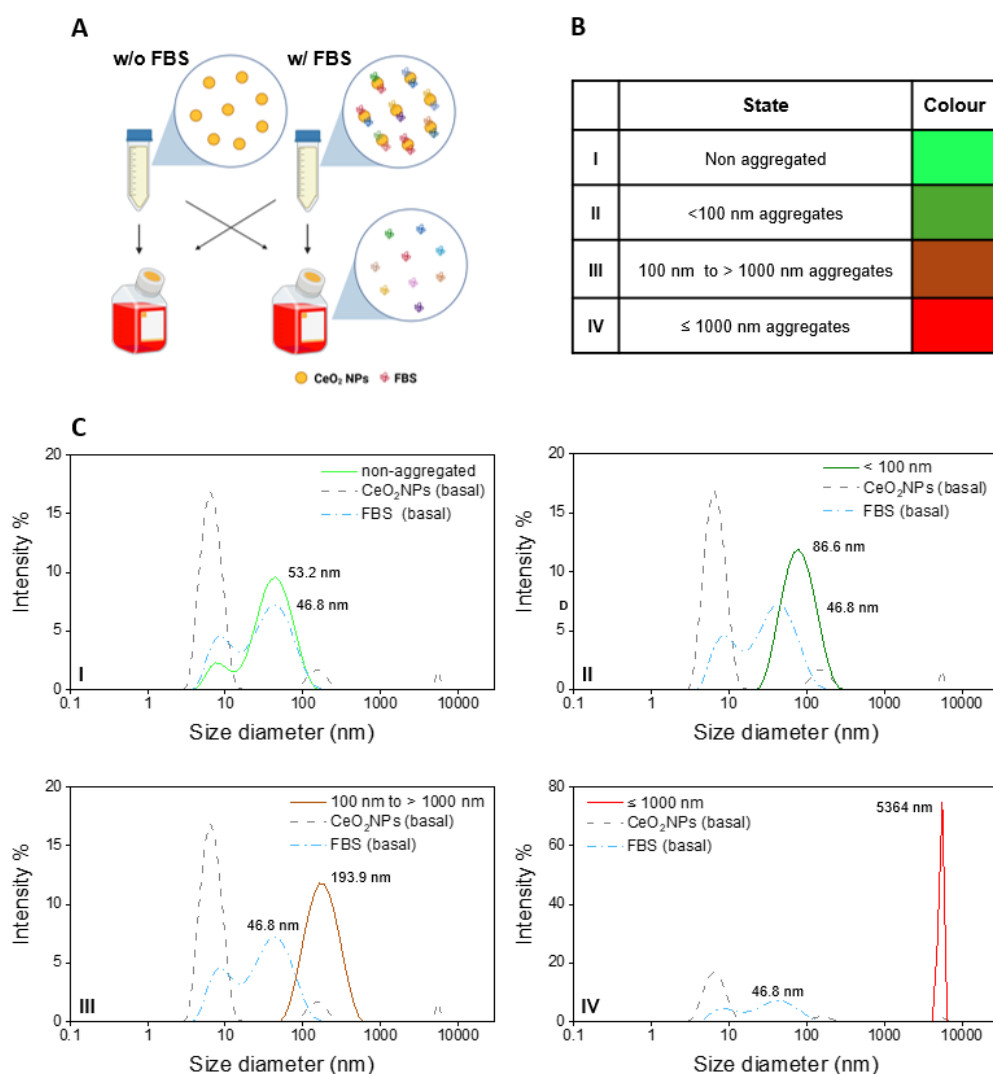


Figure 3.4. Design and classification scheme for CeO₂ nanoparticle stability in biological media. (A) Schema of the protocol: non-conjugated NPs and NPs pre-conjugated for 24 h with FBS are added to RPMI with or without FBS. **(B)** Aggregation-state classification: I. non-aggregated (green), II. <100 nm (dark green), III. 100-1000 nm (brown), and IV. ≥1000 nm (red). **(C)** DLS size distributions illustrating each state with the CeO₂ NPs (alone) and FBS (alone) controls.

Subsequently, DLS results from each condition were classified into four aggregation states based on shifts in size distributions and histogram broadening: I) non-aggregated; II) aggregates <100 nm; III) aggregates between 100 and 1000 nm; and IV) aggregates ≥1000 nm (**Figures 3.4B-C**). For comparison, several controls were included. Non-conjugated CeO₂

NPs alone (dashed grey line in **Figure 3.4C**) exhibited a characteristic DLS peak at approximately 4.5 nm, while FBS alone showed a characteristic bimodal size distribution (blue dashed line). These controls are essential because, following pre-incubation with FBS, direct monitoring of CeO₂ NPs is no longer possible. Instead, changes in the FBS protein peaks, including peak shifts and distribution broadening, serve as sensitive indirect indicators of CeO₂ NP@FBS nanoconjugate aggregation, providing a non-invasive readout of CeO₂ NP colloidal stability.

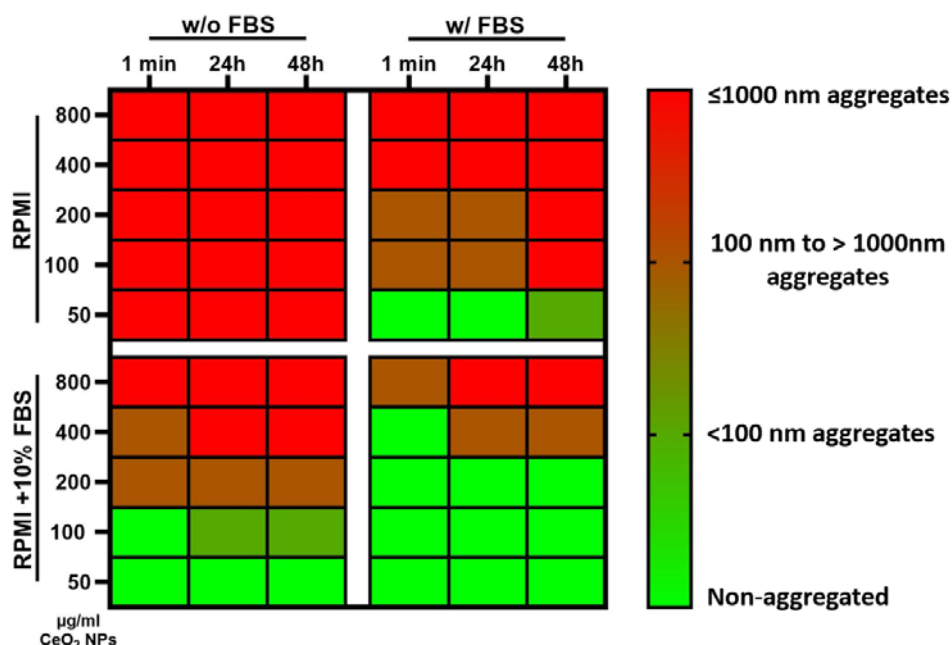


Figure 3.5. Heatmap of aggregation states for CeO₂ NPs at 50–800 µg/ml (rows) in RPMI and RPMI + 10% FBS, with and without 24 h pre-conjugation with FBS (time columns: 1 min, 24h, 48h). Colors encode states: I. stable/non-aggregated (green), II. <100 nm/partially aggregated (dark green), III. 100–1000 nm/partially aggregated (brown), and IV. ≥1000 nm/totally aggregated (red).

Using all experimental conditions, a heatmap was generated summarizing the aggregation behavior of CeO₂ NPs in RPMI with or without 10% FBS, with or without 24h pre-conjugation with FBS, across all concentrations and time points (**Figure 3.5**). Aggregation states ranged from green (non-aggregated) to red (fully aggregated), corresponding to the four predefined categories. In RPMI without FBS, both conjugated and, more markedly, non-conjugated CeO₂ NPs rapidly transitioned toward states III–IV, indicating pronounced

aggregation. In contrast, in RPMI supplemented with 10% FBS, pre-incubation with FBS biased CeO₂ NP populations toward states I-II, maintaining colloidal stability for up to 48h. This behavior is consistent with the formation of a protein coating that provides steric stabilization and prevents salt-induced aggregation. Based on these results, the working conditions selected for subsequent biological experiments were 50, 100, and 200 µg/mL CeO₂ NPs pre-conjugated with FBS for 24h and tested in RPMI + 10% FBS, as these conditions ensured stable dispersions for at least 48h (states I-II).

3.2.2 Multimodal electron microscopy analysis of CeO₂ NPs internalization in endosomes

To demonstrate the internalization, intracellular trafficking, and storage of CeO₂ NPs in trophoblast endosomes, images were acquired using TEM (**Figure 3.6A-B**), HR-TEM (**Figure 3.6C-D**), and HAADF-Scanning Transmission Electron Microscopy (STEM) (**Figure 3.7**) on a Tecnai F20 S/TEM microscope. TEM imaging enabled the identification of CeO₂ NPs confined within intracellular vesicular compartments consistent with late endosomes, with diameters in the range of 300-500 nm (**Figure 3.6A**). Higher magnification TEM images (**Figures 3.6B-C**) allowed clearer visualization of NPs localized within the endosomal lumen. In addition, HR-TEM provided a more detailed structural characterization of the internalized CeO₂ NPs (~3 nm), revealing lattice fringes corresponding to the characteristic crystallographic planes of CeO₂.

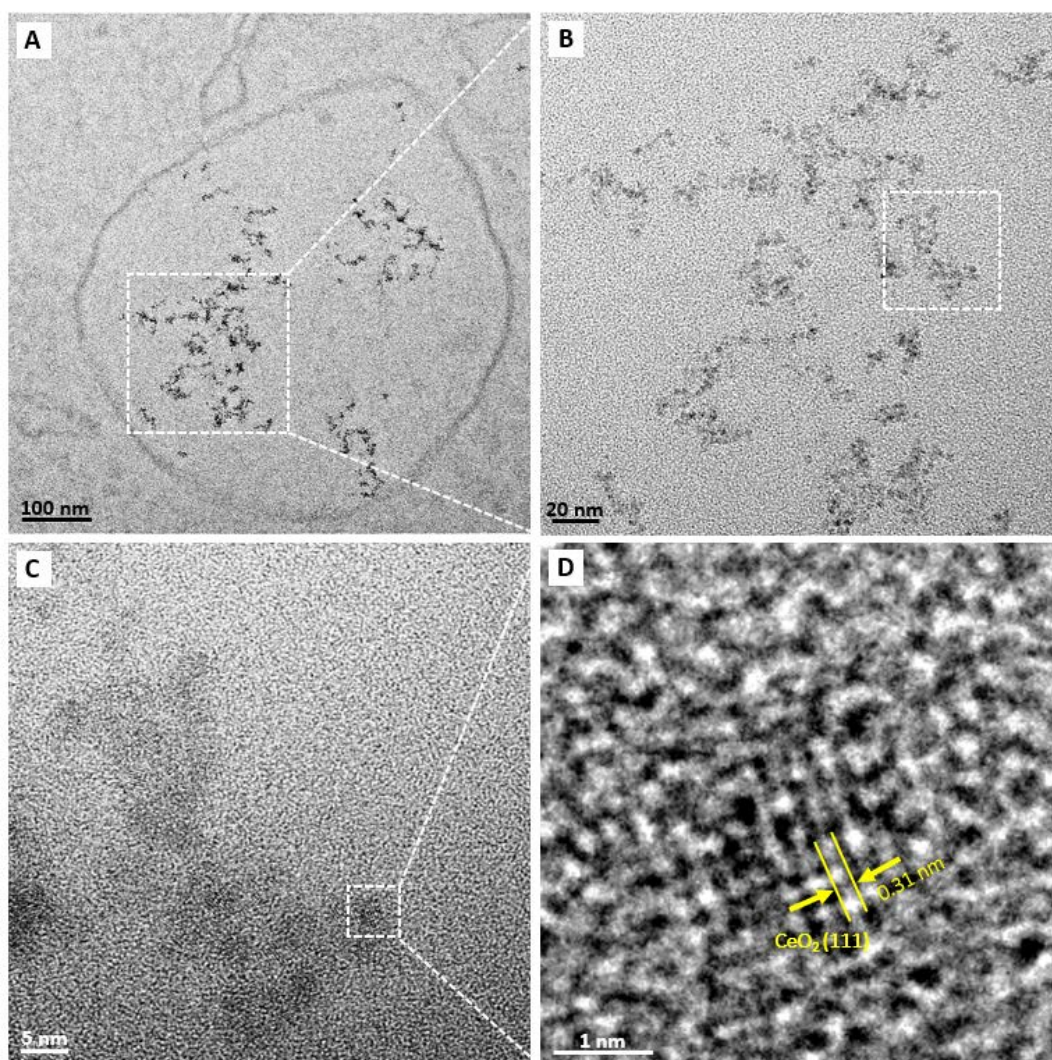


Figure 3.6. Intracellular trafficking of 3 nm CeO₂ NPs in trophoblast endosomes within the same region. Characterization was performed using a Tecnai F20 S/TEM operated in TEM and HR-TEM modes. **(A)** TEM image of a trophoblast endosome (diameter ~500 nm) containing CeO₂ NPs. Scale bar: 100 nm. **(B)** Higher-magnification TEM image of the boxed region in (A), showing CeO₂ NPs localized within the endosomal lumen. Scale bar: 20 nm. **(C)** HR-TEM image of the boxed region in (B), showing a CeO₂ NP cluster inside the endosome and revealing NPs with a size of ~3 nm. Scale bar: 5 nm. **(D)** HR-TEM image of the boxed region in (C), showing atomic lattice fringes with an interplanar spacing of 0.31 nm, corresponding to the (111) crystallographic planes of CeO₂ NPs. Scale bar: 1 nm.

In parallel, HAADF-STEM imaging provided complementary information based on Z-contrast, electrons scattered at high angles are collected by an annular detector, producing contrast in which elements with higher atomic numbers appear brighter. Accordingly, CeO₂

NPs appeared as intensely bright spots against the low-contrast organic cellular background, allowing their identification even at low magnification. Overall, HAADF-STEM images revealed several endosomal compartments distributed throughout the cytoplasm and densely loaded with CeO₂ NPs (**Figure 3.7A-B**), confirming intracellular storage of the NPs following internalization. At higher magnification (**Figure 3.7C-D**), the intraluminal distribution of the NPs was further confirmed.

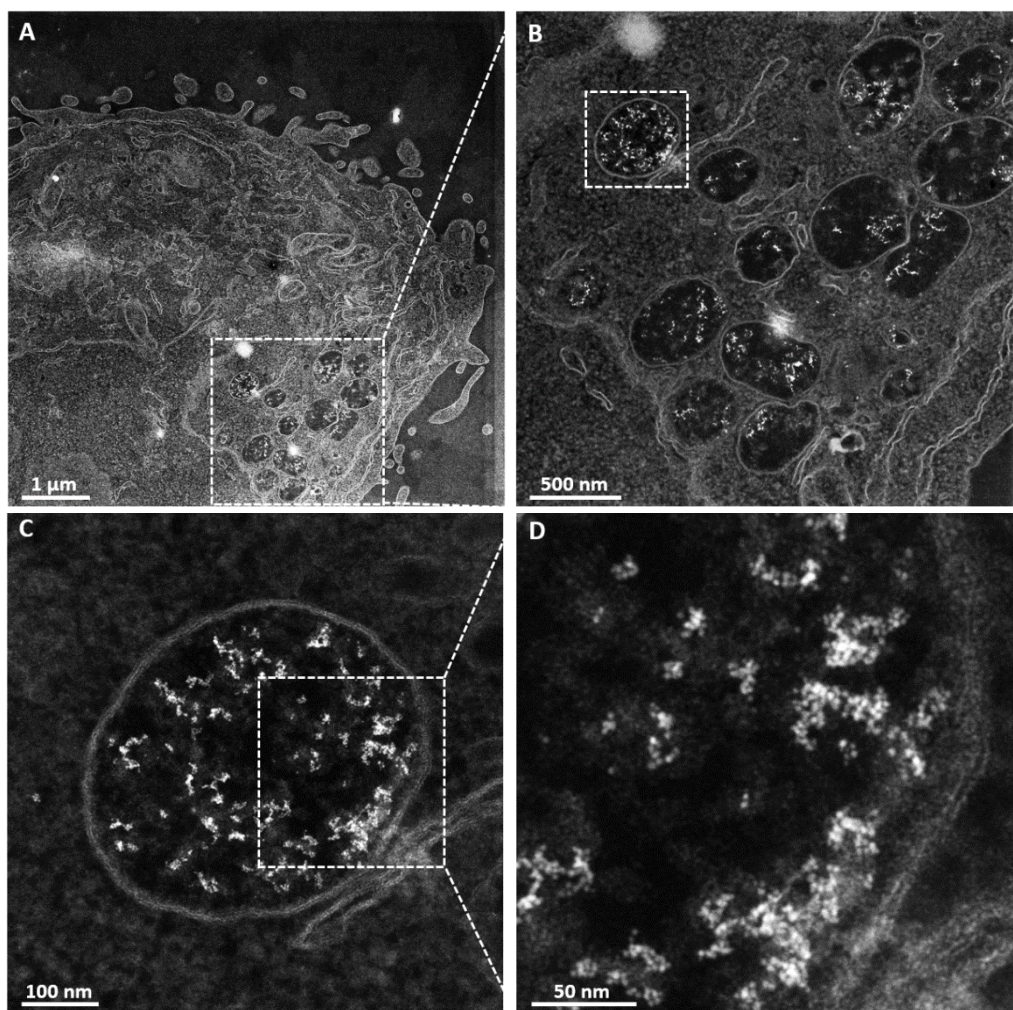


Figure 3.7. Images acquired using a Tecnai F20 S/TEM operated in HAADF-STEM mode. **(A)** Low-magnification HAADF-STEM image of a trophoblast cell showing multiple endosomal compartments with CeO₂ NPs inside. Scale bar: 1 μm. **(B)** Higher-magnification showing endosomes loaded with CeO₂ NPs within the endosomal lumen (typical diameter ~300-500 nm). Scale bar: 500 nm. **(C)** Higher-magnification HAADF-STEM image of a single endosome containing intraluminal CeO₂ NPs. Scale bar: 100 nm. **(D)** Further magnification of the boxed region in (C) highlighting clustered CeO₂ NPs as bright spots inside the vesicular lumen. Scale bar: 50 nm.

To be totally sure that the HAADF-bright intraluminal structures correspond to CeO₂ NPs, Energy-Dispersive X-Ray Spectroscopy (EDX) was performed in HAADF-STEM mode images to verify their elemental composition (**Figure 3.8**). The EDX spectrum acquired from the boxed region shows distinct characteristic cerium peaks (**Figure 3.8B**). Signals associated with Cu, Si, and Cl arise from the TEM grid, the surrounding cellular matrix, and the staining agents used during sample preparation.

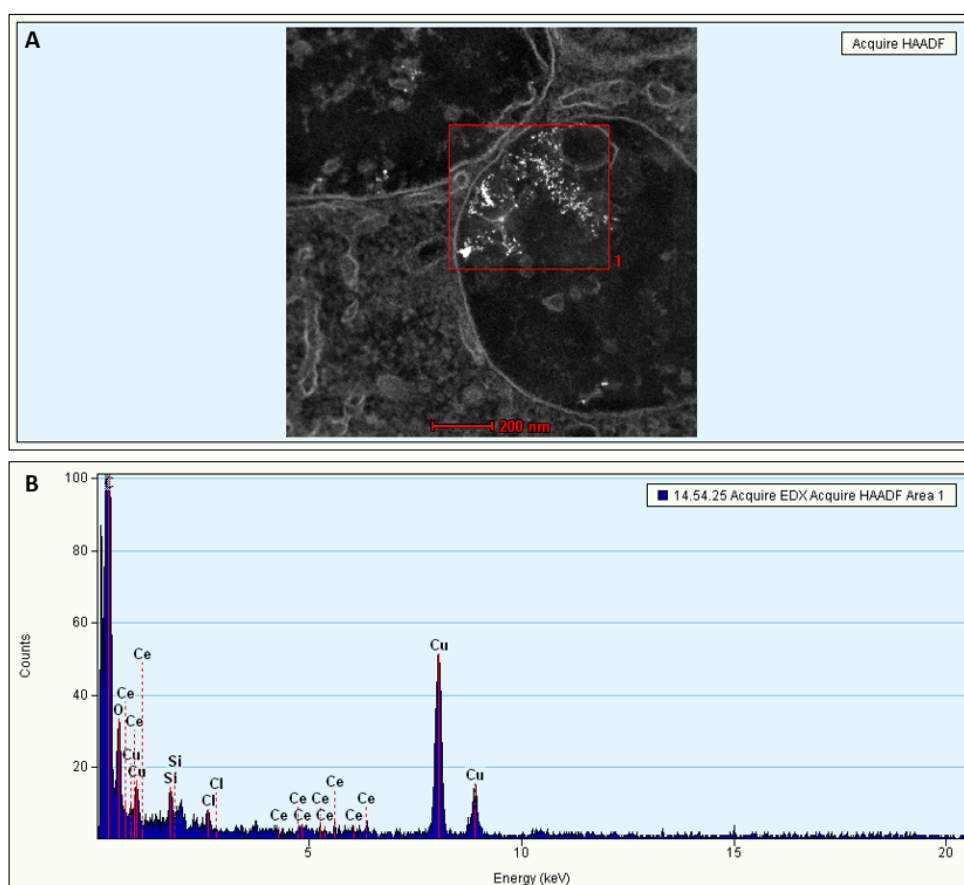


Figure 3.8. Images acquired using a Tecnai F20 S/TEM operated in HAADF-STEM mode and coupled to EDX analysis. **(A)** HAADF-STEM image showing a NP-rich region selected for elemental analysis (boxed area 1). Scale bar: 200 nm. **(B)** EDX spectrum acquired from Area 1 confirming the presence of elemental Ce.

3.2.3 Time- and dose-dependent impact of CeO₂ NPs on HTR-8/SVneo trophoblast cell viability

HTR-8/SVneo trophoblasts were first exposed to a range of sodium citrate concentrations (0-10 mM), encompassing the 2.2 mM used to disperse CeO₂ NPs, to rule out vehicle-related effects on cell viability. Cell survival was quantified with the PrestoBlue™ resazurin assay at 72 h. Vehicle exposure did not reduce metabolic readouts versus the untreated control, confirming that sodium citrate alone does not account for cytotoxicity signals in this system (Figure 3.9).

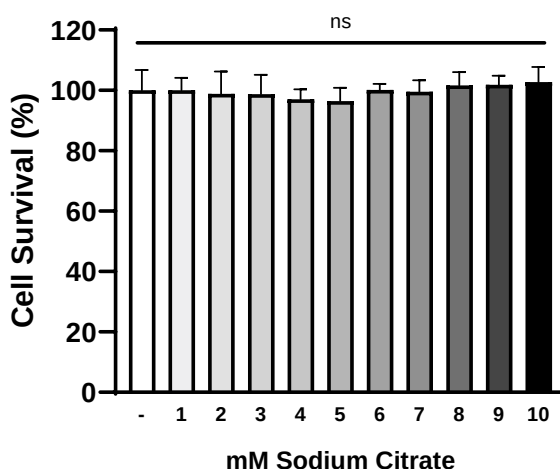


Figure 3.9. Cell survival of HTR-8/SVneo trophoblasts exposed to sodium citrate (CeO₂ NPs vehicle). Data are mean $\pm$ SD (n = 3). One-way ANOVA with Tukey's post hoc test.

Subsequently, it was important to profile dose- and time-dependent effects of CeO₂ NPs. Cells were treated with 50, 100, 200, 400, or 800 μ g/mL CeO₂ NPs (pre-conjugated with FBS over night) for 24, 48, or 72h under hypoxia (0.5% O₂) or normoxia (18% O₂). The PrestoBlue™ readout revealed progressive loss of survival at higher doses (400-800 μ g/mL) (Figure 3.10). Visible well-bottom aggregates most evident at 800 μ g/mL, could be observed (data not shown). It is a well-known phenomenon that aggregates might induce cytotoxicity in cell culture by sedimentation.⁴⁴ By contrast, 50-200 μ g/mL maintained cell viability comparable to untreated controls in both oxygen conditions. Based on these data, 100 and 200 μ g/mL were selected as the primary working concentrations.

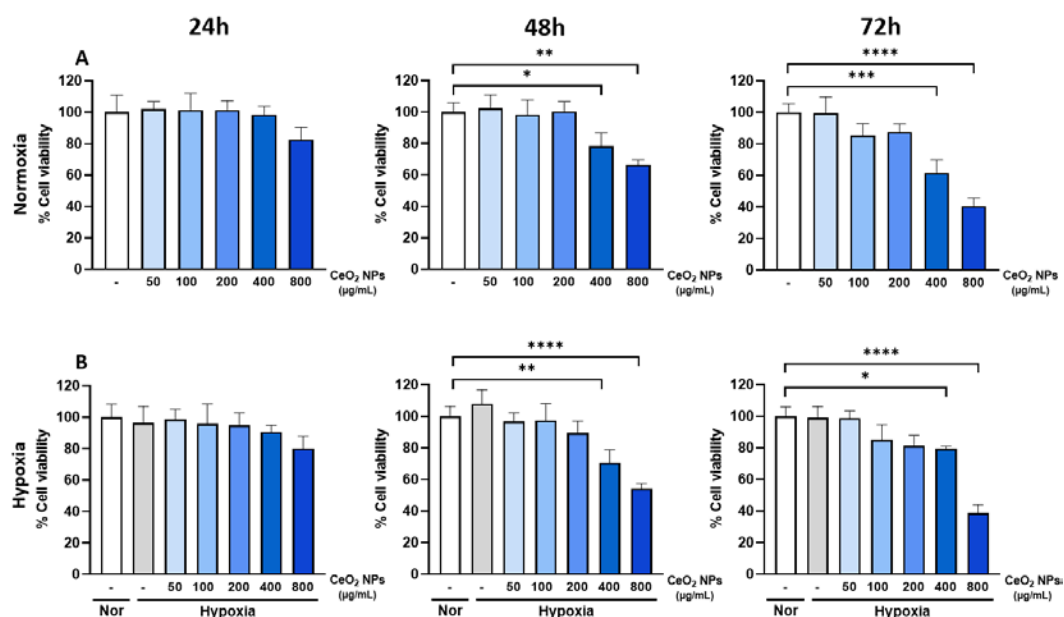
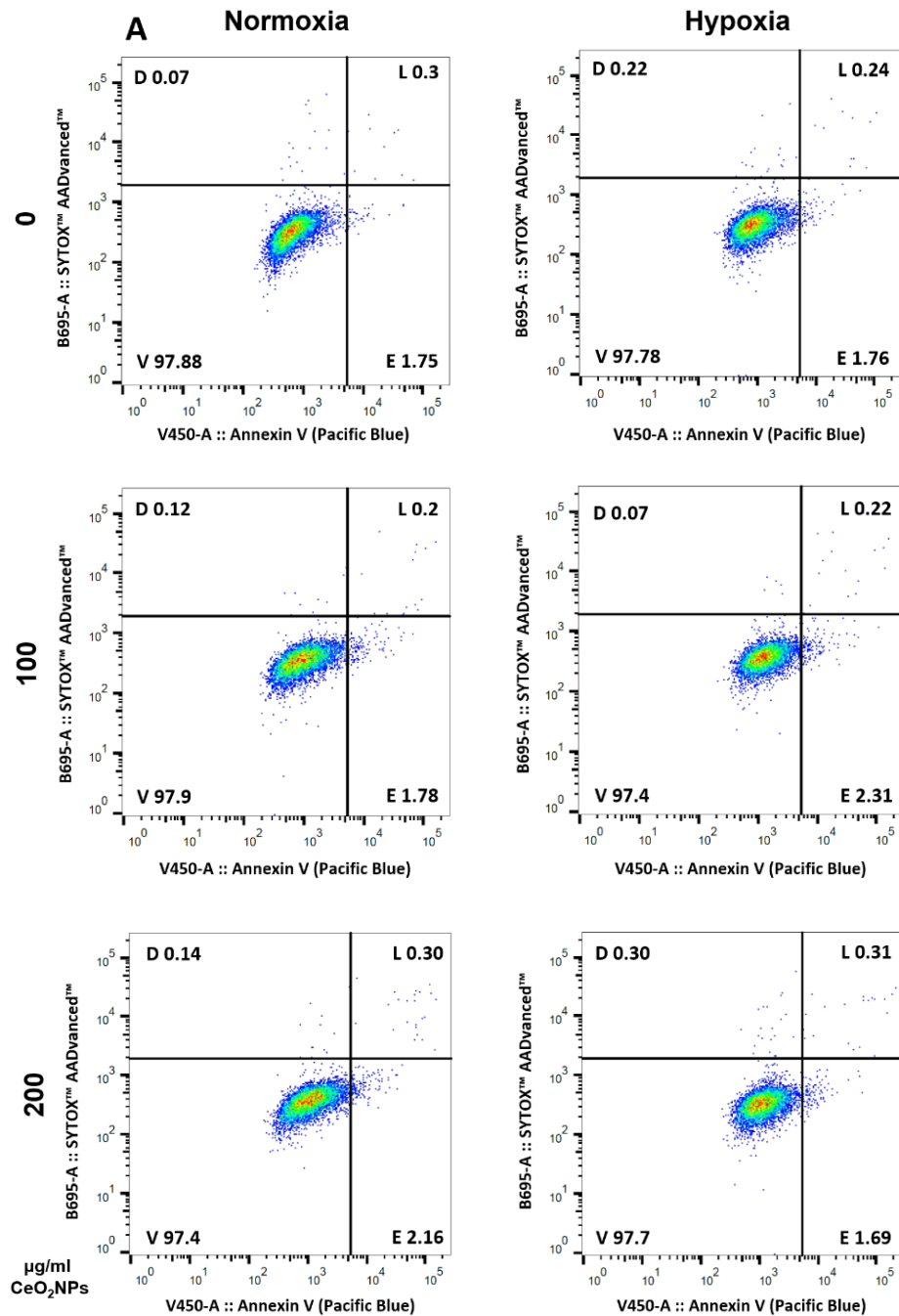


Figure 3.10. Cell survival of HTR-8/SVneo trophoblasts treated with CeO₂ NPs (50-800 µg/mL) for 24, 48, and 72h. (A) Under 18% O₂ (normoxia; control). (B) 0.5% O₂ (hypoxia; basal = normoxia (nor.)). Data are mean ± SD (n = 3). One-way ANOVA with Tukey's post hoc test; p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p ≤ 0.0001 (****).

The cytotoxicity presented endpoints were further corroborated by flow cytometry using the Pacific Blue™ Annexin V / Sytox™ AADvanced™ kit with FlowJo post-analysis. Focusing on hypoxia, no evidence of cytotoxicity was observed under 0.5% O₂ for 48h at the working doses of CeO₂ NPs (0, 100, 200 µg/mL). In hypoxia, the viable population (Annexin V⁻/Sytox⁻) remained dominant, with no increase in early apoptosis (Annexin V⁺/Sytox⁻), late apoptosis (Annexin V⁺/Sytox⁺), or primary necrosis (Annexin V⁻/Sytox⁺) compared with untreated trophoblasts. Importantly, this hypoxic profile was indistinguishable from normoxia, indicating that oxygen deprivation does not unmask a latent toxic effect of CeO₂ NPs within the working CeO₂ NPs concentrations (**Figure 3.11**). Together with the previous cell survival data (**Figure 3.10**), this supports that CeO₂ NPs up to 200 µg/mL are well tolerated even under hypoxic stress, whereas the decrease in viability seen at 400-800 µg/mL are more consistent with dose-dependent aggregation cytotoxicity.



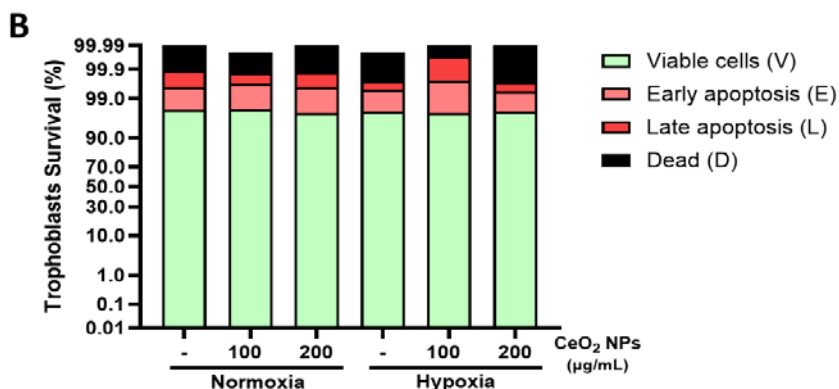


Figure 3.11. Apoptosis and cell death in HTR-8/SVneo trophoblasts treated with CeO₂ NPs (0, 100, 200 µg/mL) for 48h under normoxia (18% O₂) or hypoxia (0.5% O₂). Apoptosis assessed by flow cytometry using the Pacific Blue™ Annexin V / Sytox™ AADvanced™ Apoptosis Kit and analyzed with FlowJo. Annexin V Pacific Blue was acquired in the Pacific Blue channel (V450-A) with the violet laser (405 nm), and Sytox™ AADvanced™ was acquired in the YG610-A channel with the yellow-green laser (561 nm). **(A)** Dot plots are arranged with (left column) normoxia and (right column) hypoxia; rows correspond to 0, 100, and 200 µg/mL CeO₂ NPs, respectively. Quadrants denote viable cells (Annexin V⁻/Sytox⁻, lower left), early apoptotic cells (Annexin V⁺/Sytox⁻, lower right), late apoptotic cells (Annexin V⁺/Sytox⁺, upper right), and dead cells (Annexin V⁻/Sytox⁺, upper left). **(B)** The stacked bar graph summarizes the percentage of each population across conditions and doses. Data are mean ± SD (n = 3). One-way ANOVA with Tukey's post hoc test.

Collectively, these integrated results support the use of CeO₂ NPs within a dose of 50-200 µg/mL window for short-term experiments in HTR-8/SVneo trophoblasts across normoxic (basal) and hypoxic conditions.

3.2.4 Hypoxia at 0.5% O₂ induces time-dependent expression of inflammatory, metabolic, migration and angiogenic genes in HTR-8/SVneo trophoblasts

To establish the optimal conditions for hypoxic stimulation and according to literature, a time-course study was performed to assess the transcriptional response of HTR-8/SVneo trophoblast cells exposed to 0.5% O₂ for 3, 6, 24, and 48 hours, compared with normoxia (18% O₂). The genes analyzed, *IL6*, *IL1B*, *CCL2*, *SLC2A1*, *MMP2*, *MMP9*, *VEGFA*, and *PGF*, have been described to be altered in preeclampsia and regulated downstream by HIF-1α. (Figure 3.12).

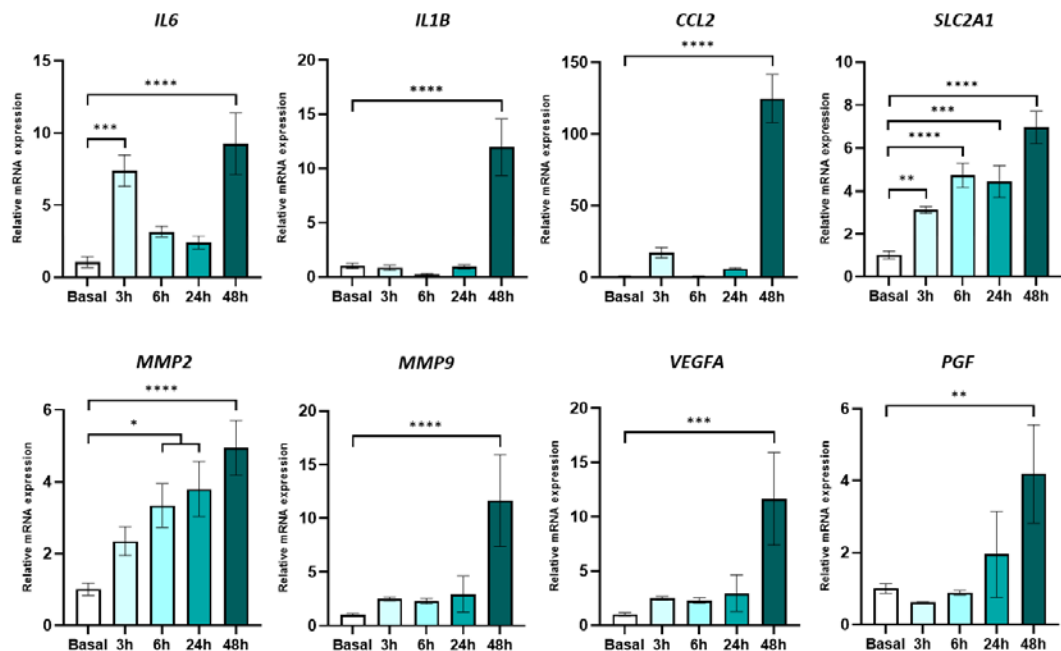


Figure 3.12. Time-course of hypoxia (0.5% O₂) effects on gene expression in HTR-8/SVneo trophoblasts. HTR-8/SVneo cells were cultured under normoxia for 48h (18% O₂; Basal) or hypoxia (0.5% O₂) for 3, 6, 24, or 48h. Relative mRNA levels of *IL6*, *IL1B*, *CCL2*, *SLC2A1*, *MMP2*, *MMP9*, *VEGFA*, and *PGF* were quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and normalized to the housekeeping gene *RPL37A*. Data are mean $\pm$ SD ($n = 3$) and are shown relative to basal (normoxia). Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

The results revealed a progressive and time-dependent increase in gene expression. Early responses (3–6h) included genes such as *IL6* and *SLC2A1*, the gene encoding GLUT1, which displayed rapid but transient upregulation. The early rise of *IL6*, a pro-inflammatory cytokine, likely reflects a prompt adaptive mechanism mediated by HIF-1 α signaling to promote immune communication and cellular stress responses. Similarly, the upregulation of *SLC2A1*, a canonical HIF-1 α target, suggests an immediate metabolic shift toward anaerobic glycolysis to sustain ATP production when oxygen is limited and efficient OXPHOS cannot be maintained. As hypoxia persisted, the transcriptional response intensified in all cytokines, factors and enzymes, with *IL1B*, *SLC2A1*, *CCL2*, *MMP9* and *VEGFA* showing strong and sustained induction, reaching their highest expression at 48 hours. Among these, *CCL2* exhibited the most remarkable increase, rising by more than

100-fold relative to normoxia (control), indicating a potent activation of chemotactic and inflammatory pathways at 48h. *IL1B* also displayed a robust rise, around 10-15-fold, reinforcing the pro-inflammatory adaptation to prolonged hypoxia. *SLC2A1* increased to approximately 7-fold by 48h supporting a sustained metabolic shift toward anaerobic glycolysis to support energy demands under low oxygen. *MMP2* and *MMP9*, key regulators of extracellular matrix remodeling and trophoblast invasiveness, increased progressively over time, with *MMP9* showing a pronounced late response of 12-folds relative to normoxia (control). Similarly, *VEGFA* and *PGF*, two central angiogenic factors, were strongly upregulated at 48h, confirming activation of the HIF-1 α -dependent angiogenic program essential for placental vascular development and oxygen delivery.

Together, these results delineate a sequential HIF-1 α -driven adaptive cascade. The marked upregulation observed at 48h demonstrates that this duration elicits maximal activation of the hypoxic response machinery in trophoblasts. Consequently, 48h of hypoxia (0.5% O₂) was selected as the optimal experimental condition, representing the time point at which HTR-8/SVneo cells exhibit the most comprehensive transcriptional activation encompassing inflammation, metabolic reprogramming, matrix remodeling, and angiogenesis. Further experiments are going to be conducted mainly at this time point.

3.2.5 Validation of the hypoxic model, 0.5% O₂ for 48h, and HIF-1 α pathway activation in HTR-8/SVneo trophoblasts

To validate the hypoxic model and confirm the consequences of oxygen deprivation, HTR-8/SVneo trophoblast cells were cultured under normoxia (18% O₂) or hypoxia (0.5% O₂) for 6, 24, and 48h. Intracellular oxygen reduction was verified with the Hypoxia Green Reagent®, which showed a clear time-dependent rise in fluorescence by flow cytometry under hypoxia compared with normoxia. The gMFI increased at 6 hours, rose further at 24 hours, and then reached a plateau through 48 hours, indicating sustained intracellular hypoxia (**Figure 3.13**).

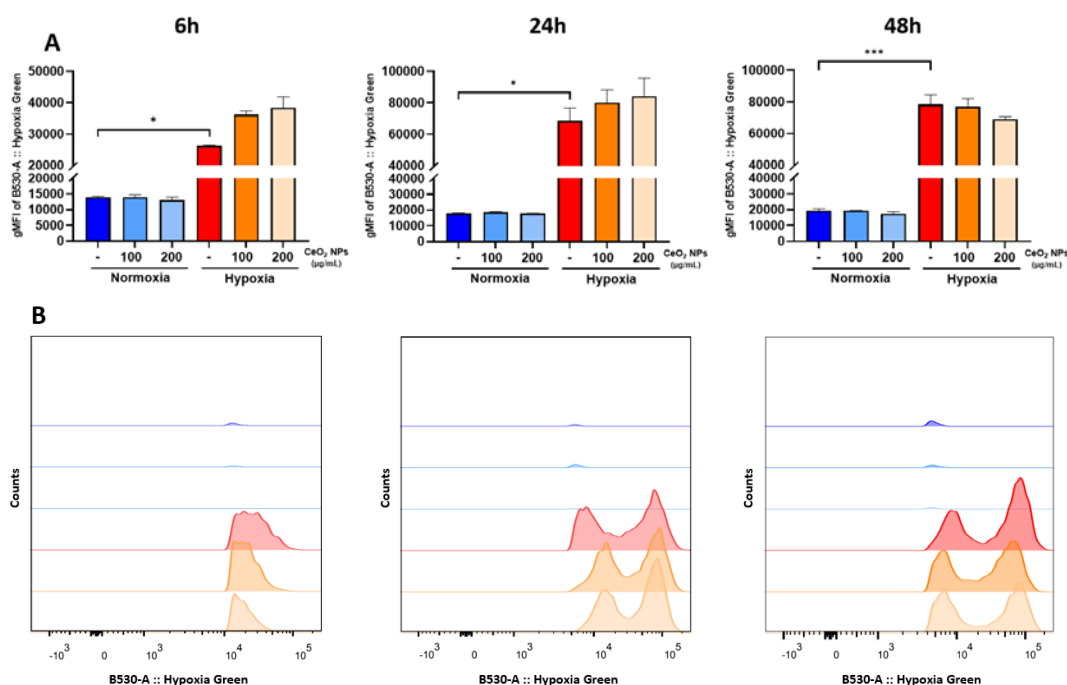


Figure 3.13. Hypoxia detection using Hypoxia Green Reagent under normoxic and hypoxic (0.5% O₂) conditions. (A) gMFI of Hypoxia Green Reagent measured by flow cytometry in the FITC channel (B530-A), excited with the blue laser (488 nm), in trophoblast exposed to normoxia or hypoxia (0.5% O₂) for 6, 24, and 48h. **(B)** Representative histograms of Hypoxia Green Reagent fluorescence acquired in the FITC channel (B530-A), excited with the blue laser (488 nm), in trophoblast exposed to normoxia or hypoxia (0.5% O₂) for 6, 24, and 48h.

Furthermore, HIF-1α protein expression was quantified by ELISA (**Figure 3.14A**). Results demonstrate that HIF-1α levels were significantly up-regulated under hypoxia compared to normoxia (****p<0.0001), consistent with its stabilization under low oxygen tensions, a phenomenon extensively documented in the literature⁴⁵ and further explained in this thesis in **Section 3.1.2.2**. Notably, treatment with CeO₂ NPs at concentrations of 100 and 200 μg/mL did not significantly alter HIF-1α protein levels under either normoxic or hypoxic conditions. This lack of effect is expected given that CeO₂ NPs primarily act through ROS modulation. Under normoxia, basal ROS levels are minimal, so CeO₂ NP-mediated ROS scavenging has no effect on HIF-1α modulation. Under hypoxia, probably HIF-1α expression is driven and maintained so robustly by oxygen deprivation itself that CeO₂ NPs

cannot modulate it at this level. Instead, as will be demonstrated throughout this thesis, CeO₂ NPs exert their regulatory effects downstream of HIF-1 α through ROS-dependent signaling pathways. In parallel, analysis of total protein concentration (**Figure 3.14B**) shows only a modest decrease of total protein levels under hypoxia, which is insufficient to account for the marked HIF-1 α up-regulation observed. This further reinforces that the substantial increase in HIF-1 α expression under hypoxic conditions reflects genuine protein stabilization and accumulation rather than alterations in total cellular protein content.

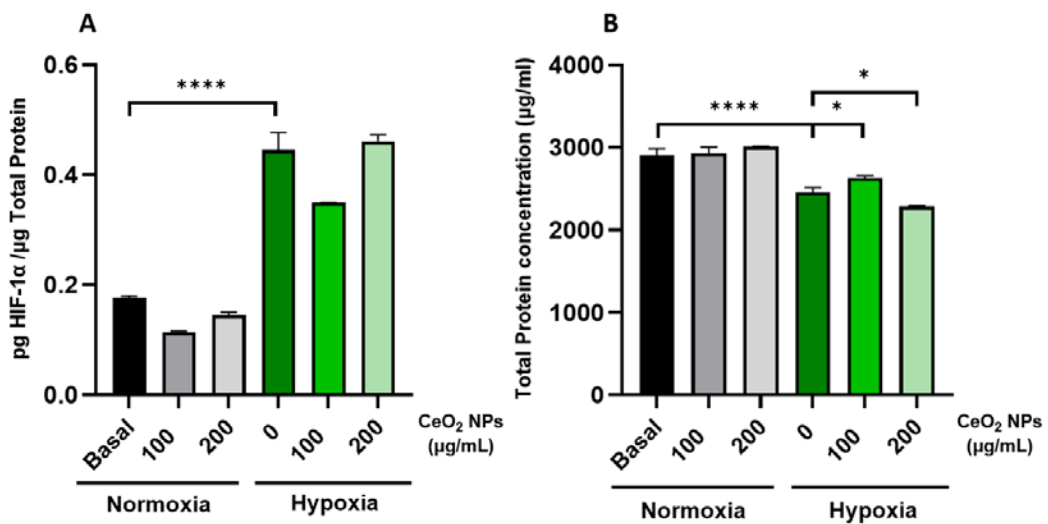


Figure 3.14. *HIF1A* gene expression and HIF-1 α protein levels in HTR-8/SVneo trophoblasts treated with CeO₂ NPs (0, 100, 200 μg/mL) for 48h under normoxia (18% O₂) or hypoxia (0.5% O₂). **(A)** Relative *HIF1A* mRNA expression normalized to the housekeeping gene *RPL37A* and relative to basal (normoxia). **(B)** HIF-1 α protein content (pg HIF-1 α /μg total protein) and relative to basal (normoxia). **(C)** Total protein concentration (μg/mL) in cell lysates determined by ELISA. Data are mean $\pm$ SD (n = 3). One-way ANOVA with Tukey's post hoc test; p $\leq$ 0.05 (*), p $\leq$ 0.01 (**), p $\leq$ 0.001 (***), p $\leq$ 0.0001 (****).

Collectively, these findings confirm robust activation of the HIF-1 α signaling pathway and validate 0.5% O₂ for 48 hours as an effective experimental condition to study hypoxia-driven molecular responses in trophoblast cells.

3.2.6 CeO₂ NPs attenuate 0.5% O₂ hypoxia-induced pro-Inflammatory signaling in HTR-8/SVneo trophoblasts

To assess the protective role of CeO₂ NPs under hypoxic, pro-inflammatory conditions, HTR-8/SVneo trophoblasts were cultured under normoxia (18% O₂) or hypoxia (0.5% O₂) for 48h and treated with 100 or 200 µg/mL CeO₂ NPs. Specifically, the inclusion of the lower concentration (100 µg/mL) was to allow the evaluation of a potential dose-dependent effect, thereby facilitating the interpretation and validation of the results. Moreover, IL-6, IL-1β, and CCL2 expression was then quantified at the mRNA level by qRT-PCR and at the protein level by ELISA (**Figure 3.15**). It is well established that, under hypoxia, HIF-1α enhances pro-inflammatory signaling primarily via NF-κB activation in an IκB kinase (IKK)-transforming growth factor β-activated kinase 1 (TAK1)-dependent manner. HIF-1α and NF-κB engage in bidirectional crosstalk, reinforcing one another and creating a positive feedback loop that amplifies inflammatory outputs.⁴⁶ Mechanistically, HIF-1α can directly induce *IL6* and *CCL2* through HREs,^{47–49} while NF-κB upregulates *IL6*, *IL1B*, and *CCL2*, leading to synergistic cytokine induction under oxygen deprivation.^{49,50} This mechanism resembles that observed in PE, where dysregulated HIF-1α/NF-κB signaling contributes to excessive inflammation and impaired placental function.⁵¹ Additionally, evidence suggests that the NLRP3 inflammasome, among other pathways, may also contribute to amplifying hypoxia-induced inflammatory responses, since the inflammatory response is inherently complex⁵²

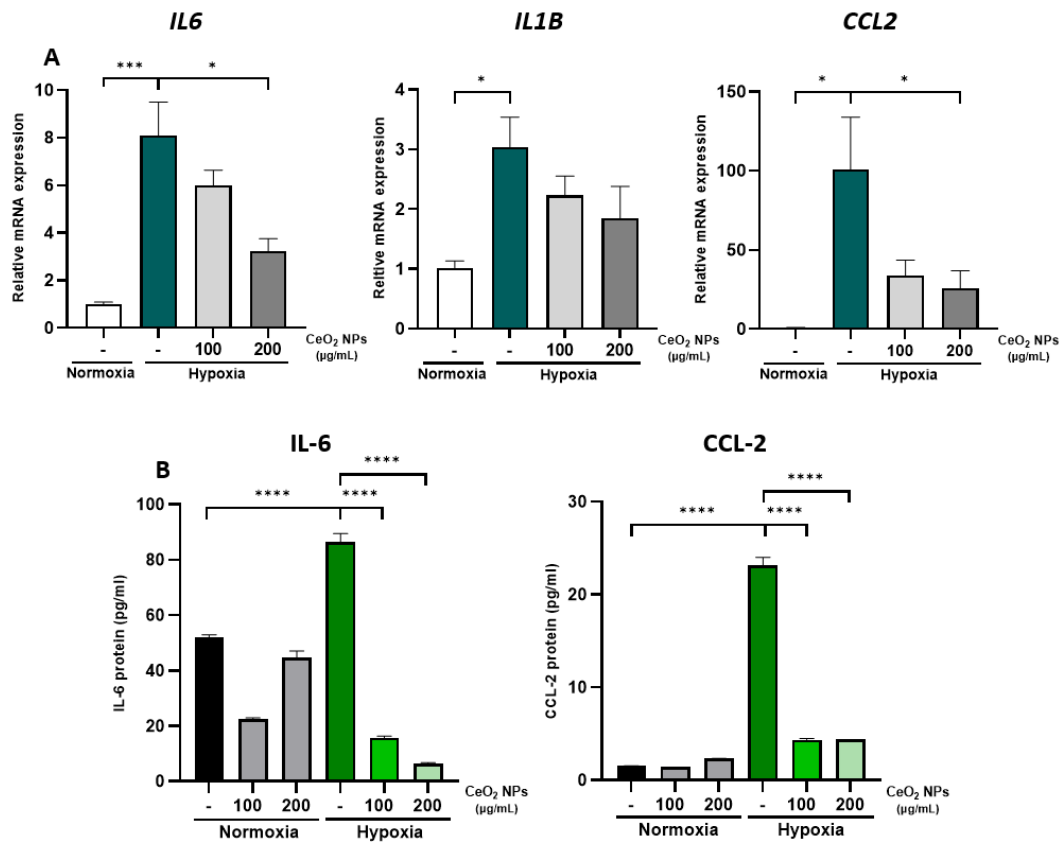


Figure 3.15. *IL6*, *IL1B*, and *CCL2* gene expression and IL-6 and CCL-2 protein levels in HTR-8/SVneo trophoblasts treated with CeO₂ NPs (0, 100, 200 μg/mL) for 48h under normoxia (18% O₂) or hypoxia (0.5% O₂). (A) Relative (left) *IL6*, (middle) *IL1B*, and (right) *CCL2* mRNA expression normalized to the housekeeping gene *RPL37A* and relative to basal (normoxia). (B) (left) IL-6 and (right) CCL-2 protein concentrations (pg/mL) measured in cell supernatants by ELISA. Data are mean ± SD (n = 3). One-way ANOVA with Tukey's post hoc test; p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p ≤ 0.0001 (****).

As expected, hypoxia alone markedly upregulated all three cytokine transcripts relative to normoxic controls. *CCL2* showed a pronounced increase, rising by approximately 100 folds compared with basal levels. *IL6* and *IL1B* also increased significantly by about 8 folds and 3 folds, respectively (Figure 3.15A). At the protein level, ELISA analysis corroborated the transcriptional data, showing enhanced secretion of IL-6 and CCL-2 after 48 hours of hypoxia, p ≤ 0.0001 (****) (Figure 3.15B). These findings confirm that transcriptional activation under low oxygen is accompanied by elevated cytokine release, reflecting a robust pro-inflammatory phenotype achieved in HTR-8/SVneo trophoblasts. Furthermore,

exposure to CeO₂ NPs at 100 or 200 µg/mL during hypoxia significantly attenuated both mRNA expression and protein secretion of IL-6, IL-1β, and CCL-2, with a more pronounced reduction at 200 µg/mL (**Figure 3.15**), with an approximate 20-fold reduction in IL-6 and CCL-2 compared with untreated hypoxic cells at the protein level (**Figure 3.15B**). This inhibitory effect highlights the ability of CeO₂ NPs to counteract hypoxia-driven inflammatory signaling, likely through modulation of canonical inflammatory pathways such as MAPKs (ERK1/2, JNK1/2, p38), NF-κB, JAK/signal transducer and activator of transcription (STAT), and PI3K/AKT, as previously demonstrated in other pathological contexts.^{53–55} Notably, potential modulation of HIF-1α should also be considered. The absence of detectable changes in total HIF-1α protein observed in **Section 3.2.6** does not exclude alterations in its transcriptional activity, since HIF-1α can be differentially regulated at the level of nuclear localization, co-activator recruitment, and target-gene selectivity.⁵⁶ Overall, these findings demonstrate that prolonged hypoxia (0.5% O₂, 48h) elicits a strong pro-inflammatory response in trophoblasts and highlights the capacity of CeO₂ NPs to suppress this response.

3.2.7 Effects of CeO₂ NPs on metabolic reprogramming of trophoblasts under severe hypoxia (0.5% O₂) for 48h

Under aberrant hypoxia (around 0.5% O₂), as in PE, limited oxygen availability constrains trophoblast function. Because oxygen normally serves as the final electron acceptor of the mitochondrial ETC, its shortage forces cells to downscale OXPHOS and increasingly rely on ATP production via anaerobic glycolysis, a compensatory pathway that helps restrain excessive ETC-derived ROS but yields only about 2 ATP per glucose, compared with roughly 38 ATP per glucose when glycolysis, the TCA cycle, and OXPHOS operate fully under aerobic conditions.³⁰ Residual ROS generated under these conditions still acts as a signaling cue, and together with oxygen depletion modulates HIF-1α stability and activity; in turn, HIF-1α fine-tunes the expression of key metabolic enzymes and transporters that coordinate this hypoxic metabolic adaptation (**Figure 3.16**). In this context, HIF-1α is well established as a

master regulator of metabolic adaptation. It increases expression of lactate dehydrogenase A (LDHA), an enzyme that catalyzes the reduction of pyruvate to lactate, thereby preventing its conversion into acetyl-CoA. Simultaneously, HIF-1 α upregulates PDK1, which phosphorylates and inhibits the pyruvate dehydrogenase complex, further blocking acetyl-CoA formation from pyruvate and reducing substrate supply to the TCA cycle, thus reinforcing reliance on anaerobic glycolysis. In parallel, HIF-1 α enhances expression of the monocarboxylate transporter MCT4, which exports lactate and protons, limiting intracellular lactic acid accumulation, preventing feedback inhibition of LDHA, and sustaining anaerobic glycolysis.^{20,21,57}

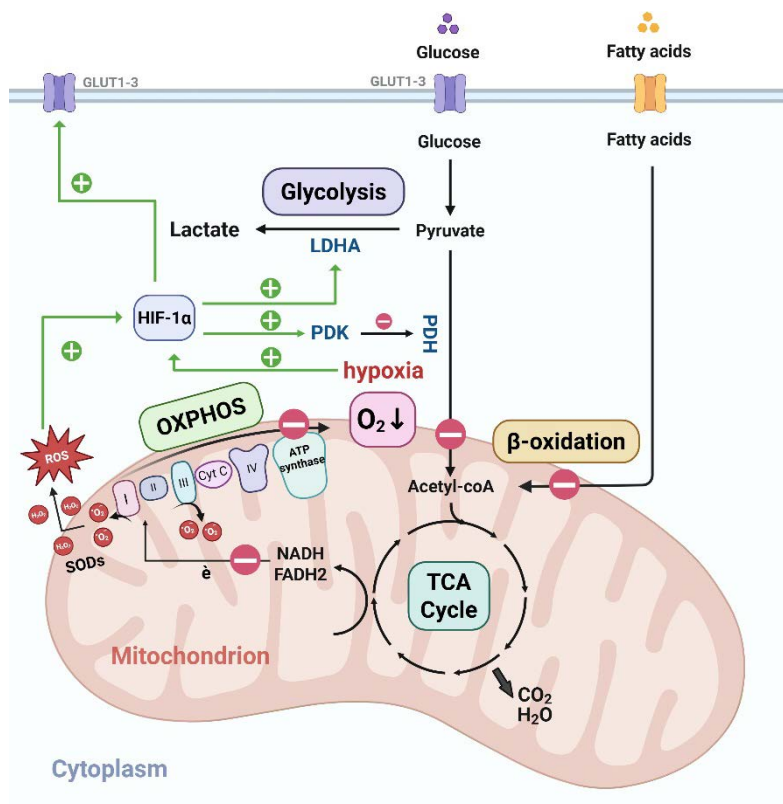


Figure 3.16. EVT_s metabolic reprogramming under aberrant hypoxia in preeclampsia

In this context, *SLC2A1* expression was evaluated by qRT-PCR in HTR-8/SVneo trophoblasts treated with CeO₂ NPs (0, 100, 200 μ g/mL) for 48h (**Figure 3.17A**). *SLC2A1* encodes GLUT-1, a direct transcriptional target of HIF-1 α whose promoter contains functional

hypoxia-response elements that drive strong transcription under low-oxygen conditions.²⁰ Accordingly, the link between HIF-1 α stabilization and *SLC2A1* induction is well established, and up-regulation of this axis is strongly implicated in the pathogenesis of PE.^{20,21} GLUT-1 expression is further enhanced by NF- κ B signaling, which can act synergistically with HIF-1 α to amplify *SLC2A1* transcription.⁵⁷ In our model, CeO₂ NPs (100 and 200 μ g/mL) significantly reduced hypoxia-induced *SLC2A1* mRNA levels ($p < 0.001$), suggesting a partial normalization of GLUT-1-mediated glucose uptake under 0.5% O₂. A decrease in GLUT-1 does not necessarily imply a shift away from anaerobic glycolysis, but may instead reflect attenuation of an exaggerated hypoxic stress response and a partial recovery of metabolic homeostasis, in line with observations from other systems where GLUT-1 down-regulation accompanies metabolic re-equilibration rather than complete reversal of the anaerobic glycolytic phenotype.^{58,59} This effect is therefore compatible with a lowering of redox- and inflammation-driven signaling by CeO₂ NPs, potentially involving ROS-sensitive pathways such as HIF-1 α and NF- κ B,^{20,53–55} although these mechanisms were not directly assessed here.

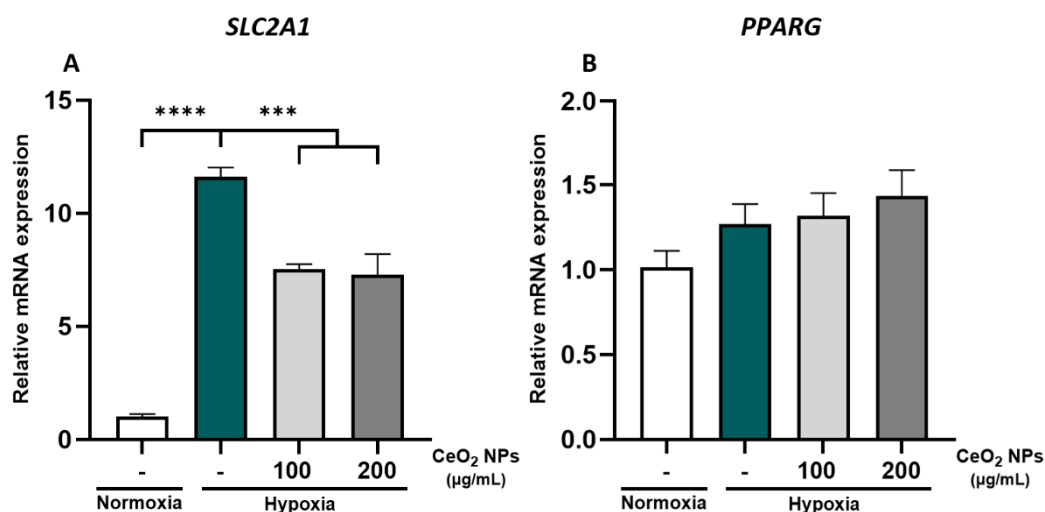


Figure 3.17. (A) *SLC2A1* and (B) peroxisome proliferator-activated receptor gamma (*PPARG*) gene expression in HTR-8/SVneo trophoblasts treated with CeO₂ NPs (0, 100, 200 μ g/mL) for 48h under hypoxia (0.5% O₂). mRNA levels were quantified by qRT-PCR, normalized to the housekeeping gene *RPL37A*, and expressed relative to basal (normoxia). Data are mean $\pm$ SD ($n = 3$). One-way ANOVA with Tukey's post hoc test; $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

In parallel, *PPARG* expression was quantified under the same experimental conditions (**Figure 3.17B**). PPAR γ is a nuclear receptor and transcription factor that regulates mitochondrial fatty-acid oxidation (FAO), mitochondrial biogenesis, antioxidant defenses, and anti-inflammatory signaling, while coordinating trophoblast differentiation, particularly in EVT_s, and metabolic adaptation to placental stress.^{60–62} Trophoblasts characteristically maintain a mixed metabolic profile, integrating anaerobic glycolysis for rapid ATP production with basal fatty acid oxidation to support biosynthetic demands and mitochondrial function.^{24,63} Furthermore, PPAR γ represents a gene of particular interest in understanding placental adaptation to hypoxic stress. Importantly, sustained PPAR γ expression under hypoxia does not necessarily indicate active reliance on FAO for ATP production, as OXPHOS is severely constrained by limited oxygen availability.⁶⁴ Although trophoblasts, particularly EVT_s, are capable of sustaining low-level OXPHOS at remarkably low oxygen tensions, this metabolic strategy requires precise redox balance to prevent excessive ROS generation when oxygen is insufficient to serve as the terminal electron acceptor.^{65,66} Beyond energy metabolism, PPAR γ also plays critical roles in mitochondrial biogenesis and antioxidant defense coordination, with multiple functions acting in parallel; therefore, it is difficult to determine with precision which mechanisms drive PPAR γ maintenance under hypoxia.^{61,67} The literature reports conflicting patterns: sustained PE or chronic hypoxia is associated with progressive PPAR γ downregulation due to chronic oxidative stress and NF- κ B-mediated inflammation, while early hypoxic stress may paradoxically upregulate PPAR γ as a survival and adaptive response.^{61,62,68} As observed in **Figure 3.17B**, *PPARG* expression was maintained under hypoxia, with a very modest dose-dependent increase following CeO₂ NPs administration. Since PPAR γ activity is regulated by ROS-sensitive pathways including NF- κ B signaling, the nanoparticles may be reinforcing the adaptive upregulation pathway by mitigating ROS accumulation, thereby supporting mitochondrial quality control and stress-adaptive mechanisms.^{61,62,66,69,70}

3.2.8 Effect of CeO₂ NPs on trophoblast metalloproteinase expression and migration under 0.5% O₂ hypoxia for 48h

Since MMPs play a key role in tissue remodeling required for placental development, a process that is altered in preeclampsia, and are also strongly expressed under hypoxic conditions, we next investigated their expression pattern in HTR-8/SVneo trophoblasts. And also, the treatment with CeO₂ NPs to assess their effect. MMPs are a family of more than 25 Zn²⁺-dependent endopeptidases that degrade collagen and other components of the extracellular matrix (ECM). Among them, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are key mediators of EVT migration and invasion, as they cleave a broad spectrum of ECM molecules, especially collagens type IV, V and XI, as well as laminin and the aggregate core protein. Tight spatial and temporal control of their activity is essential, particularly for remodeling of the maternal decidua and uterine spiral arteries during gestation, a process crucial for establishing and maintaining adequate placental perfusion.^{71,72} Further, HTR-8/SVneo EVTs were exposed to 0.5% O₂ for 48h, and *MMP* mRNA levels were quantified by qRT-PCR in cells cultured in the presence or absence of 100 or 200 µg/mL CeO₂ NPs for 48h (Figure 3.18).

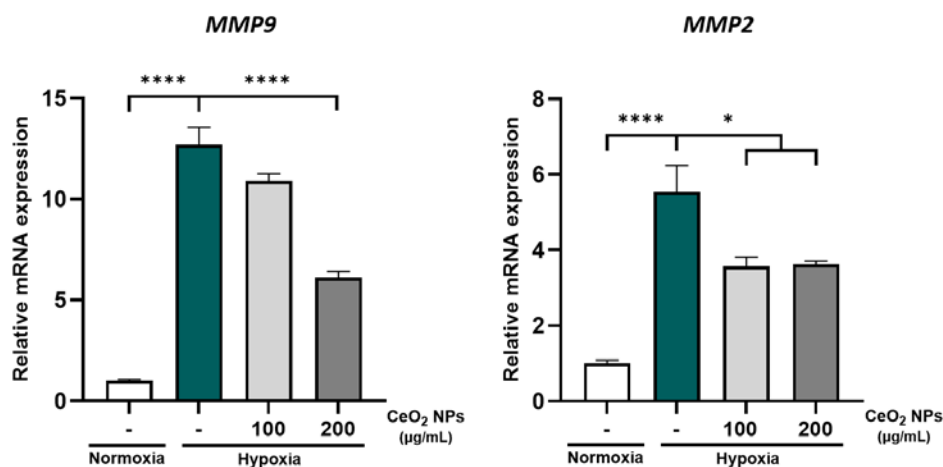


Figure 3.18. Relative mRNA expression of (left) *MMP9* and (right) *MMP2* in HTR-8/SVneo trophoblasts exposed to hypoxia (0.5% O₂) alone or treated with CeO₂ NPs (100 or 200 µg/mL) for 48h. mRNA levels were quantified by qRT-PCR, normalized to the reference gene *RPL37A*, and expressed relative to the basal (normoxic) condition. Data are presented as mean ± SD (n = 3). One-way ANOVA with Tukey's post hoc test; *p* ≤ 0.05 (*), *p* ≤ 0.01 (**), *p* ≤ 0.001 (***), *p* ≤ 0.0001 (****).

As observed, hypoxia significantly increased *MMP9* and *MMP2* mRNA levels relative to normoxic controls ($p < 0.001$). Treatment with CeO₂ NPs (100 or 200 µg/mL) under hypoxia attenuated *MMP2* and *MMP9* expression in a dose-dependent manner, with the strongest inhibition observed for *MMP9* at 200 µg/mL. These findings suggest that CeO₂ NPs, through their ability to scavenge ROS, help restore cellular homeostasis and counteract hypoxia-driven *MMP* activation. One possible explanation is that they decrease ROS levels and thereby limit redox-dependent stabilization and activation of HIF-1 α and its binding to *MMP* hypoxia-responsive element, while at the same time reducing ROS-mediated activation of NF- κ B, MAPK (such as p38 and ERK1/2) and Notch signaling pathways, all of which are important regulators of *MMP* expression.^{13,53,71–75}

Furthermore, trophoblast functionality was assessed by wound-healing migration assays performed using time-lapse video imaging, and the results corroborated the previously described molecular findings (**Figure 3.19A**). Under hypoxia (0.5% O₂), trophoblasts exhibited an exaggerated migratory response, achieving approximately 85-90% wound closure at 21 h, compared with 60% closure under normoxic conditions. In contrast, CeO₂ NPs treatment restored a more controlled, coordinated and slower migratory pattern, resulting in 70% closure at 100 µg/mL and 60% at 200 µg/mL (both $p < 0.01$), values comparable to the normoxic condition. The 200 µg/mL dose produced the most stable phenotype, effectively normalizing migration velocity and trophoblast organization (**Figure 3.19B**).

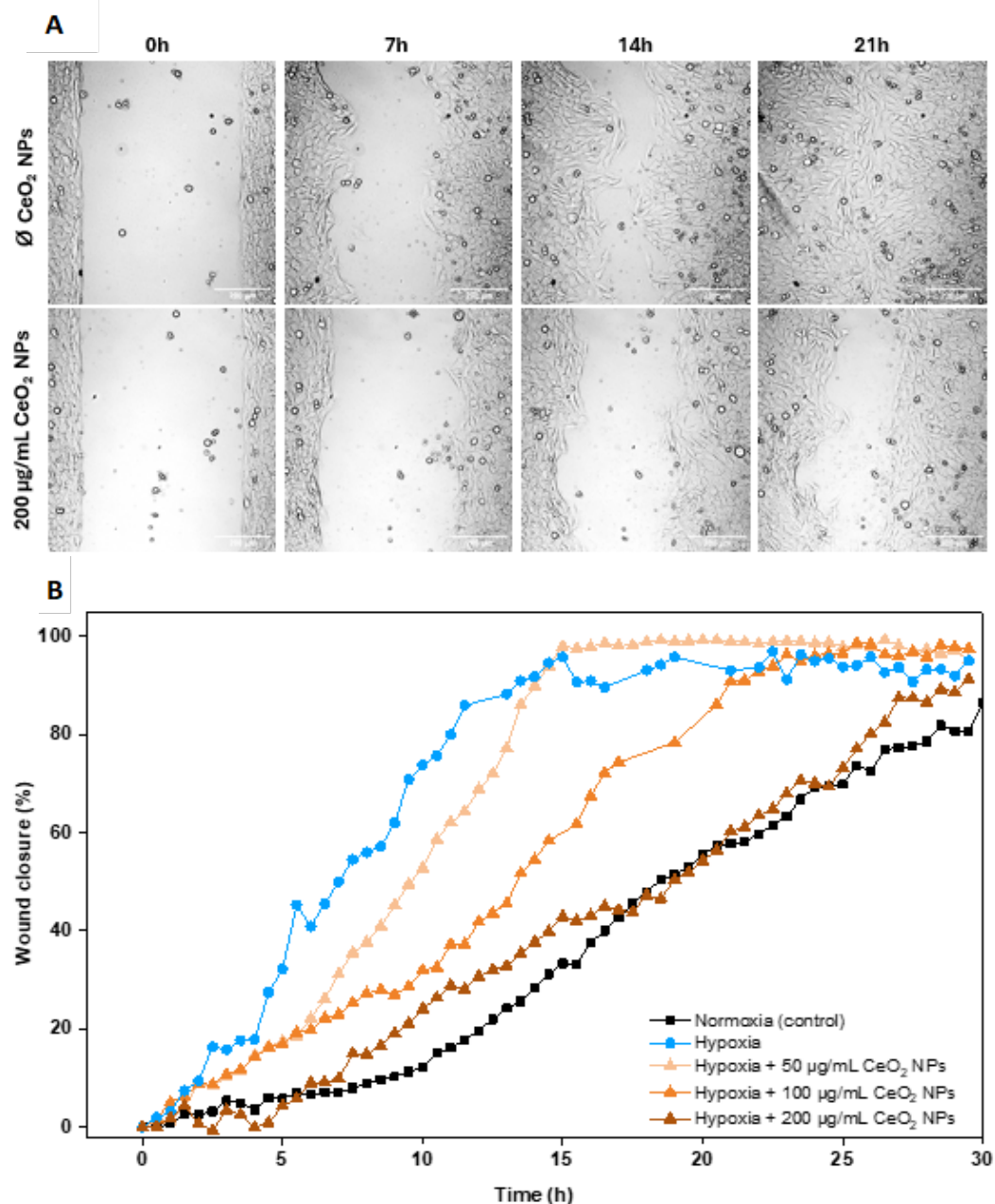


Figure 3.19. Effect of 0.5% O₂ hypoxia and CeO₂ NPs treatment on trophoblast migration (wound-healing assay). **(A)** Time-lapse imaging of HTR-8/SVneo trophoblast migration under hypoxia (0.5% O₂) with or without CeO₂ NPs. Representative frames at 0, 7, 14, and 21 h acquired on a Zeiss LSM 980 confocal microscope. The **upper panel** (no CeO₂ NPs) shows aberrant and rapid wound closure under hypoxia. The **lower panel** (CeO₂ NPs, 200 µg/mL) shows slower and more controlled closure. Scale bar, 200 µm. **(B)** Quantification of percent wound closure over time in normoxia (control), hypoxia (0.5% O₂), and hypoxia + CeO₂ NPs (50, 100, 200 µg/mL), derived from continuous time-lapse recordings on the Zeiss LSM 980.

Under hypoxia (0.5% O₂), as in our study, trophoblasts consistently show increased *MMP* expression and enhanced migration, indicating that low oxygen per se drives a pro-migratory, pro-gelatinolytic response that may reflect an early maladaptive phase of placental development rather than the fully established PE phenotype.^{71,72} In this experimental context, CeO₂ NPs effectively modulated *MMP* expression and migration, lowering *MMP2* and *MMP9* levels and normalizing trophoblast motility back towards normoxic values, in agreement with previous reports describing CeO₂ NPs as potent ROS scavengers with protective effects in wound-healing.^{54,69} When it comes to translating these findings to PE, the literature remains contradictory, as several studies report elevated circulating MMP-2 and MMP-9 in the serum or plasma of PE patients, whereas trophoblast MMP expression and migration appear reduced, highlighting the complexity of *in vivo* regulation of gelatinases across different stages.^{71,72,76}

3.2.9 Effects of CeO₂ NPs on the expression of pro-angiogenic factors VEGFA and PGF under 0.5% O₂ hypoxia for 48h

VEGF-A and PGF are key pro-angiogenic factors that regulate endothelial proliferation, migration and vascular permeability. Both factors belong to the VEGF ligand family and share substantial structural homology. PGF was originally identified in placental tissue and is predominantly expressed there during pregnancy, although it is also present in other tissues. During gestation, these molecules are essential for placental vascular development, contributing to trophoblast invasion, spiral artery remodeling and adequate uteroplacental perfusion. VEGF-A exerts its biological effects through vascular endothelial growth factor receptor 1 (VEGFR-1 or Flt-1) and vascular endothelial growth factor receptor 2 (VEGFR-2 or KDR/Flk-1), whereas PGF binds exclusively to VEGFR-1. In addition, sFlt-1, an alternatively spliced isoform of VEGFR-1, functions as a potent decoy receptor by binding and sequestering both VEGF-A and PGF.^{77,78} In preeclampsia, both VEGF-A and PGF levels have been reported as either elevated or decreased, and there is no clear consensus;^{78,79} however, studies describing reduced circulating levels support the concept

that increased sFlt-1 sequesters VEGF-A and PGF. Importantly, sFlt-1 is predominantly produced by cytotrophoblasts and syncytiotrophoblasts, with minimal expression in EVTs.^{77,80} Consistent with their EVT lineage, HTR-8/SVneo cells express very low basal levels of sFlt-1 mRNA and protein. Moreover, a study in BeWo cytotrophoblast cells showed that exogenous sFlt-1 did not decrease VEGF family levels but rather led to their upregulation.^{78,80} Even so, *VEGFA* and *PGF* gene expression was further analyzed in HTR-8/SVneo trophoblasts exposed to 0.5% O₂ for 48h (**Figure 3.20**).

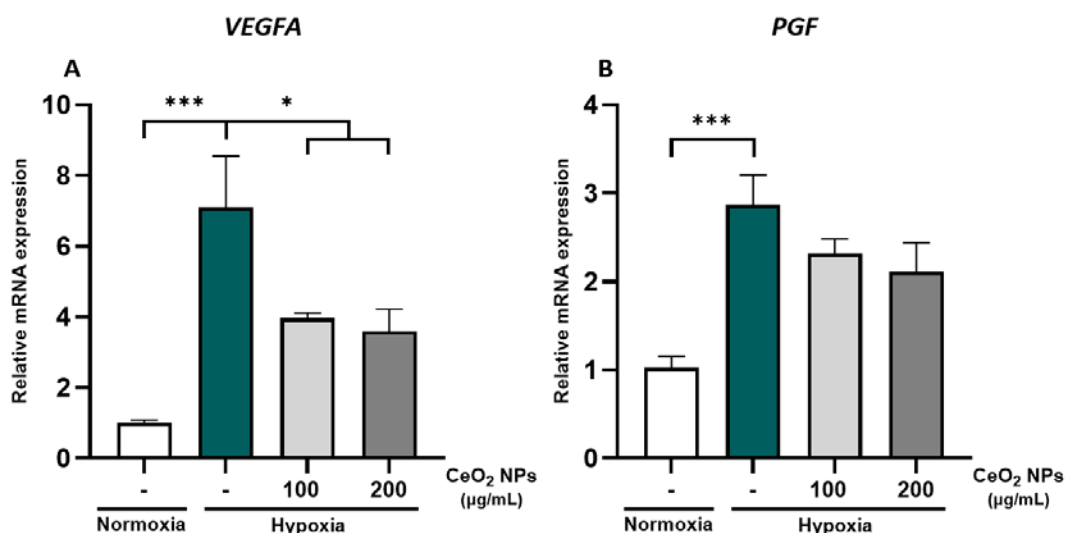


Figure 3.20. Relative mRNA expression of (A) *VEGFA* and (B) *PGF* in HTR-8/SVneo trophoblasts exposed to hypoxia (0.5% O₂) alone or treated with CeO₂ NPs (100 or 200 µg/mL) for 48h. mRNA levels were quantified by qRT-PCR, normalized to the reference gene *RPL37A*, and expressed relative to the basal (normoxic) condition. Data are presented as mean ± SD (n = 3). One-way ANOVA with Tukey's post hoc test; p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***).

Under severe hypoxia (0.5% O₂), both *VEGFA* and *PGF* were upregulated (**Figure 3.20**), with *VEGFA* showing a particularly pronounced increase (**Figure 3.20A**). Furthermore, treatment with CeO₂ NPs reversed this hypoxia-induced mRNA upregulation, indicating that CeO₂ NPs can attenuate the angiogenic response triggered by low oxygen tension. This behavior is consistent with the classical hypoxia-induced pro-angiogenic response driven by HIF-1α binding to HREs in the regulatory regions of *VEGFA* and *PGF*,^{81,82} further modulated by PI3K/Akt/mTOR and MAPK/extracellular signal-regulated kinase (ERK) signaling, inflammatory cytokines (for example IL-1β and IL-6) and hormonal cues such as estrogens,

progesterone and hCG.^{83–85} A plausible mechanistic explanation is that CeO₂ NPs, by scavenging ROS, influence these redox-sensitive pathways, thereby interfering with the transcriptional programs controlling *VEGFA* and *PGF*.

3.2.10 Effects of CeO₂ NPs on mitochondrial networks morphology and function under 0.5% O₂ hypoxia for 48h

Mitochondria are commonly classified by their morphology as (i) elongated, (ii) spherical and (iii) reticular networks; depending on the cellular environment, they dynamically transition between these forms. As highly adaptable organelles, they continuously remodel their architecture to meet (i) bioenergetic demands (sustain ATP production) and (ii) supply key catabolic intermediates for macromolecular synthesis, including nucleotides, proteins, and lipids.^{66,86} Further, the mitochondrial morphology is governed by two opposing but coordinated processes: fission and fusion. These processes are regulated physically by dynamin-related GTPases. Dynamin-related protein 1 (DRP1) mediates mitochondrial fission, whereas mitofusins (MFNs)1/2 and optic atrophy protein 1 (OPA1) orchestrate outer and inner mitochondrial membrane fusion, respectively. Moreover, the longstanding question of whether mitochondrial misshaping precedes dysfunction or vice versa remains under active debate. Current evidence indicates that each can initiate and reinforce the other, depending on the cell type and cellular context.^{86,87} Under hypoxia, however, the mechanistic sequence is comparatively well characterized. As already mentioned, mitochondria in a physiologically state consume the majority of intracellular O₂ to generate ATP through OXPHOS. However, reduced O₂ availability directly compromises electron transport, as O₂ serves as the terminal electron acceptor. When electron flow is impaired, primarily due to electron leakage at complexes I and III, proton pumping declines, causing a progressive loss of $\Delta\Psi_m$ (mitochondrial membrane depolarization) and enhanced ROS generation.⁸⁸ Consistent with this, classical studies demonstrated that defects in the ETC directly influence mitochondrial morphology.⁸⁷ In hypoxia, these abnormalities manifest as pronounced mitochondrial fragmentation and the appearance of donut-shaped

mitochondria, both hallmarks of $\Delta\Psi_m$ dissipation.⁸⁸ Mitochondrial dysfunction has also been associated with pregnancy complications; such as PE, where the first reported link between mitochondrial abnormalities and PE was published in 1989, when a family with mitochondrial dysfunction exhibited an unusually high incidence of the disorder. Since then, numerous studies have implicated impaired mitochondrial function in pregnancy pathologies.⁸⁹

Mechanistically, the loss of $\Delta\Psi_m$ is accompanied by alterations in intracellular Ca^{2+} and ROS, which jointly modulate DRP1, OPA1, and MFN1/2; this signaling cascade is observed in most conditions in which $\Delta\Psi_m$ is compromised. Increased cytosolic Ca^{2+} activates CaMKI α , which phosphorylates DRP1 at serine (Ser)616 to promote its pro-fission, fragmentation, conformation, and simultaneously activates the phosphatase calcineurin, which dephosphorylates DRP1 at Ser637, removing an inhibitory phosphate and facilitating DRP1 recruitment to the mitochondrial outer membrane. Elevated ROS further fine-tune this fission response by acting on upstream kinases and phosphatases (including ERK, PKC, and calcineurin), thereby reinforcing DRP1 activation. In parallel, a drop in $\Delta\Psi_m$ directly suppresses mitochondrial fusion by activating inner-membrane proteases, most notably metalloendopeptidase 1 (OMA1), which cleave long, fusion-competent OPA1 isoforms into short, fusion-deficient forms.^{90–93} Moreover, FUN14 domain-containing 1 (FUNDC1), one of the mitochondrial outer membrane proteins, has been recently reported to be a new DRP1 receptor that is specifically required for mitochondrial fragmentation in response to hypoxia. Collectively, FUNDC1 specifically mediates DRP1 recruitment and facilitates DRP1-dependent mitochondrial fission under hypoxic conditions.⁹¹

Furthermore, HIF-1 α emerges as a key factor governing mitochondrial behavior under hypoxic conditions and appears to play a dual role by (i) triggering mitochondrial fission and, at the same time, (ii) adapting mitochondrial function to the hypoxic environment. Several studies have focused on the role of HIF-1 α in the induction of mitochondrial fission under hypoxic conditions. It has been shown that HIF-1 α directly regulates DRP1

expression by binding to a hypoxia-responsive element within the DRP1 promoter. Additionally, HIF-1 α controls complex IV cytochrome c oxidase (mitCOX) subunit composition by promoting COX4-1 degradation and upregulating COX4-2 expression, thereby shifting mitochondrial respiration and favoring a metabolic transition toward anaerobic glycolysis.⁹³ This is consistent with our previous observations of increased GLUT-1 expression, which is closely associated with enhanced glycolytic flux under hypoxic conditions.

Interestingly, mitochondria may also undergo hyperfusion under hypoxic conditions when the level of damage is mild. This adaptive response enhances cellular resistance by allowing mitochondria to (i) optimize ATP production, (ii) promote more efficient electron transfer, (iii) use the remaining OXPHOS capacity more effectively, (iv) maintain membrane potential ($\Delta\Psi_m$), and (v) dilute partially damaged components through content mixing.^{91,92,94} In line with this, several studies have reported mitochondrial hyperfusion as a protective mechanism: for example, during nutrient depletion, the formation of a hyperextended mitochondrial network selectively protects mitochondria from autophagic degradation and increases cell survival. In contrast, inhibition of elongation forces mitochondria to consume ATP generating unsustainable ROS levels, ultimately leading to cell death. Notably, it has also been reported that centenarians exhibit an increased proportion of hyperfused mitochondria, suggesting that mitochondrial hyperfusion contributes to enhanced bioenergetic resilience against oxidative stress.⁹⁴

Building on this mechanistic framework, and given that hypoxia-driven mitochondrial fragmentation and loss of $\Delta\Psi_m$ are key features of trophoblast dysfunction in PE, the mitochondrial morphology and integrity of trophoblasts exposed to 0.5% O₂ and treated with 100 or 200 $\mu\text{g/mL}$ CeO₂ NPs for 48h were further investigated (**Figure 3.21**).

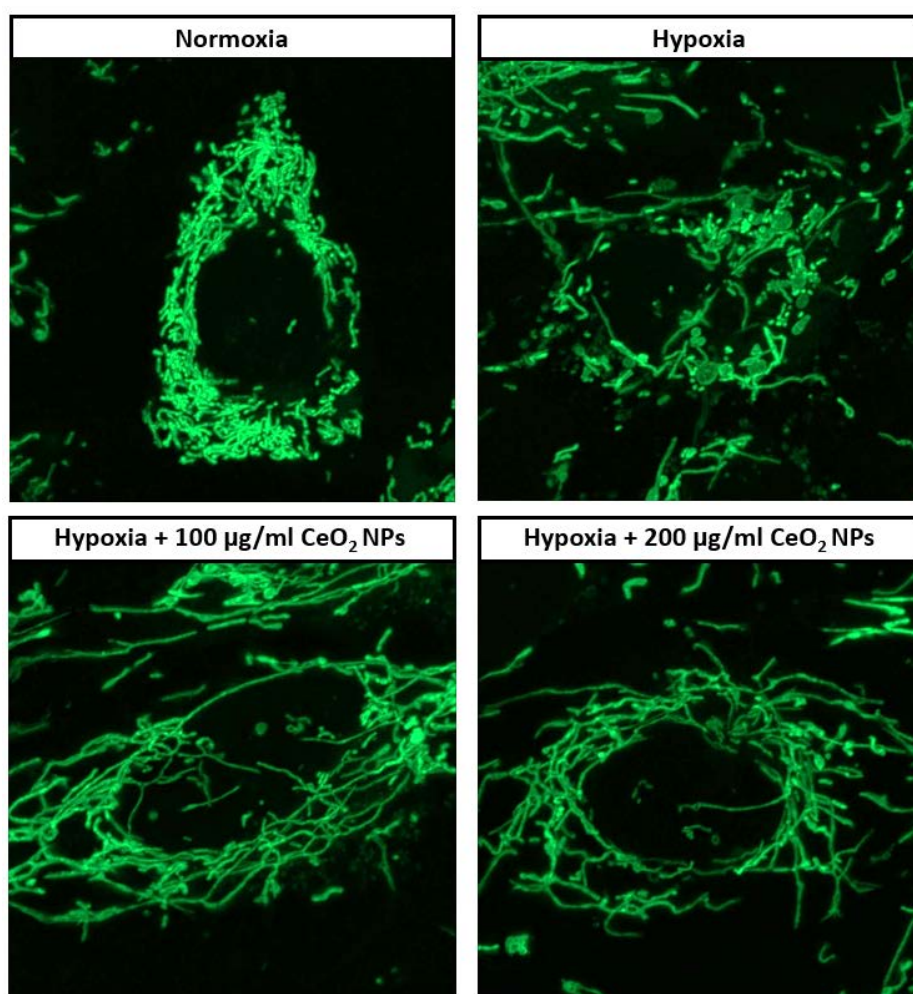


Figure 3.21. Confocal fluorescence microscopy (LSM 980 with Airyscan processing) images stained with MitoTracker™ Green to assess mitochondrial morphology and mass under different conditions: normoxia (top left), hypoxia (top right), hypoxia treated 100 µg/mL CeO₂ NPs (bottom left), and hypoxia treated 200 µg/mL CeO₂ NPs (bottom right).

The analysis was performed by confocal microscopy using MitoTracker Green, which labels mitochondrial mass, followed by quantitative assessment of multiple structural parameters, including the number of long mitochondrial branches (>100 µm/mitochondrion), mitochondrial sphericity, three-dimensional (3D) mitochondrial volume, mean object integrated density (sample intensity [a.u.] per volume [µm³]), and lacunarity, a measure of spatial heterogeneity closely linked to mitochondrial health and metabolic activity (Figure 3.22).

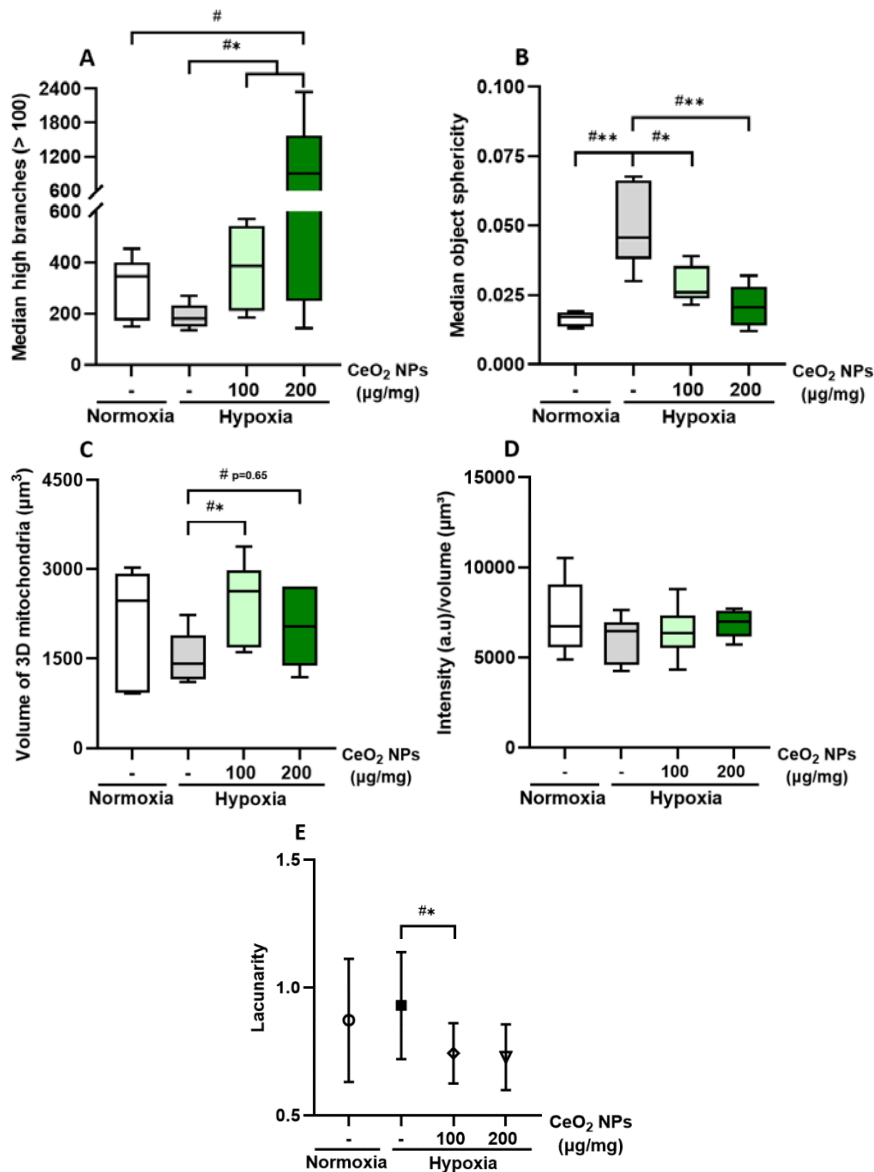
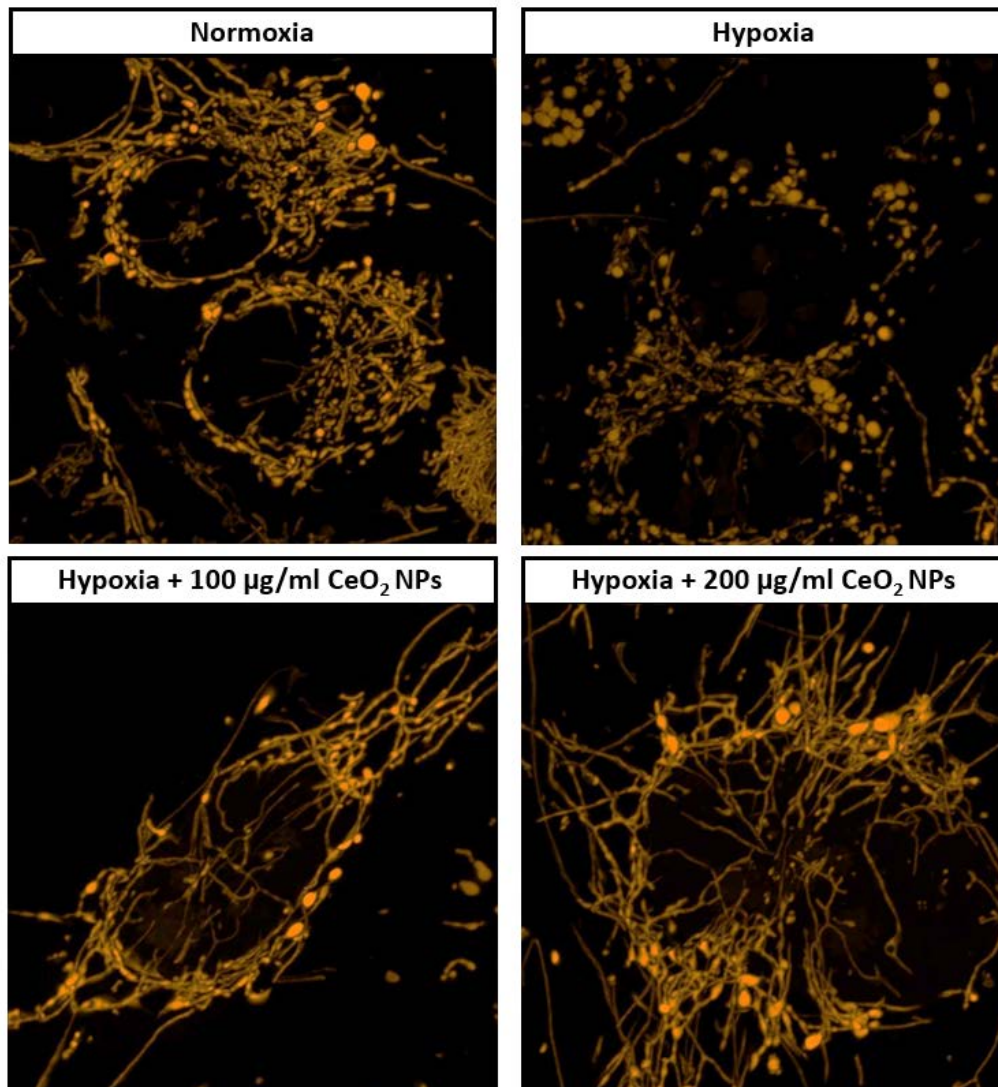


Figure 3.22. Effects of CeO₂ NPs on mitochondrial network architecture under normoxia and hypoxia (A) Median number of high-order branches (>100) per trophoblast. (B) Median mitochondrial object sphericity. (C) Volume of 3D mitochondria (µm³). (D) Fluorescence intensity normalized by mitochondrial volume (a.u./µm³). (E) Lacunarity of the mitochondrial network. Trophoblasts were cultured under normoxia or 0.5% hypoxia for 48h and treated with or without CeO₂ NPs (100 or 200 µg/mL). (n=6.000 ramified elements/condition) Data in panels A-D are represented as Tukey-style box-and-whisker plots. Panel E shows mean values with Tukey error bars. Statistical significance was assessed using (i) Kruskal-Wallis One-way ANOVA followed by Mann-Whitney U tests; significance levels are indicated as *p < 0.05, **p < 0.01, and (ii) bootstrap resampling (10.000 iterations) with 95% confidence intervals (CI) to estimate population-level effects; the # symbol indicates significance according to the bootstrap test.

To complete our analysis, mitochondrial membrane potential ($\Delta\Psi_m$) was quantified by flow cytometry using MitoTracker Orange (Figure 3.23).

A



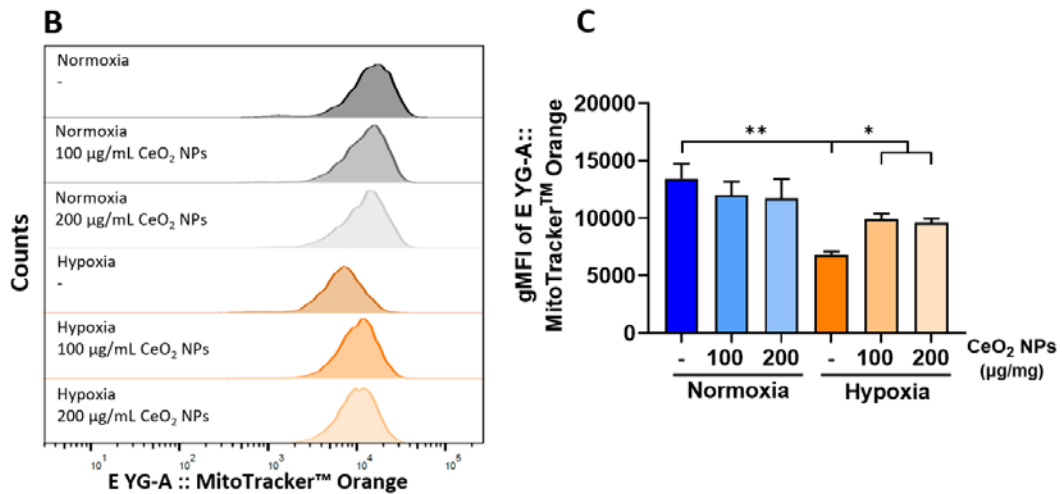


Figure 3.23. Mitochondrial membrane potential ($\Delta\Psi_m$) assessment using MitoTracker™ Orange CMTMRos under normoxic and 0.5% O₂ hypoxic conditions, with or without CeO₂ NPs. (A) Confocal fluorescence microscopy (LSM 980 with Airyscan processing) images of trophoblasts stained with MitoTracker™ Orange CMTMRos, a cell-permeant dye that accumulates in active mitochondria in a membrane potential ($\Delta\Psi_m$)-dependent manner, under normoxia (top left), hypoxia (top right), hypoxia treated with 100 µg/mL CeO₂ NPs (bottom left), and hypoxia treated with 200 µg/mL CeO₂ NPs (bottom right). **(B)** Representative histograms of MitoTracker™ Orange CMTMRos fluorescence acquired in the E YG-A channel, excited with the yellow-green laser (561 nm), in trophoblasts exposed for 48h to normoxia, normoxia + 100 µg/mL CeO₂ NPs, normoxia + 200 µg/mL CeO₂ NPs, hypoxia (0.5% O₂), hypoxia + 100 µg/mL CeO₂ NPs, or hypoxia + 200 µg/mL CeO₂ NPs. **(C)** gMFI of MitoTracker™ Orange CMTMRos measured by flow cytometry in the E YG-A channel, excited with the yellow-green laser (561 nm), in trophoblasts exposed for 48h to normoxia, normoxia + 100 µg/mL CeO₂ NPs, normoxia + 200 µg/mL CeO₂ NPs, hypoxia (0.5% O₂), hypoxia + 100 µg/mL CeO₂ NPs, or hypoxia + 200 µg/mL CeO₂ NPs. Kruskal-Wallis One-way ANOVA followed by Mann-Whitney U tests; significance levels are indicated as **p* < 0.05, ***p* < 0.01.

By examining the confocal images (**Figures 3.21** and **Figure 3.23A**), it is evident that under normoxic conditions trophoblasts display elongated well-preserved mitochondria. This morphology is indicative of preserved $\Delta\Psi_m$, efficient electron transport, and a metabolic profile relying predominantly on OXPHOS, and likely supported by FAO, which is known to be active in trophoblasts.⁹⁵ In contrast, hypoxic exposure induced a pronounced shift toward mitochondrial fission, with many mitochondria appearing fragmented, discontinuous, and in some instances adopting a characteristic donut-like shape.^{66,94} The quantitative morphometric analyses corroborated these qualitative observations. Hypoxic trophoblasts exhibited a significant reduction in the number of long mitochondrial branches and an increase in sphericity (*p* < 0.01), consistent with a fragmented phenotype.

These changes reflect a metabolic transition towards anaerobic glycolysis and reduced OXPHOS efficiency. Hypoxic cells also showed a slight tendency toward reduced mitochondrial volume, which may suggest the initiation of mitophagy, a process frequently, though not universally, reported in conditions of mitochondrial compromise.⁹⁶ Another informative measured parameter was lacunarity, which describes the heterogeneity and discontinuity of a sample. As expected, lacunarity increased under hypoxia, consistent with mitochondrial fragmentation and disorganization.

Notably, treatment with 100 $\mu\text{g/mL}$ CeO_2 NPs, and even more prominently with 200 $\mu\text{g/mL}$, effectively preserved mitochondrial structural integrity and fission. Mitochondria exposed to both NPs doses have longer mitochondrial branches ($p < 0.05$) (**Figures 3.22A**) and significantly reduced sphericity (**Figure 3.22B**), particularly at 200 $\mu\text{g/mL}$ ($p < 0.01$), indicating lesser fragmentation. Mitochondrial volume also increased slightly relative to hypoxia (alone) (**Figure 3.22C**), and lacunarity decreased (**Figure 3.22E**), reflecting a more homogeneous and continuous network consistent with a hyperfused state. This hyperfusion phenotype is well aligned with a known protective mitochondrial response that enhances cellular resilience under hypoxia by optimizing ATP production, improving electron transfer efficiency, sustaining $\Delta\Psi\text{m}$, and enabling content mixing between partially damaged mitochondria. Therefore, by decreasing ROS levels, CeO_2 NPs enhance the ability of trophoblasts to resist hypoxia-induced mitochondrial damage.

To go beyond the analysis of mitochondrial morphology, mitochondrial membrane potential ($\Delta\Psi\text{m}$) was assessed using MitoTracker™ Orange (**Figure 3.23**). The imaging data (**Figure 3.23A**), the fluorescence histograms (**Figure 3.23B**), and the gMFI values (**Figure 3.23C**) collectively indicate that $\Delta\Psi\text{m}$ is decreased under hypoxia, in agreement with the fragmented mitochondrial phenotype described above. Taken together, the loss of $\Delta\Psi\text{m}$ and the shift toward a fragmented network reinforce the idea of a metabolic transition relying on anaerobic glycolysis and reduced OXPHOS. Moreover, trophoblasts treated with CeO_2 NPs at both concentrations (100 and 200 $\mu\text{g/mL}$) displayed a right-shift of the

histogram and increased gMFI relative to hypoxia (alone), supporting the notion that CeO₂ NPs preserve mitochondrial bioenergetics by protecting $\Delta\Psi_m$ and therefore attenuating ROS-driven mitochondrial fission. Supporting the notion that CeO₂ NPs act upstream by reducing ROS levels; this alleviation of oxidative stress helps to preserve $\Delta\Psi_m$ and, as a consequence, limits ROS-driven mitochondrial fission.

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4.1 Introduction

4.1.1. Rat reproductive biology and pregnancy timeline

Rats exhibit an estrous cycle of about 4-5 days and are polyestrous, meaning they come into heat repeatedly throughout the year at regular intervals.^{1,2} Moreover, light (photoperiod) strongly influences the hypothalamus and regulates the timing of ovulation.³ The luteinizing hormone (LH) surge, which triggers ovulation, normally occurs in the late afternoon and is controlled by circadian rhythms, being activated as the light period ends.⁴ Consequently, this temporal pattern has practical implications, males are typically placed into the female's cage during the evening and removed the next morning, ensuring that mating coincides temporally with the natural LH surge and thereby maximizing the probability of copulation near ovulation.⁵ Furthermore, placing the male into the female's cage, rather than moving the female, also reduces her stress, since she remains in her own territory. Therefore, minimizing stress is essential because it can suppress LH release and therefore prevent ovulation.^{5,6}

Ovulation occurs approximately 9-10 hours after the onset of estrus. To facilitate accurate tracking of reproductive timing and physiological state, the stage of the estrous cycle can be reliably determined through vaginal cytology, which reveals four morphologically distinct phases (**Figure 4.1**).^{1,6,7} During proestrus, the smear is dominated by round or polygonal nucleated epithelial cells, with few leukocytes and only occasional cornified cells, as rising estrogen levels stimulate proliferation and maturation of the vaginal epithelium.⁷ In contrast, in estrus, elevated estrogen levels generate the characteristic cytology, the smear is dominated by cornified, anucleate squamous cells organized into dense sheets, with minimal leukocyte infiltration, a pattern that coincides with the peri-ovulatory window.^{6,7} Following the ovulation, the metestrus phase is marked by falling estrogen and rising progesterone levels after ovulation generate a mixed cytological pattern: cornified squamous cells, nucleated epithelial cells, and abundant infiltrating leukocytes are observed.¹ Finally, diestrus, progesterone dominance drives the characteristic leukocyte-

dominated smear, reflecting a phase when the vaginal epithelium is thinner and undergoing tissue remodeling and repair.^{6,7,1}

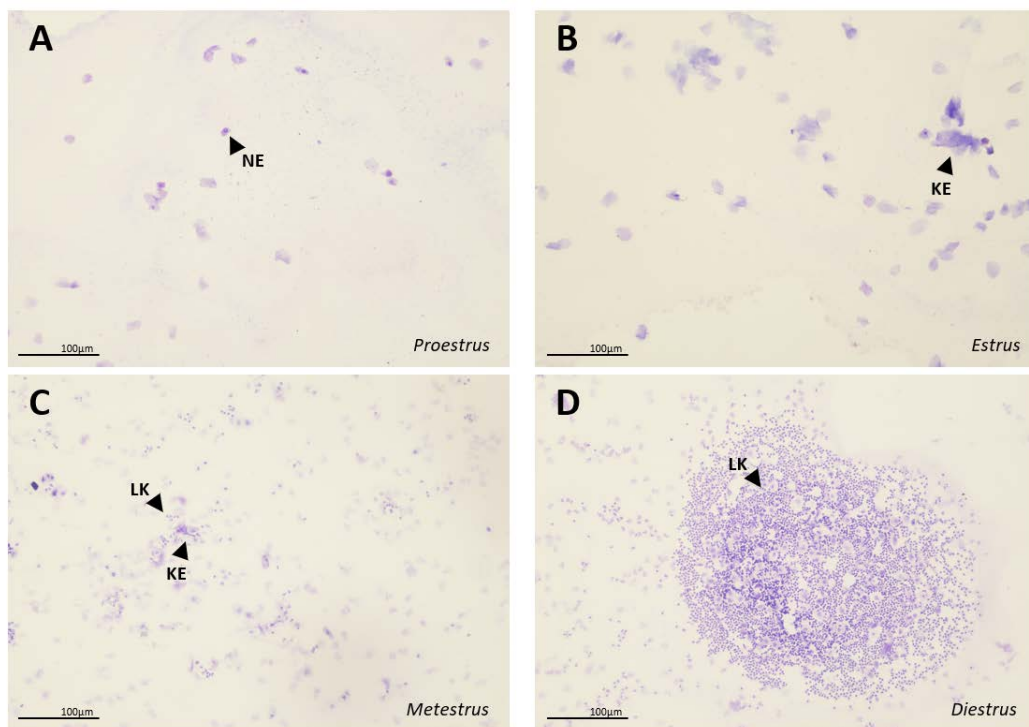


Figure 4.1. Representative vaginal cytology images illustrating the four stages of the rat estrous cycle. The main cell types observed include NE=nucleated epithelial cells), KE=keratinized/cornified epithelial cells, and LK=leukocytes. All images were captured at 10× magnification using a microscope from the VHIR animal facility, and samples were stained with crystal violet. **(A)** Proestrus. **(B)** Estrus. **(C)** Metestrus **(D)** Diestrus. Scale: 100µm.

These cytological markers thus enable precise identification of the optimal mating window and subsequent pregnancy confirmation. Although mating confirms copulation, it does not always guarantee a successful pregnancy.^{1,6,7} Typically ,time-mating is usually confirmed by the presence of a vaginal plug, essentially coagulated seminal material, and this day is recorded as gestational day (GD) 0.

Rat pregnancy lasts about 21-23 days and can be conceptualized as three roughly one-week “trimesters” for an easier comparison with women pregnancy.⁸ During the first week (GD0-7), fertilization, early embryo development, and implantation occur. The female

typically shows no external changes because embryos are still microscopic. This represents the most sensitive period of pregnancy: stress, additional handling, transport or cage changes can lead to embryo loss or pregnancy failure.^{8,9} For this reason, manipulation of pregnant females is not recommended during the first 7-10 days of gestation. Additionally, when early pregnancy loss occurs, rats usually do not expel the fetuses; instead, the embryos are resorbed, although slight vaginal bleeding is often observed. In the second week of pregnancy (GD7-14), organogenesis takes place. The uterus enlarges and shifts ventrally, and the female begins to appear larger, especially in the abdominal area. Appetite also increases markedly. Finally, during the third week (GD14-21), fetuses grow and mature rapidly. The abdomen becomes round and prominent, often described as looking “larger than a tennis ball”, the hips widen, nipples become more visible, and nest-building behavior begins. Therefore, caretakers should therefore provide nesting material during late gestation. Parturition is generally rapid, lasting about 90 minutes, and litters typically range from 3 to 18 pups. Newborn rats weigh around 5-6 g and are altricial: they are born with closed eyes and ears, poor thermoregulatory ability, and depend on the mother and littermates for warmth. The ears open around day 4, fur is fully developed by days 7-10, and the eyes open by the end of the second week.⁹

4.1.2. Preclinical rat models mimicking human preeclampsia background: mechanisms and features

Several animal models of PE in rats have been developed to reproduce the main pathophysiological mechanisms of the human disease, including systemic inflammation, placental ischemia, defective angiogenesis, and endothelial dysfunction.^{10,11} Among these, the reduced uterine perfusion pressure (RUPP) model induces placental ischemia of mechanical origin through the surgical placement of clips on the uterine arteries on gestational day 14.¹¹⁻¹³ This procedure leads to maternal hypertension, proteinuria, FGR and marked endothelial dysfunction, faithfully mimicking the hemodynamic and placental alterations observed in human PE.^{13,14} Another widely used model is the one induced by

LPS, a component of the outer membrane of Gram-negative bacteria.^{10,15,16} Two main serotypes are commonly used, O55:B5 and O111:B4, depending on the intended immune response.^{16,17} Both activate the toll-like receptor 4 (TLR4) receptor, but the O111:B4 serotype exhibits greater pyrogenic potency due to structural differences in its lipid A moiety, which confers higher affinity for the TLR4/ myeloid differentiation factor 2 (MD2) complex and triggers a stronger inflammatory response, with significant increases in several cytokines (e.g., IL-6, CCL-2, IL-8).^{10,15,16} Systemic administration of LPS during gestation produces generalized inflammation, hypertension, proteinuria, FGR, and endothelial dysfunction, in addition to renal and placental damage in some cases.^{10,15}

The nitric oxide deficiency model is based on the administration of the competitive NO synthase inhibitor L-NAME in drinking water at doses between 40 and 125 mg/kg/day starting from gestational days 9 or 10 (Shu et al., 2018; Xu et al., 2024).^{11,18} This treatment blocks the conversion of L-arginine to NO, causing systemic and placental vasoconstriction, severe hypertension (greater than 30 mmHg), reduced uteroplacental perfusion, FGR, increased sFlt-1 and endothelin-1 levels, and generalized endothelial dysfunction.^{18,19} Likewise, the exogenous administration of anti-angiogenic factors such as sFlt-1 or soluble endoglin reproduces the alteration in the VEGF/PGF-sFlt1 balance characteristic of PE, resulting in hypertension, glomerular proteinuria, and endothelial damage.^{20,21} Similarly, administration of TNF- α , a proinflammatory cytokine, intravenously or intraperitoneally during mid-gestation produces a systemic inflammatory state, increased IL-6 and IL-1 β levels, endothelial dysfunction (reduced NO bioavailability and increased platelet adhesion), along with mild hypertension and glomerular proteinuria mediated by activation of NF- κ B in the vascular endothelium and placenta.^{10,22} Finally, the use of polyinosinic-polycytidylic acid (Poly I:C), a synthetic analog of double-stranded RNA, allows modeling of antiviral immune responses by acting as a toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I) agonist, inducing innate immune activation, systemic inflammation, and placental functional alterations.^{10,23}

In this context, the LPS O111:B4 model was selected to evaluate CeO₂ NPs because it provides a robust and reproducible induction of systemic inflammation that closely mimics the inflammatory profile observed in PE. Specifically, this serotype's potent TLR4 activation elicits marked cytokine elevation and pronounced endothelial dysfunction, thus creating an oxidative-inflammatory environment that is particularly suitable for assessing the antioxidant and anti-inflammatory potential of CeO₂ NPs as a possible tool to treat PE.

4.1.3. PE-like model with LPS B111:O4 serotype at GD14-15

In our study, a PE-like phenotype is induced in pregnant rats by administering LPS serotype O111:B4 at GD 14 or 15, mimicking a late-onset inflammatory model of PE. LPS is the major glycolipid component of the outer membrane of gram-negative bacteria and a potent activator of the innate immune system. It functions as a classical pathogen-associated molecular pattern (PAMP) and is recognized primarily by the TLR4/MD-2 complex, which is expressed on maternal immune cells (monocytes, macrophages, dendritic cells), endothelial cells, trophoblasts, and vascular smooth muscle cells.^{15,24,25} Before reaching its receptor, circulating LPS binds to two accessory molecules: LPS-binding protein (LBP), a protein that captures LPS in the bloodstream, and CD14, a co-receptor that facilitates transfer of LPS to the TLR4/MD-2 complex. CD14 exists as both a membrane-bound form on myeloid cells and as a soluble form in plasma. Together, LBP and CD14 function as a delivery system that enhances LPS recognition and drives efficient activation of TLR4-mediated signaling.

Upon LPS O111:B4 engages TLR4/MD-2, two major intracellular pathways are activated: (i) the myeloid differentiation primary response 88 (MyD88)-dependent and (ii) the TIR-domain-containing adapter-inducing interferon- β (TRIF)-dependent pathways. The MyD88 pathway rapidly activates NF- κ B and multiple MAPK (ERK, JNK and p38), which in turn stimulate transcription factors such as activator protein-1 (AP-1) and promote expression of numerous inflammatory genes including TNF- α , IL-6, IL-1 β , CCL-2, COX-2 and inducible nitric oxide synthase (iNOS). Parallel, the TRIF pathway induces interferon regulatory factor

3 (IRF3) and interferon regulatory factor 7 (IRF7) activation, leading to production of type I interferons (e.g., IFN- β) and contributing to the sustained, late-phase activation of NF- κ B. In addition to these canonical pathways, LPS can also provide a priming signal for the NLRP3 inflammasome, facilitating maturation of IL-1 β and amplifying inflammatory responses.^{15,24,26,27} Altogether, this signaling network produces a robust systemic inflammatory burst that closely resembles the exaggerated maternal inflammatory state described in advanced human PE. Consistent with this mechanism, low-dose LPS exposure during mid-to-late gestation (GD14-15) in pregnant rats has been shown to increase (i) maternal systolic blood pressure, (ii) induce proteinuria and renal dysfunction, (iii) trigger a systemic cytokine cascade, (iv) activate the maternal endothelium, and (v) impair spiral artery remodeling and placental development, collectively reproducing key clinical and pathophysiological features of PE.^{15,28} Comparative evaluations of rodent PE models consistently report that LPS-based models are among the most effective for mimicking immune-driven, inflammatory forms of the disease.¹⁰

The choice of GD14-15 for LPS administration is critical. By this stage of gestation, the embryo has already been implanted; therefore, LPS exposure is not expected to interfere with implantation itself. In rats, spiral artery remodeling predominantly occurs between GD14 and GD19.²⁹ However, despite this remodeling window, we did not observe significant placental damage in our study. This finding underscores the predominant maternal systemic inflammation induced by LPS-TLR4 engagement,^{10,30} with limited direct impact on placental architecture under our experimental conditions. Importantly, this pattern mirrors the pathophysiology of human late-onset PE, characterized by maternal endothelial dysfunction and systemic inflammation, rather than placental defective architecture typical of early-onset disease.³¹⁻³³ Thus, our model more accurately recapitulates the maternal inflammatory features of late-onset PE. Accordingly, our pilot study, the most prominent LPS effects were observed at the maternal level, including elevated circulating cytokines, renal histological alterations, and increased arterial blood pressure, which will be detailed throughout this chapter.

4.2 Results and Discussion

4.2.1. Experimental design and work flow

This study was conducted using Sprague-Dawley females' rats (10 weeks old, 280-300 g), obtained from Charles River Laboratories (France). All procedures were performed in accordance with institutional guidelines and were approved by the local ethics committee (*protocol "14/22 CEEA: Biodistribución y efecto terapéutico de nanopartículas de CeO₂ en modelos de rata de pre-eclampsia"*). Animals had free access to standard chow and water and were provided with wooden sticks for environmental enrichment, in order to minimize stress and to control dental health. Female dams were paired with fertile males at a 2:1 ratio, O/N. The presence of a vaginal plug the following morning was considered confirmation of successful mating and was designated as GD 0, as previously explained in **section 4.1.1**. The subsequent experimental workflow is depicted in **Figure 4.2**.

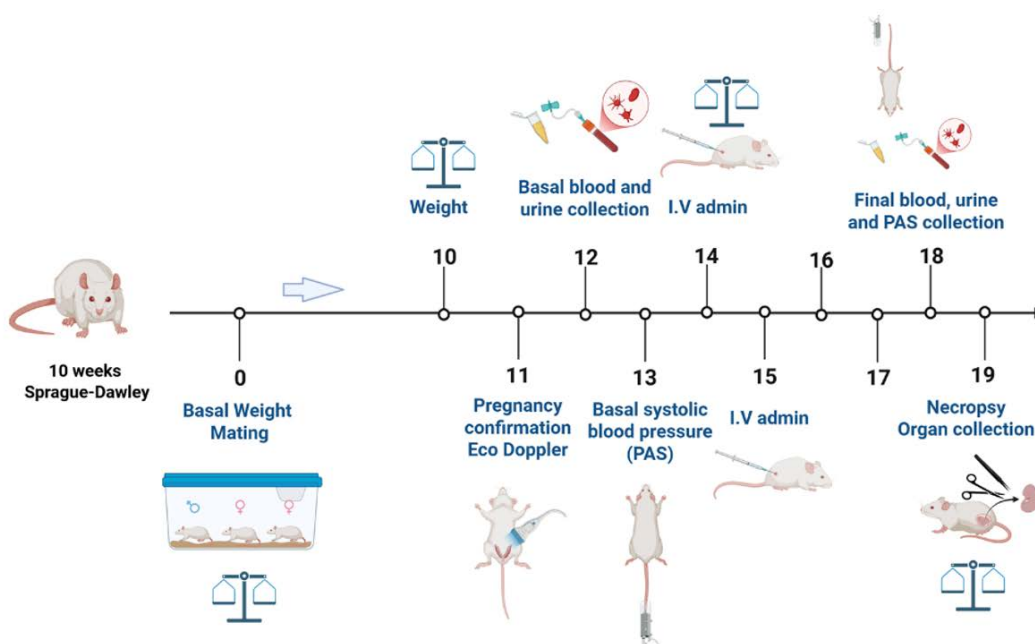


Figure 4.2. Experimental timeline of the preliminary pilot study evaluating the protective effects of CeO₂ NPs in an LPS-induced preeclampsia model.

Prior to mating, body weight was recorded and subsequently monitored throughout gestation (GD0, GD10, and GD19), as it serves as an indicator to confirm and follow pregnancy. After the first weight measurement and mating, a gap between GD0 and GD10 was intentionally left. This period corresponds to the first trimester of pregnancy, a critical window during which fetal loss is more likely to occur. Our initial aim in this experimental pilot study was to establish an early PE model based on published protocols in which low-dose LPS injections were administered on GD5, corresponding to the period of placental formation. To this end, different LPS doses (10, 1, and 0.5 $\mu\text{g LPS kg}^{-1}$ body weight (bw)) and administration routes (I.V. = intravenous; I.P. = intraperitoneal) were tested to optimize the model (**Table 4.1**). However, these early administrations resulted in rapid pregnancy loss in our case, occurring within 2 to 12 hours post-injection, or with fetuses that were not yet fully developed and already undergoing resorption. In addition, fetuses appeared dark in color, a characteristic sign of LPS exposure (**Figure 4.3**)^{34,35}



Figure 4.3. Representative macroscopic appearance of darkly colored uterine horns containing aborted or resorbed fetuses following maternal exposure to different concentrations of LPS. Panels are ordered from more to less advanced gestational stage: (A) most advanced fetal structures, (B) intermediate stage, and (C) earliest or least developed resorption sites. Photographs were taken in the animal facility of the VHIR.

Because our main objective was to evaluate whether CeO_2 NPs could attenuate inflammation and oxidative stress at both the systemic level and within placental tissue, we ultimately adopted a late-PE model.^{15,28,36–43}

As detailed in **Table 4.1**, in the late-gestation model, animals tolerated LPS doses of 1–10 $\mu\text{g LPS kg}^{-1}$ bw but experienced significant pregnancy loss, consistent with previous studies reporting dose-dependent fetal toxicity at higher LPS concentrations.^{10,44} In contrast, when

the dose was reduced to 0.5 µg LPS kg⁻¹ bw, rats tolerated the LPS injection and maintained pregnancy without fetal loss. Consequently, a final LPS dose of 0.5 µg LPS kg⁻¹ bw was selected for this primary pilot study, which was designed to assess whether CeO₂ NPs could mitigate LPS-induced oxidative stress and inflammation in the PE preclinical model. In parallel, CeO₂ NP dosing was defined based on both the available literature and our group's previous experience.²⁴ A final working concentration of 630 µg CeO₂ NPs kg⁻¹ bw was selected as a conservative yet pharmacologically relevant dose, balancing safety with sufficient antioxidant capacity for this pilot study.^{45–54} Note that concentrations exceeding 1000 µg CeO₂ NPs kg⁻¹ bw are widely reported to induce toxicity.⁴⁹

Doses (µg LPS kg ⁻¹ bw)	Admin. route	Model	Status	Dead dams (only LPS admin.)
10	I.P.	Early PE	Aborted	0/4
1	I.P.	Early PE	Aborted	1/6
1	I.V.	Early PE	Aborted	0/6
0.5	I.V.	Early PE	Aborted	0/6
10	I.V.	Late PE	Aborted	1/6
1	I.V.	Late PE	Aborted	0/6
0.5	I.V.	Late PE	At necropsy	0/9

Table 4.1. Rats reaching necropsy after LPS treatment across PE models and administration routes: Exploration of early and late PE models.

Additionally, minimal mortality was observed in the pregnant dams across all conditions (Table 4.1); however, all animals displayed behavioral and physical signs derived from LPS administration. These signs will be further described in Section 4.2.3.

Following the timeline, at GD11, pregnancy was confirmed using high-resolution ultrasonography Doppler imaging (echo-Doppler), which allowed *in vivo* visualization of the gestational structures in a non-invasive manner. During the examination, the fetus (F) could be clearly identified inside the uterine cavity (U), indicated by the blue delineated area. (see Figure 4.4). The ultrasound probe was gently placed over the lower abdominal region of the anesthetized rat, that had previously been shaved enabling the acquisition of

sagittal and transverse images of the uterus. This technique provided reliable confirmation of successful implantation and fetal development and allowed to exclude early embryonic resorption or spontaneous abortion, which are common risks during the first trimester (day 0 - 10 in rats).

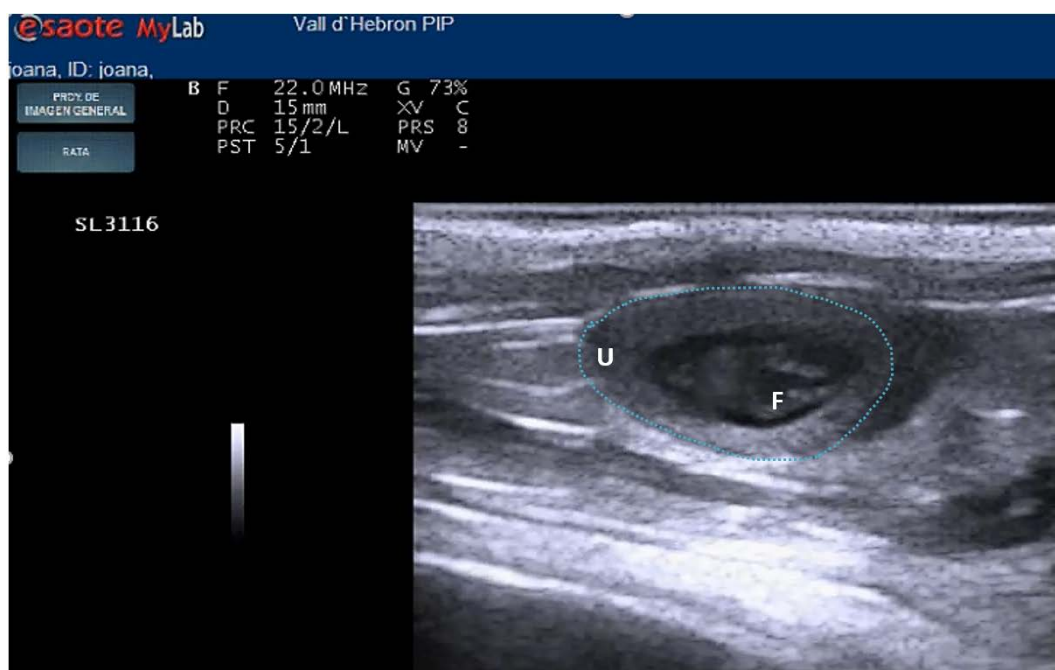


Figure 4.4. Ultrasound doppler image of a pregnant rat on GD11 showing the uterus cavity (U) and fetus (F) within the blue delineated area.

Following pregnancy confirmation, rats were randomized into four experimental groups:

Group	Description
1	Saline-treated (control)
2	LPS-administered
3	CeO ₂ NPs + LPS-administered (prophylactic CeO ₂ NPs)
4	LPS + CeO ₂ NPs-administered (treatment CeO ₂ NPs)

Table 4.2. Experimental groups used in the pilot study: (1) Control, (2) LPS-administered, and (3) prophylactic or (4) treatment CeO₂ NPs-administered.

Prior to any injection, baseline measurements were obtained. The first morning urine was collected and blood samples were drawn into heparinized tubes at GD12. Systolic blood pressure (SBP) was assessed at GD13 using a tail-cuff blood pressure measurement system coupled to a pressure transducer. Subsequently, on GD14 and GD15, rats received an intravenous tail-vein injection of either saline (control) or LPS solution. In the prophylactic CeO₂ NP group, nanoparticles were injected one day before LPS administration, while in the treatment group, they were injected one day after LPS exposure. On GD18, final urine and blood samples were collected and SBP was measured again. At GD19, animals were euthanized and necropsy was performed. During this postmortem examination, organs were harvested for cerium quantification by ICP-MS, and placentas and kidneys were collected and fixed for subsequent hematoxylin-eosin (H&E) analyses.

4.2.2. Dose administration schedule and sample preparation

Several injectable formulations were prepared and administered according to the experimental design (**Table 4.3**). The experimental design comprised four groups designed to compare basal pregnancy conditions, LPS-induced inflammation, and the protective efficacy of CeO₂ NPs administered either before (prophylaxis: CeO₂ NPs + LPS) or after (treatment: LPS + CeO₂ NPs) the inflammatory challenge. Each group received specific combinations of 1X PBS (sham control), LPS (inflammation inducer), or CeO₂ NPs at defined gestational days. The first administration took place on GD 14, followed by a second administration 24 hours later on GD 15. All injections were performed i.v. at a fixed volume of 0.5 mL per dose. In the prophylaxis group, CeO₂ NPs were delivered prior to LPS exposure, whereas in the treatment group, CeO₂ NPs were administered after the LPS challenge. This dosing strategy enabled direct comparison of basal conditions, LPS-induced inflammatory responses,¹⁰ and the potential preventive versus treatment effects of CeO₂ NPs on pregnancy outcomes.

	Basal	LPS	Prophylaxis (CeO ₂ NPs + LPS)	Treatment (LPS + CeO ₂ NPs)
Dams/group	4	4	3	2
Admin. I (GD 14)	1X PBS	1X PBS	630 µg CeO ₂ NPs kg ⁻¹ bw	0.5 µg LPS kg ⁻¹ bw
Route	I.V.	I.V.	I.V.	I.V.
mL	0.5	0.5	0.5	0.5
Admin. II (GD 15)	1X PBS	0.5 µg LPS kg ⁻¹ bw	0.5 µg LPS kg ⁻¹ bw	630 µg CeO ₂ NPs kg ⁻¹ bw
Route	I.V.	I.V.	I.V.	I.V.
mL	0.5	0.5	0.5	0.5

Table 4.3. Experimental design of animal groups, treatments and administrations.

Regarding the preparation of injectable solutions, 1X PBS was used for basal control injections. For LPS formulations, the desired concentration of 0.5 µg LPS kg⁻¹ bw was freshly dissolved in 1X PBS 24h prior to injection. The LPS serotype used was *Escherichia coli* O111:B4, a well-established endotoxin that activates the TLR4/MD-2/CD14 receptor complex and triggers a robust pro-inflammatory response characterized by strong induction of cytokines (TNF-α, IL-1β, IL-6, IL-8) and chemokines (CCL2), as detailed in **Section 4.1.2**. This serotype is commonly employed in pregnancy inflammation models due to its reproducibility and potent systemic immunostimulatory properties. Moreover, the injectable containing the CeO₂ NPs was formulated by conjugating the NPs with rat serum albumin (RSA) following the same strategy described in **Section 3.2.2 (Figure 4.5)**. Because CeO₂ NPs are intended for intravenous (i.v.) administration, exposure to the high ionic strength of blood promotes their aggregation, thereby compromising NP stability and potentially triggering unwanted immune responses. Surface passivation with albumin was therefore essential to prevent NP agglomeration and enhance biocompatibility. CeO₂ NPs at the injectable dose of 630 µg kg⁻¹ bw were pre-functionalized with RSA to achieve this stabilization. Albumin coating provides electrostatic repulsion that counteracts van der Waals attractions and prevents NP agglomeration, ensuring that all NPs follow a similar absorption, distribution, metabolism, and excretion (ADME) profile. This uniform behavior reduces inter-group variability and mitigates undesirable immunogenicity,^{55–60} as nanoparticle aggregation has been correlated with enhanced immune responses.^{61,62} Albumin is particularly well-suited for this purpose. It is a ubiquitous, non-immunogenic

protein that is abundant in circulation and has high affinity for particle surfaces, making it an excellent stabilizer for CeO₂ NPs.^{63,64}

Considering the geometric ratios, CeO₂ NPs with a diameter of 3 nm present a surface area of 19.63 nm² per particle. In comparison, a single RSA protein has dimensions of approximately 6-8 nm, corresponding to an estimated surface area of 30-35 nm² depending on molecular orientation.⁶³ Under these conditions, the theoretical coverage suggests a 1:1 ratio, where one RSA molecule is sufficient to fully coat one CeO₂ NP. To ensure complete saturation of the NP surface and avoid partially coated populations, conjugation was performed using a two-fold molar excess of RSA relative to the theoretical requirement.^{64,65} This strategy follows the approach successfully implemented in our previous albumin-functionalization studies, in which an excess of protein is used rather than the minimum required amount. As in those protocols, CeO₂ NPs were always added to the protein solution prepared in 7.4mM 10 mM phosphate buffer (PB) rather than 1X PBS, as PB has significantly lower ionic strength.^{66,67} This ensured that albumin remained in large excess throughout the adsorption process and prevented NP aggregation upon administration.⁶⁸

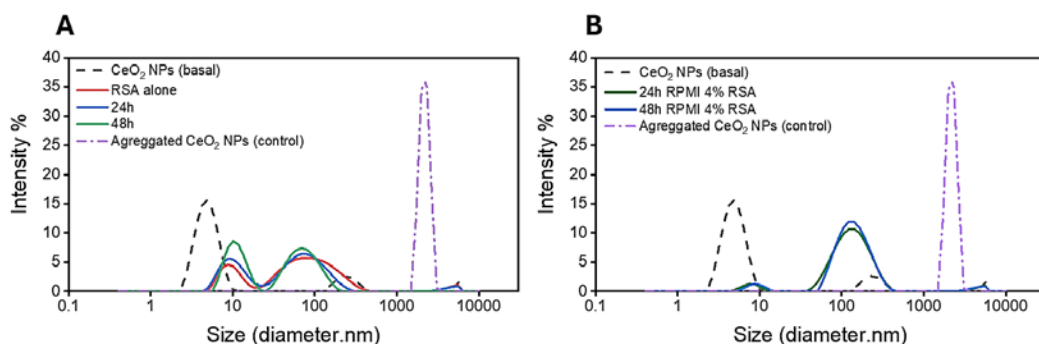


Figure 4.5. Stability study of the conjugated injectable CeO₂@RSA nanoconjugates formulation. (A) Hydrodynamic size distribution of the injectable CeO₂-RSA NPs over time. **(B)** Stability of the injectable CeO₂-RSA NPs formulation in RPMI medium containing 4% RSA after 24h and 48h.

The stability of the CeO₂@RSA nanoconjugate was characterized through DLS analysis. CeO₂ NPs were conjugated with RSA at a 2-fold molar ratio. As shown in **Figure 4.5A**, control samples included non-conjugated CeO₂ NPs (basal, dashed black line) displaying the

characteristic ~ 4.5 nm peak, and RSA alone (solid grey line). The conjugated CeO_2 @RSA nanoconjugates were evaluated at two time points: 24h (solid blue line) and 48h (solid red line) post-conjugation, and compared against an aggregation control consisting of CeO_2 NPs in 1X PBS (dashed purple line). 1X PBS has significantly higher ionic strength than PB, as discussed previously, which promotes NP aggregation and serves as a positive control for destabilization. The results demonstrated that CeO_2 @RSA nanoconjugates showed no significant aggregation compared to the basal control across both time points, confirming effective RSA-mediated stabilization. To further assess stability under physiologically relevant conditions, the CeO_2 @RSA formulation was subsequently evaluated in a protein-rich medium mimicking the *in vivo* environment of rat plasma. As shown in **Figure 4.5B**, the CeO_2 @RSA nanoconjugates were incubated in RPMI medium supplemented with 4% RSA for 24h and 48h. This concentration was selected because serum albumin in rats typically ranges from 3.8-4.8 g/dL (approximately 4%), making 4% RSA a physiologically relevant approximation of the albumin concentration expected in rat circulation⁶⁹ Under these albumin-rich conditions, the CeO_2 @RSA nanoconjugates maintained colloidal stability, being substantially smaller than the aggregated control (dashed purple line). This stability profile in RPMI + 4% RSA confirms that the nanoconjugate formulation is resistant to aggregation in a biologically relevant medium, supporting its suitability for intravenous administration to rats.

4.2.3 Behavioral and physical assessments of pregnant rats under LPS-induced preeclampsia and the therapeutic potential of CeO_2 NPs

Prior to biochemical analyses and biodistribution studies, clinical parameters were recorded during the *in vivo* phase to assess the general well-being of the pregnant dams. Clinical signs were evaluated using a 0-10 scoring scale based on rodent welfare assessment systems described by Fentener van Vlissingen et al. (2015).⁷⁰ In this system, behavioral and physical signs were scored 24 hours after each injection, GD14 and GD15 (**Figures 4.5**), conceptually ranging from absent (0) to severe (7-10).

Behavioral observations and physical signs served as sensitive indicators of well-being, highlighting the extent of LPS-induced damage (Figure 4.6 and Figure 4.7). LPS administration precipitated a drastic deterioration in the animals' clinical condition compared to the basal group, characterized by a sharp increase in signs of pain and general discomfort. This sickness behavior was clearly manifested through profound lethargy, a severe loss of exploratory activity (rearing), and the adoption of an arched posture, a classic clinical sign of abdominal pain and systemic illness, along with a mild reduction in feeding speed (Figure 4.6).

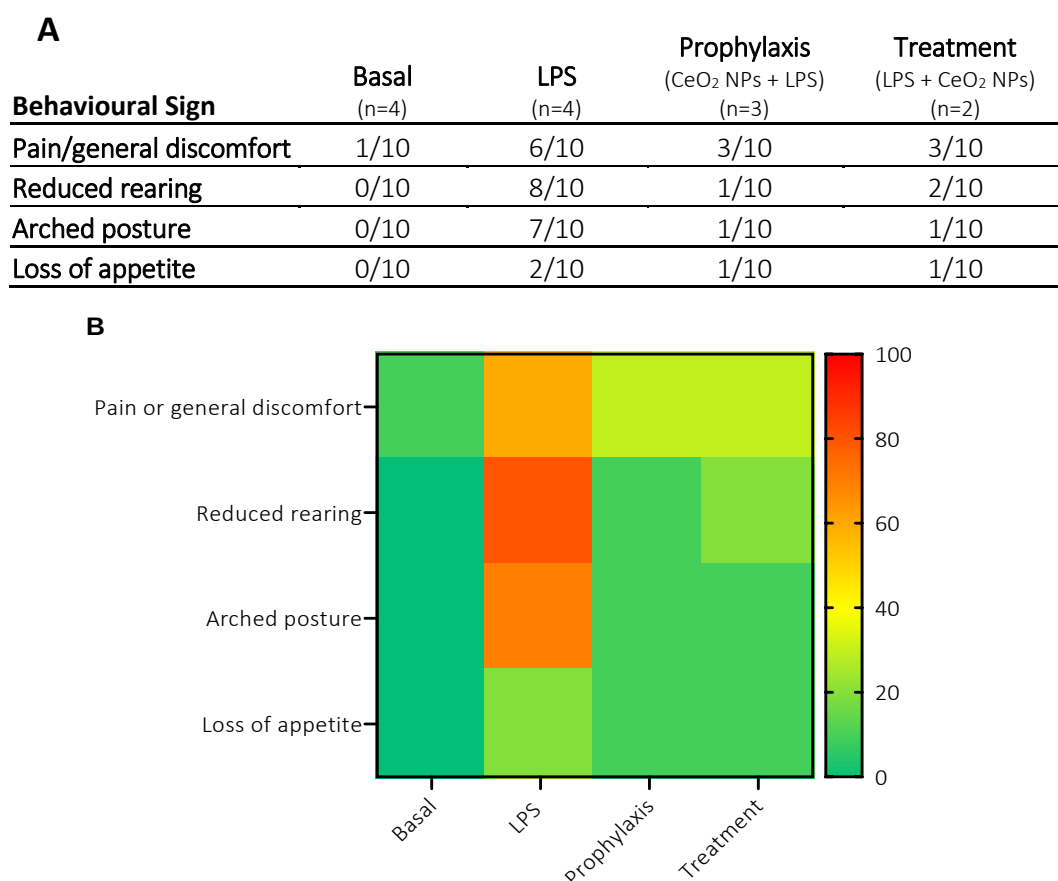


Figure 4.6. Behavioral alterations induced by LPS and modulation by CeO₂ NPs. (A) Clinical scores for each behavioral sign in the different experimental groups, as summarized in the accompanying table. Behavioral signs were scored on a 0-10 scale, where 0 = absent, 1-3 = mild, 4-6 = moderate, and 7-10 = severe. **(B)** Heatmap visualization of the clinical scores shown in panel A.

Parallel to these behavioral changes, physical signs provided complementary evidence of severe systemic stress consistent with the pathological effects of LPS in pregnant dams. Ruffled fur and slitted eyes, universal signs of poor condition and distress in rodents, were manifested severely in nearly all animals exposed to LPS. Furthermore, markers of physiological stress were significantly exacerbated; specifically, porphyrin secretion (chromodacryorrhea), a reddish pigment produced by the Harderian glands, increased beyond the mild baseline levels induced by routine handling. The inflammatory state also disrupted thermoregulation and caused signs of peripheral vascular compromise, evidenced by mild tail necrosis. No abdominal hematomas or vaginal bleeding were detected (Figure 4.7).

A

Physical Sign	Basal (n=4)	LPS (n=4)	Prophylaxis (CeO ₂ NPs + LPS) (n=3)	Treatment (LPS + CeO ₂ NPs) (n=3)
Ruffled fur	0/10	9/10	4/10	5/10
Weight loss	0/10	0/10	0/10	0/10
Slitted eyes	0/10	9/10	1/10	2/10
Vaginal bleeding	0/10	0/10	0/10	0/10
Porphyrin	2/10	5/10	3/10	3/10
Altered temperature	0/10	4/10	2/10	2/10
Abdominal hematoma	0/10	0/10	0/10	0/10
Tail necrosis	0/10	3/10	1/10	2/10
Tail infection	0/10	0/10	0/10	0/10

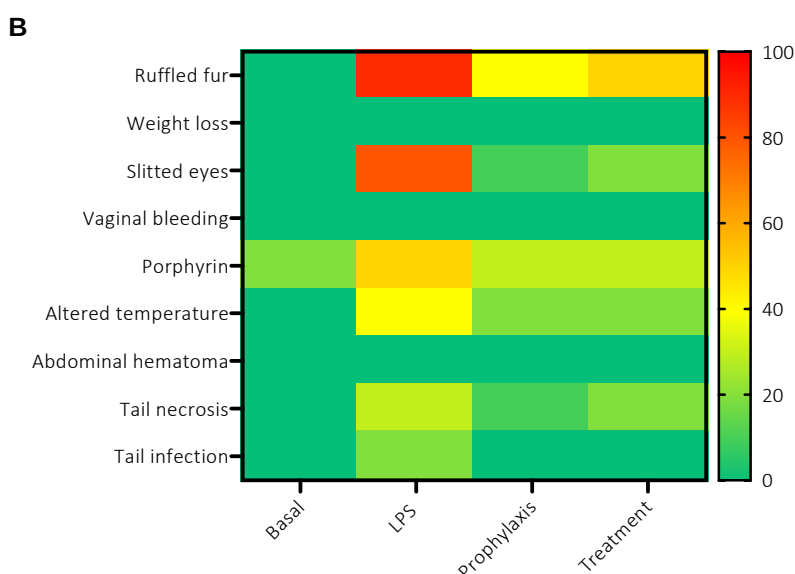


Figure 4.7. Physical alterations induced by LPS and modulation by CeO₂ NPs. (A) Clinical scores for each physical sign in the different experimental groups, as summarized in the accompanying table. Physical signs were scored on a 0-10 scale, where 0 = absent, 1-3 = mild, 4-6 = moderate, and 7-10 = severe. **(B)** Heatmap visualization of the clinical scores shown in panel A.

In contrast, the administration of CeO₂ NPs effectively attenuated this underlying inflammatory distress, preventing the development of severe pathological alterations and improving the animals' general well-being. This therapeutic effect was evident across the majority of parameters analyzed. CeO₂ NPs preserved the dams' mobility and vitality, marked by a reduction in postural deficits, and led to dramatic improvements in eye morphology and coat condition. Physiologically, animals that received CeO₂ NPs showed reduced porphyrin hypersecretion and stabilized body temperature, while partially preventing peripheral necrosis. Taken together, the heatmaps clearly illustrate this overall trend (**Figure 4.6B** and **Figure 4.7B**), whereas LPS induces a severe physical and behavioral alterations, CeO₂ NPs efficiently revert these indicators toward milder values closer to the control group. While both therapeutic regimens proved beneficial, the prophylaxis group consistently offered superior protection, suggesting that the pre-emptive presence of CeO₂ NPs effectively prevent the inflammatory cascade and oxidative stress from the onset. It is worth noting that, for ethical reasons and to minimize the number of animals used, a group

receiving NPs without LPS was not included. However, in the prophylaxis group, no behavioral alterations attributable to CeO₂ NPs were detected during the 24-hour period prior to LPS injection.

4.2.4 Fetal and maternal weight outcomes in an LPS-induced gestational model of PE and the effect of CeO₂ NPs

In order to further evaluate the impact of maternal inflammation on pregnancy outcomes in our LPS-induced gestational model of PE, two key parameters commonly used in LPS-induced gestational preclinical models were quantified. (i) The fresh weight of the pups as an indicator of FGR (**Figure 4.8A**) and (ii) the progression of maternal body weight throughout gestation (**Figure 4.8B**). Although these measures are widely reported across studies, the literature reveals substantial variability in the magnitude and direction of the effects induced by LPS, and no clear consensus has been reached regarding the extent of fetal or maternal alterations.^{8,15,18,21,44} Therefore, we report here the outcomes specifically observed under our experimental conditions.

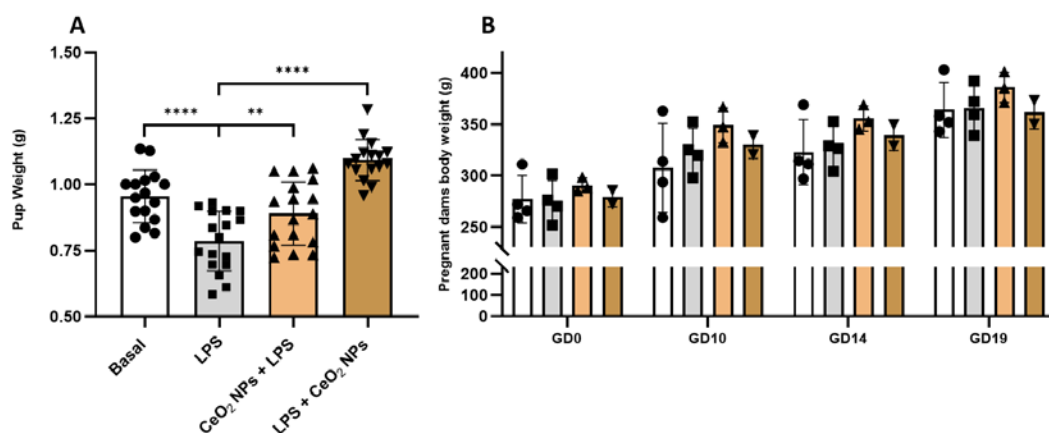


Figure 4.8. Fetal and maternal weight (g) outcomes in the LPS-induced gestational model of PE. (A) Fresh pup weight at necropsy in the Basal, LPS, CeO₂ NPs + LPS (prophylaxis), and LPS + CeO₂ NPs (treatment) groups. **(B)** Pregnant dams body weight throughout gestation (GD0, GD10, GD14, GD19). Data are presented as mean $\pm$ SD. Statistical analysis: one-way ANOVA followed by Holm-Šidák post hoc test; significance levels are indicated as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p < 0.0001$ (****).

As shown in **Figure 4.8A**, maternal exposure to 0.5 µg LPS kg⁻¹ bw resulted in a clear reduction in fetal growth. Control dams (basal group) exhibited an average pup weight of approximately 0.95 g, whereas LPS-treated animals showed a decreased mean of 0.79 g, corresponding to a 17% reduction in fetal growth. Prophylactic administration of CeO₂ NPs prevented this effect, displaying a mean pup weight of 0.9 g, representing an improvement of 14% relative to the LPS group. The treatment administration of CeO₂ NPs produced an even more pronounced effect, with a mean pup weight of 1.1 g, corresponding to an approximate 39% increase compared with LPS. These results suggest that CeO₂ NPs prevent from developing LPS-induced FGR under both experimental conditions. Furthermore, maternal body weight progression was evaluated throughout the *in vivo* experiment (**Figure 4.8B**) and did not differ significantly among groups. Nevertheless, it is interesting to note that all groups exhibited two characteristic phases of gestational weight increase: an approximate 40 g rise between GD0 and GD10, followed by another 40 g (approx.) increase between GD14 and GD19, with a very stable plateau from GD10 to GD14. Taken together, these results show that while LPS significantly impaired fetal growth, maternal weight gain patterns were largely preserved, and CeO₂ NPs provided a notable protective effect in preventing FGR.

4.2.5 Systolic blood pressure changes in an LPS-induced gestational model of PE and the effect of CeO₂ NPs

To complement the evaluation of fetal and maternal weight outcomes, SBP was assessed as a key hemodynamic parameter in the LPS-induced gestational model of PE (**Figure 4.9**). SBP variation (Δ SBP) was calculated as the difference between late gestation and baseline values for each dam, allowing comparison of treatment-induced changes across groups.

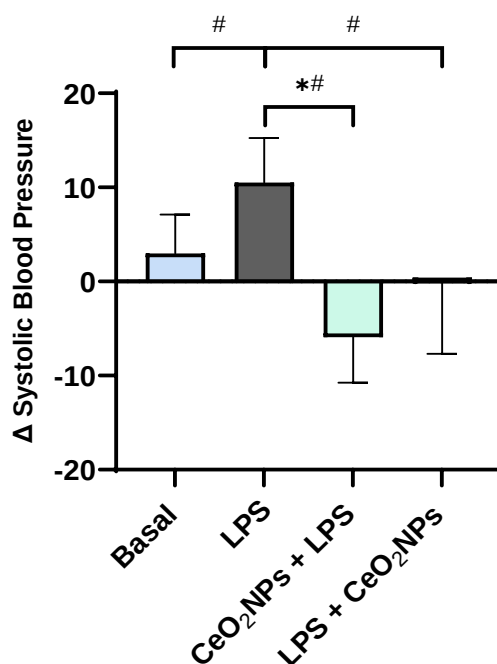


Figure 4.9. Changes in systolic blood pressure (Δ SBP) in the LPS-induced gestational model of PE. Measurements taken in the Basal, LPS, CeO₂ NPs + LPS (prophylaxis), and LPS + CeO₂ NPs (treatment) groups. Data are presented as mean $\pm$ SD. Statistical analysis was performed using a Kruskal-Wallis one-way ANOVA followed by pairwise Mann-Whitney U tests; significance levels are indicated as (*) $p < 0.5$. In addition, a bootstrap resampling analysis (10,000 iterations, 95% CI) was conducted to estimate population-level differences; the # symbol denotes significance according to the bootstrap test.

As shown in **Figure 4.9**, dams in the basal group exhibited only a modest increase in SBP over gestation, consistent with normal physiological adaptation to pregnancy. In contrast, LPS exposure induced a marked rise in SBP, with an average Δ SBP of approximately +10 mmHg, confirming the development of a hypertensive phenotype in this gestational model. Prophylactic administration of CeO₂ NPs prior to LPS challenge (CeO₂ NPs + LPS group) effectively prevented the LPS-induced hypertensive response, resulting in a Δ SBP close to 0 mmHg and values comparable to basal conditions.

When CeO₂ NPs were administered after LPS exposure (LPS + CeO₂ NPs treatment group), Δ SBP also remained near 0 mmHg, indicating normalization of the LPS-induced increase in systolic pressure to levels similar to the basal group. These findings indicate that LPS

triggers a significant rise in systolic blood pressure in pregnant dams, and that CeO₂ NPs exert a notable protective effect, both in prophylaxis and treatment regimens, by preventing the development of gestational hypertension in this model.

4.2.6 Serum cytokine profile in the LPS-Induced PE model and nodulation by CeO₂ NPs

Maternal serum cytokines, CCL-2, IL-6 and TNF- α , were quantified in preripheral blood (?) to evaluate the systemic inflammatory response induced by LPS and the potential immunomodulatory and anti-inflammatory effect of CeO₂ NPs, either as prophylaxis or treatment. In this pilot study, LPS serotype O111:B4 was administered on GD14 and GD15, as previously described in **Section 4.1.2** and **4.2.2**. Blood was collected 24h after the last injection in heparinized tubes, centrifuged to obtain the serum fraction, and stored at -20°C until analysis. ELISA readouts were normalized to total serum protein content. Consistent with activation of the TLR4-MyD88 pathway and downstream NF- κ B and MAPK (ERK, JNK and p38) signaling, LPS exposure triggered measurable changes in inflammatory mediators, particularly CCL-2 and IL-6, both of which are associated with acute systemic inflammation and monocyte recruitment (**Figure 4.10**).^{15,24,26,27}

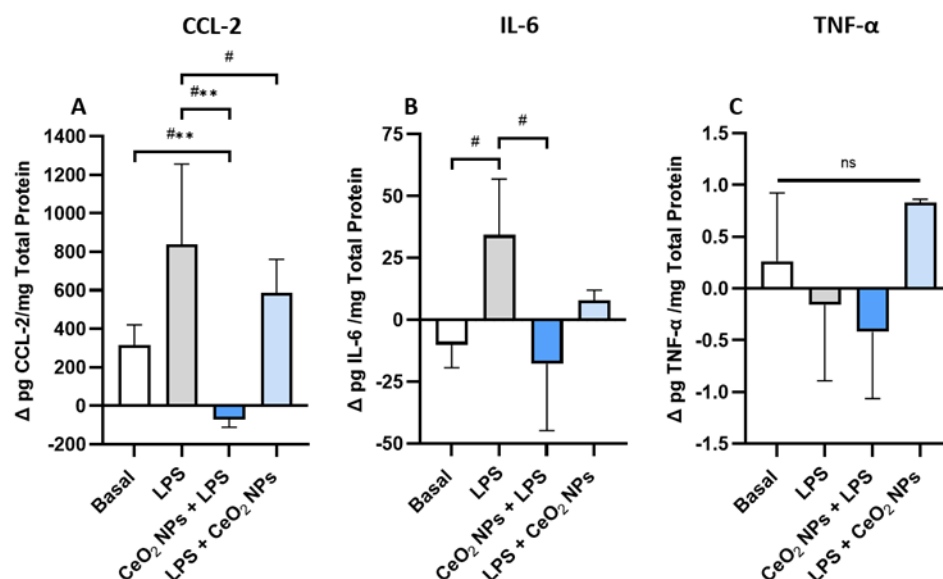


Figure 4.10. Changes (Δ) in maternal serum cytokine concentrations between pre (basal) and post (24h after the last administration). (A) CCL-2, (B) IL-6, and (C) TNF- α were quantified by ELISA in isolated maternal serum samples stored at -20°C until analysis. Cytokine values are expressed as Δ (post-pre) and were normalized to total serum protein content (pg cytokine/mg total protein). Data are presented as mean $\pm$ SD. Statistical analysis was performed using a Kruskal-Wallis one-way ANOVA followed by pairwise Mann-Whitney U tests; significance levels are indicated as (*) $p < 0.1$ and (**) $p < 0.05$. In addition, a bootstrap resampling analysis (10,000 iterations, 95% CI) was conducted to estimate population-level differences; the # symbol denotes significance according to the bootstrap test.

As shown in **Figure 4.10A-B**, LPS exposure produced an overall upward shift in both serum CCL-2 and IL-6 compared with the basal group, consistent with an induced systemic pro-inflammatory response. For IL-6, this increase was statistically supported, by bootstrap (95% CI) for the basal vs LPS groups, indicating a significant elevation of IL-6 in LPS-treated dams. For CCL-2, a comparable increase was observed at the descriptive level, although this change did not reach statistical significance, despite a clear upward trend. In the prophylaxis group (CeO₂ NPs + LPS), both cytokines showed the strongest attenuation. CCL-2 levels were markedly reduced relative to LPS, with the decrease reaching statistical significance ($p < 0.05$) and also confirmed by bootstrap resampling, bringing concentrations close to baseline values. IL-6 followed the same qualitative pattern, serum IL-6 decreased in the prophylaxis group, and bootstrap confidence intervals again supported a statistically significant reduction. The treatment group (LPS + CeO₂ NPs), in which CeO₂ NPs were

administered after LPS, showed a more moderate attenuation. CCL-2 levels were partially reduced, and although the effect was less pronounced than in the prophylaxis group, the reduction reached statistical significance in the bootstrap resampling analysis. IL-6 also exhibited a downward trend relative to the LPS group, but again the magnitude of this decrease was smaller than that observed under prophylactic administration. Overall, these data indicate that the most pronounced and statistically supported effects occur in the prophylaxis group (CeO₂ NPs + LPS), particularly for CCL-2, reinforcing the notion that pre-exposure to CeO₂ NPs is more effective at attenuating the LPS-induced systemic inflammatory response than post-insult. A milder, yet still observable, modulatory effect was also present in the treatment group.

Additionally, TNF- α levels did not differ significantly between groups (**Figure 4.10C**). TNF- α typically peaks within approximately 3h after LPS exposure and is known to have a very short-lived expression peak,⁷¹ whereas serum in this study was collected at 24h after administration, which likely explains the absence of detectable changes. Moreover, because additional handling in the dams was found in preliminary tests to increase miscarriage rates, an earlier blood-collection time point was deliberately avoided in order to preserve pregnancy.

4.2.7. Maternal-fetal biodistribution of cerium ions in an LPS-induced rat model of preeclampsia: prophylaxis versus therapeutic

Understanding the absorption, distribution, metabolism, and excretion (ADME) of CeO₂ NPs is fundamental for evaluating their pharmacokinetics and safety, particularly in pregnancy, where both maternal and fetal exposure must be rigorously assessed. However, the specific literature addressing the ADME of CeO₂ NPs during gestation is still limited. Some experimental studies have reported adverse pregnancy outcomes following CeO₂ NP exposure, and several works describe measurable nanoparticle or cerium content in the placenta, whereas far fewer provide robust data regarding fetal levels. It is important to emphasize that nanoparticle-related toxicity in pregnancy is highly dependent on

formulation and exposure parameters, including (i) nanoparticle size, (ii) surface coating, (iii) synthesis route, (iv) excipients, (v) surface conjugation, and (vi) injection route, which together determine particle charge and biological interactions.^{72,73} Dose is also critical, as CeO₂ NPs administered at inappropriate or excessively high concentrations may become toxic and have been associated with pregnancy impairments and miscarriage in some reports, as occurs with other drugs. Importantly, the toxicological thresholds reported in the literature vary widely: some studies have described systemic toxicity at doses of 1 mg CeO₂ NPs kg⁻¹ bw,⁴⁹ as mentioned previously, whereas others reported no adverse effects until doses exceeded 300 mg CeO₂ NPs kg⁻¹ bw. These discrepancies reflect the profound influence of NPs physicochemical properties (size, surface chemistry, Ce³⁺/Ce⁴⁺ ratio), administration route, and biological context on toxicity outcomes, indicating that toxicity outcomes are strictly determined by the particular formulation and dosage of the CeO₂ NPs.^{48,49}

For any drug or biomedical material, ADME provides the framework for determining:

- Absorption (A): How a substance enters the body and reaches the bloodstream.⁷²
- Distribution (D): How it distributes among organs and tissues once in the circulation.^{72,73}
- Metabolism (M): Whether and how it undergoes chemical or enzymatic transformation (for example in the liver), which can activate, inactivate, or increase its solubility.⁷²
- Excretion (E): How it is ultimately removed from the body, mainly via urine (kidney) or bile/feces (liver), and to a lesser extent through other routes (sweat, breath, milk).^{72,74}

In the case of our study, which uses intravenously administered CeO₂ NPs, absorption (A) is bypassed because the entire injected dose (ID) reaches the bloodstream immediately. Therefore, the most relevant ADME components are distribution (D) and excretion (E), together with overall clearance behavior. Once in circulation, nanoparticle biodistribution

is governed by (i) hydrodynamic size, (ii) surface charge, (iii) proteins at the surface, and (iv) the anatomical and physiological characteristics of individual organs and tissues.^{72,74,75}

Excretion can follow two main pathways: (i) hepatobiliary clearance, in which particles processed by the liver are excreted into bile and ultimately eliminated in feces; and (ii) renal elimination, which is largely restricted to dissolved ionic species or very small particles capable of crossing the glomerular filtration barrier.^{75,76}

Rodent studies have repeatedly shown that intravenously administration of CeO₂ NPs accumulate preferentially in the liver, typically around 80% of the injected dose with the spleen acting as the secondary major site of deposition, largely independent of physiological or disease state.^{50,51,54,77} When nanoparticles follow the hepatobiliary route, circulating material is recognized and sequestered by liver and spleen macrophages of the mononuclear phagocyte system (MPS), including Kupffer cells in the liver and splenic macrophages, which remove a substantial fraction of the material from the bloodstream and thereby limit its access to renal filtration.^{64,78} The sequestered material is then processed in the liver, transported into bile, passed into the intestines, and ultimately excreted in feces. In the case of NPs coated with albumin, like CeO₂ NPs@RSA, this clearance tendency is further supported by the role of albumin, which can promote interaction with albumin-binding endothelial and hepatic receptors such as glycoprotein 60 (gp60), secreted protein acidic and rich in cysteine (SPARC), and the neonatal Fc receptor (FcRn), which are highly expressed in the liver and spleen.^{78,79} Such receptor-mediated processes are thought to favor hepatic uptake and help explain why hepatobiliary and MPS-driven elimination often outweigh renal pathways.

To characterize the ADME profile of nanocerium in pregnancy, we investigated the general and maternofetal biodistribution of intravenously administered CeO₂ NPs in a Sprague Dawley pregnant rat model of LPS-induced inflammation, with a special focus on whether CeO₂ NPs reach the placenta or fetus and how maternal inflammation influences this process (**Figure 4.11**). CeO₂ NPs of approximately 3 nm primary size were synthesized under sterile, endotoxin-free conditions and coated with RSA. Each rat received a 0.5 mL

intravenous injection of the CeO₂ NP suspension, delivering a total dose of 189 µg CeO₂ NPs (equivalent to 153.8 µg Ce), which corresponds to approximately 630 µg CeO₂ NPs kg⁻¹ bw or 512.5 µg Ce kg⁻¹ bw. This route of administration ensured immediate systemic availability, bypassing epithelial absorption barriers and minimizing variability in bioavailability.

To quantify cerium accumulation across maternal and fetal tissues and evaluate whether systemic inflammation alters nanoparticle deposition, two complementary experimental schemes were analyzed by ICP-MS. In the prophylaxis group, CeO₂ NPs were administered on GD 14, followed 24h later by LPS on GD 15. Under these conditions, maternal tissues had not yet undergone LPS-induced injury when NPs entered the circulation; thus, vascular permeability and macrophage activation were expected to be relatively low, predicting more limited organ uptake. In the treatment group, the order was reversed: LPS was administered first, and CeO₂ NPs were injected 24h later. Here, NPs encountered an already inflamed maternal milieu characterized by endothelial dysfunction, increased vascular permeability, conditions predicted to significantly alter biodistribution, potentially increasing retention in organs of the mononuclear phagocyte system.²⁸

In both groups, all organs, placentas, and fetuses were collected on GD 19, immediately frozen at -20 °C, and stored until analysis. Cerium quantification was performed by ICP-MS, the gold standard for trace elemental analysis due to its high sensitivity. Cerium concentrations were calculated by interpolation against a calibration curve prepared from a 1000 µg/gram certified cerium standard. Prior to measurement, tissues underwent complete mineralization using an acidic mixture of 45% HNO₃, 5% H₂O₂ and 50% H₂O in a microwave digestion system for 120 min at 200 °C, ensuring full dissolution of both organic matrices and CeO₂ NPs, thus NPs are converted into soluble ionic ceria.

The resulting biodistribution profiles under both prophylactic and treatment conditions were highly consistent with the classical behavior of intravenously administered CeO₂ NPs

described in the literature and previously observed by our group (Figure 4.11).^{50,51,54,77} In all animals, regardless of administration scheme, the liver was the dominant site of accumulation, capturing approximately 80.1% of the injected dose in the prophylaxis group (Figure 4.11A) and 89.6% in the treatment group (Figure 4.11B). This strong hepatic retention reflects the central role of the hepatobiliary system in NP clearance. The spleen acted as the second major repository, although with much lower accumulation (3% in both groups). The relative distribution among the remaining tissues followed a conserved descending pattern in both schemes: lung > kidney > placenta > blood > brain > fetus.

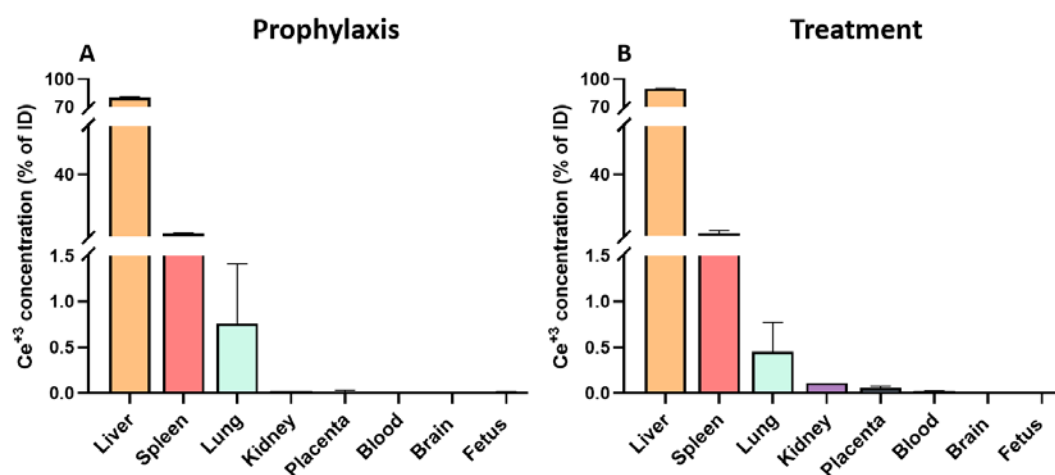


Figure 4.11. Biodistribution of elemental cerium in maternal organs in 10-week-old pregnant Sprague-Dawley rats with LPS-induced inflammation treated with intravenous CeO₂ NPs given either before or after LPS. **(A)** Ce concentrations in maternal organs (n=3) in the prophylaxis group (CeO₂ NPs → LPS). **(B)** Ce concentrations in maternal organs (n=2) in the treatment group (LPS → CeO₂ NPs).

Excretion patterns further supported predominant hepatobiliary clearance. Detectable cerium levels were found in feces samples, confirming elimination via the biliary route, whereas urinary cerium levels were minimal, indicating negligible renal filtration of intact nanoparticles. It is important to note that excreta were collected only on the day of necropsy (GD 19), rather than continuously throughout the experiment, in order to minimize animal handling and stress, thereby reducing the risk of spontaneous abortion in these sensitive pregnancies. Although this sampling strategy precludes a mass-balance calculation of total excretion, the results remain highly valuable as they qualitatively

reinforce the hepatobiliary excretion hypothesis, consistent with the high cerium content observed in fecal matter (Figure 4.12).

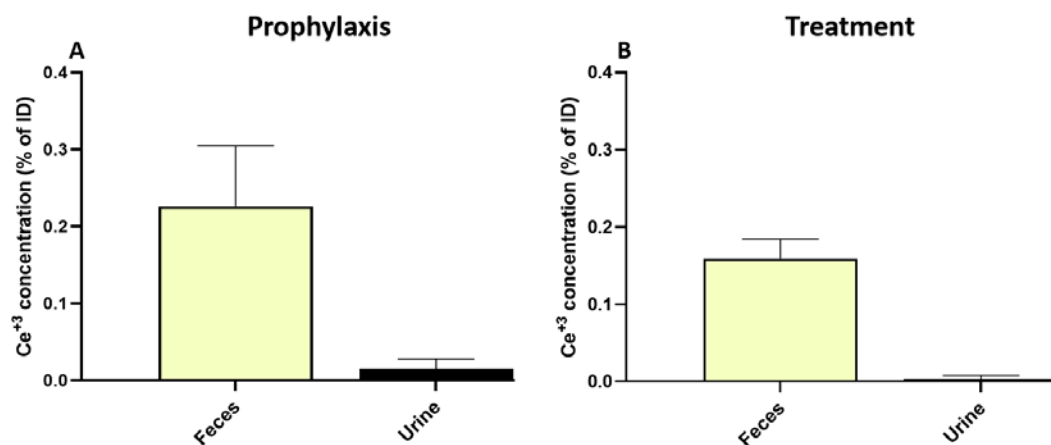


Figure 4.12. Biodistribution of elemental cerium in maternal organs in 10-week-old pregnant Sprague-Dawley rats with LPS-induced inflammation treated with intravenous CeO₂ NPs given either before or after LPS. (A) Ce concentrations in maternal organs (n=3) in the prophylaxis group (CeO₂ NPs → LPS). (B) Ce concentrations in maternal organs (n=2) in the treatment group (LPS → CeO₂ NPs).

Collectively, these findings confirm that CeO₂ NPs behave as long-lived particulate entities primarily processed by the liver and spleen, with slow secondary elimination through bile. The total recovery of the injected dose across the analyzed tissues was approximately 84% (Figures 4.11A, 4.12A, 4.13A) in the prophylaxis group and 93% in the treatment group (Figures 4.11B, 4.12B, 4.13B). The unrecovered fraction likely resides in compartments not analyzed in this study, such as the bone marrow, skeletal muscle, or skin, or corresponds to the cumulative fraction excreted via feces over the 5-day period prior to sampling.

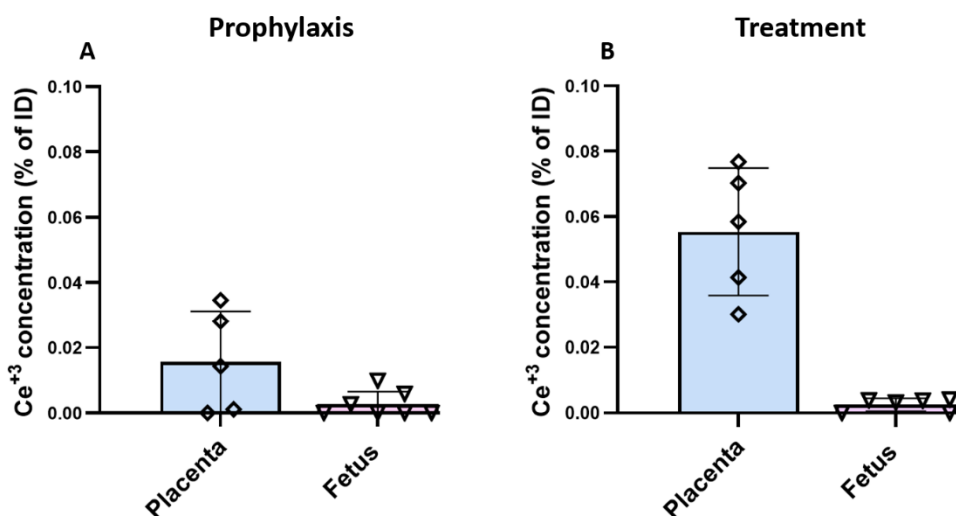


Figure 4.13. Biodistribution of elemental cerium in placenta and fetus in 10-week-old pregnant Sprague-Dawley rats with LPS-induced inflammation treated with intravenous CeO₂ NPs given either before or after LPS. (A) Ce concentrations in placenta and fetus (n=3) in the prophylaxis group (CeO₂ NPs → LPS). (B) Ce concentrations in placenta and fetus (n=2) in the treatment group (LPS → CeO₂ NPs).

From a reproductive toxicology perspective, the most critical observation relates to the maternofetal interface. In both prophylaxis and treatment groups, placental cerium levels remained low (Figure 4.13), approximately 0.015% ID (Figure 4.13A) and 0.05% ID (Figure 4.13B) respectively, while fetal cerium content remained at background levels (<0.02% ID) in both cases (Figure 4.13). Thus, the prophylaxis group, where NPs encounter first a non-inflamed maternal environment, showed placental values indistinguishable from background, indicating minimal interaction with placental tissue. In contrast, placental accumulation increased slightly in the treatment group. Moreover, CeO₂ NPs are not detected in the fetus in our study, with measured values remaining within the range of analytical noise.

Physically, NPs reach the placenta via the maternal spiral arteries, which open into the labyrinth zone (the functional equivalent of the human intervillous space) filled with maternal blood. This blood does not mix with the fetal circulation; instead, it bathes the trophoblast layers. Unlike the single-layered human syncytiotrophoblast, the rat placenta is trichorial. Layer I is highly fenestrated, but Layers II and III contain transtrophoblastic

channels with reported diameters of ~20-25 nm.⁸⁰ These dimensions are functionally analogous to the human trans-syncytial nanopores (~10-60 nm),⁸¹ and theoretically permit the passive entry of small NPs into the placental tissue. Crucially, even if NPs bypass the trophoblast layers, they encounter the fetal mesenchyme and then the final and most restrictive barrier, the fetal capillary endothelium. Similar to humans, this endothelium possesses continuous adherent and tight junctions with a conserved functional pore radius of only ~3.5-6 nm.⁸² This strict size exclusion effectively prevents the fetal transfer of the majority of NPs. Regarding our CeO₂NPs@RSA formulation, although the primary CeO₂ core is ~3 nm, the adsorption of RSA creates a protein corona. Since albumin has a hydrodynamic diameter of ~7.2 nm (radius ~3.6 nm), the effective hydrodynamic diameter of the CeO₂NPs@RSA complex increases significantly (>15 nm), thereby exceeding the cutoff of the fetal endothelial pores.^{58,62,62,83} As an additional consideration, under inflammatory conditions, hemodynamic changes and endothelial dysfunction, such as PE, some NPs may extravasate from the spiral arteries into the decidua basalis (equivalent to the human placental bed). This compartment is densely populated by macrophages, which could internalize the NPs arriving in this region and contribute to their retention within maternal tissues.⁸⁴

Collectively, the rapid clearance of NPs by the liver (80-90% retention), the sequestration by decidual macrophages, and the size-exclusion limit of the fetal endothelium explain the absence of fetal bioaccumulation despite placental uptake.⁸⁵⁻⁸⁷

4.2.8 Histological evaluation of kidney tissues and urinary albumin levels in an LPS-induced gestational model of PE and the therapeutic effect of CeO₂ NPs

In our rat model, we did not observe abortions or overt pregnancy loss after CeO₂ NP injection, suggesting that, under our experimental conditions, CeO₂ NPs did not induce gross embryotoxicity. Because renal structural alterations are a hallmark of PE,^{25,36,38,43} renal histopathology was examined microscopically using H&E staining, in which connective tissue appears pink and cell nuclei stain purple, to evaluate across all

experimental groups the effects of LPS-induced inflammation and the potential protective role of CeO₂ NPs (**Figure 4.14**). For this purpose, kidneys were collected directly into 4% paraformaldehyde (PFA) and stored at 4 °C to allow proper fixation for 72h.

In the control group, kidney architecture was largely preserved: glomeruli and tubules maintained normal morphology, with intact glomerular structures, no swelling, no reduction in Bowman's space, no inflammatory infiltration, and a structurally conserved tubular epithelium without evidence of necrosis or brush-border loss. Vascular congestion was minimal and the interstitial space appeared compact, indicating an absence of renal injury and confirming the expected healthy baseline status in the control animals. In contrast, kidneys from LPS-only animals showed a pattern compatible with mild to moderate LPS-induced renal injury. Although the overall architecture remained recognizable and no necrosis was observed, several features indicated renal alterations, including dilatation of the tubular lumen and swollen glomeruli with reduced Bowman's space and partial occlusion of capillary loop spaces, which are characteristic alterations of LPS-induced renal damage in PE models and other models involving kidney injury.^{88–91} animals receiving CeO₂ NPs, either as prophylaxis (CeO₂ NPs + LPS) or as treatment (LPS + CeO₂ NPs), renal histology tended to be better preserved compared with the LPS-only group, with tubules frequently showing conserved morphology and limited luminal dilatation, and glomeruli exhibiting a more normal appearance with better preservation of Bowman's space.

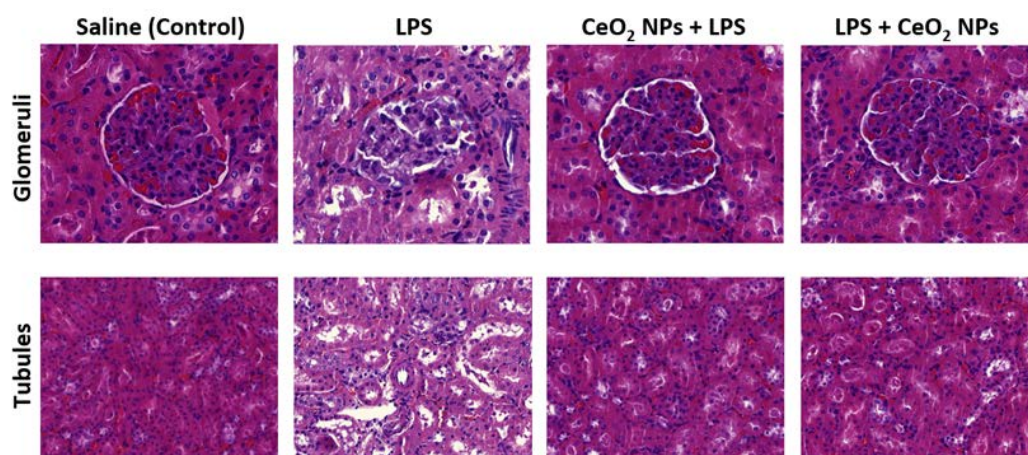


Figure 4.14. Representative histological images (H&E staining, 400× magnification) of renal glomeruli and tubules in rat kidneys from different treatment groups: Saline (Control), LPS, CeO₂ NPs + LPS, LPS + CeO₂ NPs.

Because increased protein excretion in urine reflects damage to the glomerular filtration barrier and is a hallmark of PE,^{15,25,39,89,92} proteinuria was assessed (**Figure 4.15**). Ideally, the gold standard for quantifying proteinuria involves 24-hour urine collection using metabolic cages. However, to avoid inducing excessive stress in pregnant dams, which could potentially trigger spontaneous abortions or confound the inflammatory and hypertensive parameters of the PE model, we opted for a spot urine collection protocol.⁹³ Consequently, protein excretion was normalized to urinary creatinine (Protein/Creatinine ratio, P/C ratio), a widely accepted approach in both clinical practice and experimental studies when 24-hour urine collection is not feasible,⁹² as it corrects protein levels for variations in urine concentration. LPS-treated rats (LPS and LPS+CeO₂ NPs groups) exhibited urinary creatinine levels approximately 60 mg higher than controls (data not shown), indicating that the true degree of proteinuria in these inflammatory groups is actually slightly higher than suggested by the normalized P/C values.

For the analysis, dams were placed in plastic cages without bedding, and first-morning urine samples were collected and stored at -20 °C until analysis. Protein concentration was determined using the Bradford reagent containing Coomassie Brilliant Blue G-250. Baseline samples were collected before administrations on GD12, and final samples were collected

after administrations (saline, LPS, or CeO₂ NPs, depending on the experimental group) on GD18.

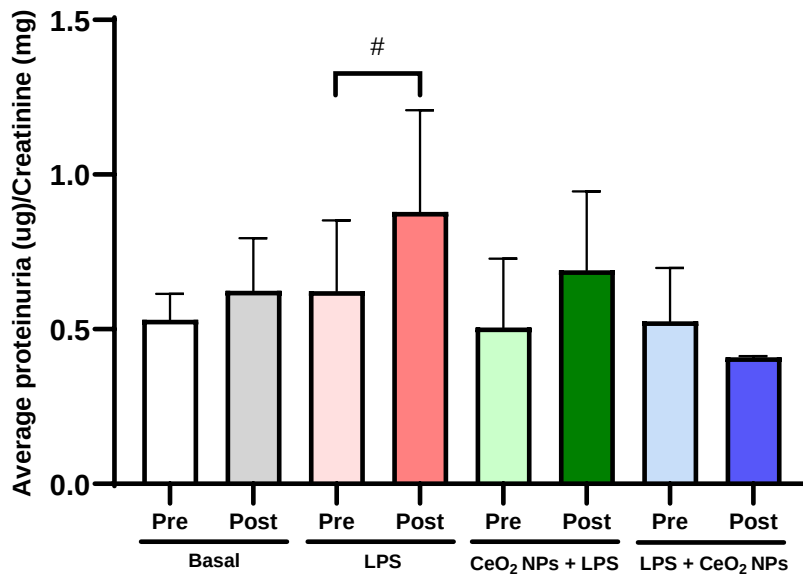


Figure 4.15. Average proteinuria (µg)/creatinine (mg) in each experimental group before and after administrations. Urine samples collected at GD12 represent pre-administration (Pre) values, whereas samples collected at GD18 correspond to after administrations (Post). Bootstrap resampling analysis (10.000 iterations, 95% CI) was performed to estimate population-level differences; the # symbol denotes significance according to the bootstrap test.

Average proteinuria (µg)/creatinine (mg) was quantified in each experimental group before and after the administrations, with urine samples collected at GD12 representing pre-administration values and samples collected at GD18 corresponding to post-administration values (**Figure 4.15**). A tendency toward increased proteinuria was detected in the LPS-treated group compared with the basal condition, consistent with the renal dysfunction typically associated with preeclampsia. This trend was supported by bootstrap resampling analysis (10.000 replicates, 95% CI), which indicated a population-level increase in proteinuria. It seems that animals receiving CeO₂ NPs, either as prophylaxis or as treatment, exhibited attenuated proteinuria levels relative to the LPS group, with values closer to the basal condition; however, a larger sample size would be required to confirm this protective effect.

Overall, these findings suggest that renal dysfunction was already beginning to develop with the applied low LPS dose of $0.5 \mu\text{g}\cdot\text{kg}^{-1}$ bw, primarily affecting renal architecture and showing a mild tendency toward impairment of glomerular filtration capacity. CeO_2 NPs, in both prophylactic and therapeutic regimens, appeared to mitigate these effects. Because glomerular damage does not occur as an immediate primary consequence of LPS exposure, CeO_2 NPs administered even 24h after LPS may still be able to counteract emerging injury. It is also plausible that, if the experiment had been extended and the rats euthanized several days later, more pronounced differences between groups would have been observed not only regarding renal dysfunction, but also across other parameters analyzed in this chapter. However, some rats could have gave birth before the end of the experiment.²⁵

4.2.9. Placenta evaluation in an LPS-induced gestational model of PE and the effect of CeO_2 NPs

In line with the microscopic findings observed in the kidneys, placentas were also analyzed. While placental histology did not reveal observable alterations in the present study, additional staining's such as PAS or immunostainings for the macrophage marker CD68 and α -SMA could be performed in future work to further characterize subtle or cell-specific changes (these analyses are not included in this thesis). Furthermore, macroscopic evaluation of the placentas (**Figure 4.16**) showed that saline-treated controls exhibited a homogeneous, normal coloration, whereas placentas from LPS-treated rats were markedly darker, consistent with vascular congestion and inflammation; this discoloration was clearly attenuated in animals receiving CeO_2 NPs, with the most evident protective effect in the CeO_2 + LPS NPs group.

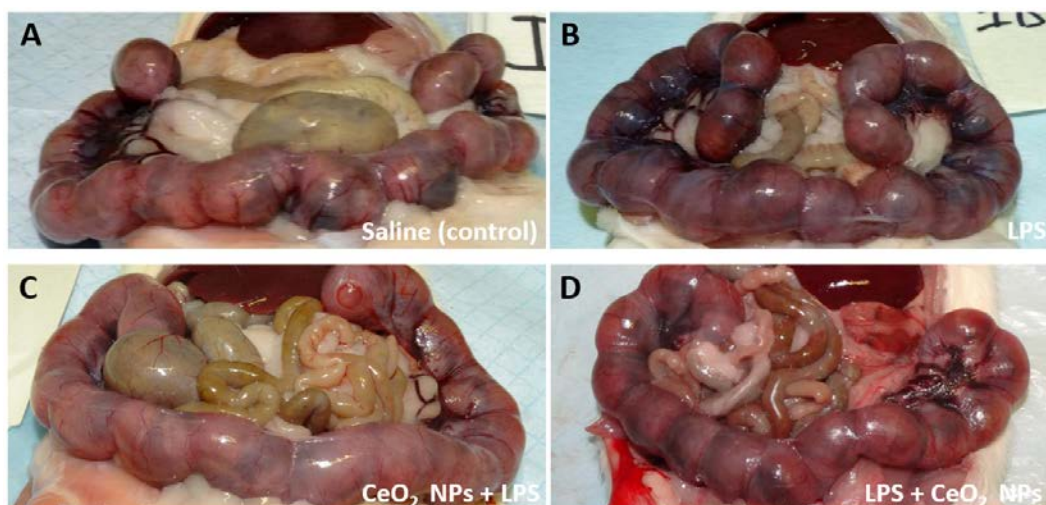


Figure 4.16. Representative macroscopic appearance of uterine horns with placentas and fetuses from pregnant rats under the different experimental conditions. (A) Saline (control). (B) LPS. (C) CeO₂ NPs + LPS (prophylaxis). (D) LPS + CeO₂ NPs (treatment). Photographs were taken in the animal facility of the VHIR.

Furthermore, a similar treatment-related pattern was observed in the intestines (**Figure 4.16**): control rats showed a normal pink coloration, while LPS-treated animals presented pronounced intestinal darkening, congestion, and inflammation (data not shown), which are classic signs of LPS-induced systemic inflammation and indicate severe gut alterations such as barrier dysfunction, edema, and immune activation via TLR4/NF- κ B signalling, as widely reported in animal models.⁹⁴ This vascular congestion and discoloration were visibly reduced in rats treated with CeO₂ NPs, especially in the CeO₂ NPs + LPS group. In line with these findings, it was also recorded a greater amount of feces in the cages of LPS-treated rats, suggesting an inflammatory LPS-induced alteration of intestinal function that was mitigated when CeO₂ NPs administration.

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5.1 Protocols

3 nm CeO₂ NPs synthesis

CeO₂ NPs were synthesized via a wet-chemistry route based on base-induced precipitation of cerium (III) chloride heptahydrate (CeCl₃·7H₂O, Aldrich, 228931) in the presence of sodium citrate tribasic dihydrate (SC, ACS reagent, ≥99.0%, S4641-1KG, Sigma-Aldrich, Merck) as a stabilizing agent. Cerium (III) nitrate hexahydrate (Ce (NO₃)₃·6H₂O, 238538, Sigma-Aldrich) was also evaluated as an alternative cerium precursor. Subsequently, 50 mL of tetramethylammonium hydroxide (TMAOH, 25% in water, 42052-0010, Thermo Scientific, formerly Acros Organics; 81 mM) were added to 100 mL of an aqueous solution containing CeCl₃·7H₂O (30 mM) and SC (60 mM), yielding final concentrations of 27 mM TMAOH, 10 mM Ce³⁺ and 20 mM SC after mixing. The reaction mixture was stirred overnight at room temperature and then transferred to a 250 mL three-neck round-bottom flask and heated under reflux at 100 °C for 4h. Before biological experiments, the NP suspension was purified using 3 kDa centrifugal filter units (Amicon® Ultra-15 3K, Merck) at 5000xg for 20 min, and the retained NPs were re-dispersed in 2.2 mM SC.

Bacterial endotoxin evaluation (LAL assay)

Nanoparticle synthesis and purification were carried out using sterile procedures and non-pyrogenic materials. To confirm the absence of endotoxin contamination, CeO₂ NP batches were routinely analyzed for endotoxin levels at the Echevarne analytical laboratory (Barcelona).

UV-visible spectroscopy

UV-Vis absorbance spectra were acquired at room temperature using a Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Measurements were performed between 250 and 800 nm in 1.5 mL quartz cuvettes with a 10 mm path length (Hellma Analytics, Müllheim, Germany) or disposable plastic cuvettes (BRAND® UV

cuvettes, semi-micro, BRAND GMBH + CO KG, Wertheim, Germany), depending on the experiment.

UV-vis spectroscopy measures the wavelength-dependent extinction of light in the ultraviolet and visible regions. The recorded signal accounts for both absorption and scattering contributions. The resulting spectra provide qualitative and quantitative information on electronic transitions and overall optical properties of the samples.

Optical band gap determination

The optical band gap is defined as the minimum energy difference between the top of the valence band and the bottom of the conduction band required to promote an electron from the valence to the conduction state. This energy threshold corresponds to the fundamental absorption edge in the UV-visible spectrum, where a sharp increase in absorbance is observed. To determine the band gap energy, the method proposed by Tauc et al. was employed. This method relates the optical absorption coefficient to the photon energy using the following equation:

$$\alpha h\nu^{\left(\frac{1}{n}\right)} = A(h\nu - E_g)$$

Where:

- h Planck's constant
- ν is the photon frequency
- E_g is the optical band gap energy
- A is a proportionality constant
- α is the absorption coefficient

The absorption coefficient is calculated from the experimental absorbance data using the Beer-Lambert relation:

$$\alpha = \frac{2.303A}{d}$$

where A represents the absorbance measured in the UV-vis spectrum and d is the path length of the cuvette (typically 1 cm).

The exponent n determines the nature of the electronic transition: $n = 1/2$ for direct allowed transitions, $n = 3/2$ for direct forbidden transitions, $n = 2$ for indirect allowed transitions, $n = 3$ for indirect forbidden transitions.

In the case of CeO₂ NPs, the strong absorption peak observed at approximately 320 nm is attributed to a charge-transfer transition from O²⁻ (2p) states to empty Ce⁴⁺ (4f⁰) levels, resulting in a Ce 4f¹ configuration. Although ceria can exhibit direct transitions, this study analyzes the data assuming an indirect allowed transition ($n=2$). Therefore, the band gap was estimated by plotting $(\alpha h\nu)^{1/2}$ versus photon energy ($h\nu$) and extrapolating the linear region to the x-axis; the intercept corresponds to E_g .

Dynamic light scattering

The hydrodynamic size of the nanoparticles was determined using a Malvern Zeta Sizer Nano ZS (Malvern Instruments, UK) equipped with a 532 nm laser and operating at a fixed scattering angle of 173° (backscatter detection). Measurements were performed in triplicate at RT using either disposable or quartz cuvettes with a 1 cm path length.

DLS is a non-invasive technique that determines the size distribution of submicron particles by analyzing the fluctuations in scattered light intensity caused by Brownian motion. These fluctuations allow for the determination of the particles' translational diffusion coefficient. The hydrodynamic diameter d_H is then derived from this diffusion coefficient using the Stokes-Einstein relationship, which inversely relates particle size to diffusion speed while accounting for the viscosity and temperature of the medium.

It is important to note that d_H refers to the diameter of a theoretical sphere that diffuses at the same rate as the particle being measured. Consequently, this value reflects not only the metallic core but also the entire solvated structure, including surface ligands and the

electric double layer. Data analysis typically yields both intensity and number distributions. The intensity distribution is highly sensitive to larger particles or aggregates due to their stronger light scattering, whereas the number distribution corrects for this bias to reflect particle abundance. Comparing both distributions thus provides a comprehensive view of sample polydispersity and aggregation state.

Zeta-potential analysis

To assess the surface charge and colloidal stability of the nanoparticles, zeta-potential measurements were conducted. This parameter is a key indicator of electrostatic stabilization; generally, values falling outside the range of -30 mV to +30 mV imply sufficient inter-particle repulsion to prevent aggregation, thus suggesting a stable colloidal system. However, since the zeta potential reflects the particle-fluid interface properties, reported values are strictly dependent on environmental factors such as pH, ionic strength, and solution composition.

Zeta potential measurements were performed using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), based on the principle of electrophoretic light scattering (ELS). In this method, an electric field is applied to the sample, inducing the migration of charged particles, and the instrument measures their electrophoretic mobility (particle velocity under the applied field) to calculate the zeta potential. For the experimental procedure, aliquots of the nanoparticle suspension were loaded into disposable folded capillary cells (model DTS1060, Malvern Panalytical, Malvern, UK) and analyzed at controlled RT.

Electron microscopy characterization (TEM, HR-TEM, and HAADF-STEM imaging)

Characterization was carried out using three complementary imaging modes: standard TEM, HR-TEM, and HAADF-STEM. Measurements were performed on a JEOL JEM-1010 operating at 100 kV (for standard TEM) and a FEI Tecnai G2 F20 operating at 200 kV (for HR-TEM and HAADF-STEM).

- TEM: Standard TEM was used to obtain an overview of particle morphology and dispersion. In this technique, a broad beam of electrons is transmitted through the ultra-thin specimen. Contrast is generated primarily by the scattering of electrons by the sample's mass and thickness (mass-thickness contrast), producing a 2D projection of the nanoparticles. While effective for general observation, the resolution of standard TEM is often limited when resolving the fine structural details of ultra-small (~3 nm) nanoparticles.
- HR-TEM: To resolve atomic-scale features, HR-TEM was performed on the FEI Tecnai G2 F20. Unlike conventional amplitude-contrast imaging, this technique utilizes phase-contrast imaging, which relies on the interference of the electron beam with the crystalline lattice of the sample. This allows for the direct visualization of crystal planes (lattice fringes), providing critical information about the crystallinity and structural integrity of the CeO₂ NPs.
- HAADF-STEM: To further analyze size distribution without the interference of diffraction effects, HAADF-STEM was employed. In this mode, a finely focused electron beam raster-scans the sample, and an annular detector collects electrons scattered at high semi-angles. The signal intensity is incoherent and proportional to the square of the atomic number (Z) of the scattering elements (Z-contrast). Consequently, the heavier Cerium atoms (Z=58) appear significantly brighter than the lighter carbon support or organic ligands. This high Z-contrast makes HAADF-STEM an indispensable tool for clearly distinguishing the metallic cores from the background, facilitating accurate and robust polydispersity analysis.

Finally, the morphological characteristics and size distribution of the CeO₂ NPs were quantitatively analyzed using ImageJ analysis software. To ensure statistical significance, the average diameter was calculated by measuring a minimum of 500 individual particles from the acquired micrographs.

Prior to analysis, the purified nanoparticle suspension was dried, and the resulting solid was ground into a fine powder. The powder was deposited onto a zero-background silicon holder to minimize signal interference.

X-ray diffraction: crystallite size and lattice parameter calculation

Prior to analysis, the purified NPs suspension was dried, and the resulting solid was ground into a fine powder. The powder was deposited onto a zero-background silicon holder to minimize signal interference. XRD patterns were collected using a Malvern PANalytical X'Pert PRO diffractometer operating at 40 kV and 40 mA, utilizing Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$). Diffractograms were recorded in the angular range (2θ) of 20° to 95° . Then crystalline phase was identified by comparing the obtained diffraction peaks with the standard reference pattern for cubic fluorite (FCC) CeO₂ ICDD-Powder Diffraction File reference (PDF No. 00-034-0394).

Quantitative structural analysis was performed to determine the average crystallite size and lattice parameters. The average crystallite size (D) was estimated from the line broadening of the most intense diffraction peak using the Scherrer equation:

$$D = \frac{K\lambda}{\beta \cos(\theta)}$$

Where:

- D is the average crystallite size (nm).
- K is the shape factor (typically taken as 0.9).
- λ is the X-ray wavelength ($1.5406 \text{ \AA}$).
- β is the line broadening at FWHM in radians.
- θ is the Bragg angle.

Furthermore, the lattice parameter (a) for the cubic crystal structure was calculated based on Bragg's Law ($2d\sin(\theta) = n\lambda$). For a cubic system, the interplanar spacing (d) is related to

the Miller indices (h, k, l) of the reflection planes. Consequently, the lattice parameter was derived using the following relationship:

$$a = \frac{d\sqrt{h^2 + k^2 + l^2}}{\sqrt{h^2 + k^2 + l^2}}$$

This calculation allows for the assessment of potential lattice expansion or contraction relative to bulk ceria, often associated with the presence of Ce^{+3} cations or surface defects in nanomaterials.

pH measurement

pH measurements were performed at room temperature under continuous stirring using a PH BASIC 20 pH meter (Crison Instruments, Barcelona, Spain), previously calibrated at three points with standard buffer solutions (pH 4.01, 7.00 and 9.21)

Tyndall effect

The Tyndall effect was used as a qualitative method to confirm nanoparticle formation in the colloidal suspensions. In this phenomenon, a beam of light passing through a colloidal dispersion is scattered by the dispersed particles, making the light path visible, in contrast to true molecular solutions where no visible scattering occurs.

Catalase-like redox activity assay of CeO_2 NPs

The catalase-like redox activity of CeO_2 NPs was evaluated by monitoring the decomposition of hydrogen peroxide in the presence of the nanoparticles. Hydrogen peroxide (30% w/w H_2O_2 solution, Sigma-Aldrich, Merck) was used to prepare a reaction mixture containing 10 mM H_2O_2 and 10 mM CeO_2 NPs (1:1 molar ratio), by adding 1 μL of the 30% stock (≈ 9.8 M) to 1 mL of CeO_2 NP suspension in a closed 1 cm path length cuvette suitable for UV-Vis measurements. Samples were protected from light during preparation and storage to prevent H_2O_2 photodegradation. Measurements were performed in triplicate at room temperature using closed disposable cuvettes, and catalase-like redox

activity was evaluated by analyzing the time-dependent evolution of the UV–Vis spectra and subsequent data treatment through Tauc plots, as described in the Methods section on optical band gap determination.

ABTS antioxidant activity assay of CeO₂ NPs

The ABTS radical cation decolorization assay was used to evaluate the antioxidant capacity of the CeO₂ NPs. The ABTS•⁺ radical cation was generated by reacting 7 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; A1888, Sigma-Aldrich, Merck) dissolved in 10 mM PB (prepared from sodium phosphate dibasic, Na₂HPO₄, S5136, and sodium phosphate monobasic, NaH₂PO₄, 71496, Sigma-Aldrich, Merck) with 2.45 mM potassium persulfate and incubating the mixture in the dark at room temperature (RT) overnight until a stable dark blue-green chromophore developed. The following day, a mixture of 80 µL of CeO₂ NP suspension (1.72 mg/mL), 50 µL of 10 mM PB, and 35 µL of ABTS•⁺ solution was added in this order to the desired number of wells of a 96-well plate, yielding an absorbance close to 0.7 at 734 nm, which is required for reliable measurements. Absorbance at 734 nm was recorded using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The ABTS•⁺ solution was always prepared 24h in advance and used the following day; solutions stored for up to 48–72h in the dark at RT also retained suitable performance, although it is recommended to prepare the ABTS•⁺ reagent freshly for each experiment.

Cell culturing

HTR-8/SVneo trophoblast cells were maintained in 75 cm² Nunclon™ Δ tissue culture flasks (Thermo Fisher Scientific) in ATCC-modified RPMI 1640 medium (RPMI 1640 Medium, HEPES, liquid, ATCC-compatible, 22400-089, Gibco, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Fetal bovine serum, qualified, heat inactivated, Brazil origin, 10500-064, Gibco, Thermo Fisher Scientific) and 25 mM HEPES (HEPES 1 M Buffer Solution, 15630-080, Gibco, Thermo Fisher Scientific) at

37 °C in a humidified atmosphere containing 5% CO₂. Under hypoxic conditions, HTR-8/SVneo cells were cultured in an InvivoO₂® 300 hypoxia workstation (Baker Ruskinn, The Baker Company) with the oxygen concentration set to 0.5% for 48h, using culture medium that had been pre-equilibrated under hypoxia for 24h before use to allow deoxygenation. 1X PBS (Gibco, Thermo Fisher Scientific) was used for washing steps.

Protein conjugation and aggregation monitoring of CeO₂ NPs with FBS (Chapter 3) and RSA (Chapter 4)

CeO₂ NPs were pre-conjugated with proteins to form a stabilizing coating before their use in physiological media, in order to prevent NP aggregation, which is associated with reduced biocompatibility and can even induce distress and mortality in cells and animals (further detailed in **Section 3.2.1**). In cell culture media, CeO₂ NPs were mixed with FBS at a 1:2 NP:protein ratio and incubated for 24h; the resulting nanoconjugate was then diluted 1:10 in the culture medium. For the RSA assay, CeO₂ NPs were conjugated with rat serum albumin (RSA; Sigma-Aldrich, Merck, A9731) at a 1:2 CeO₂ NPs@RSA ratio; however, as discussed in **Section 4.2.2**, for 3 nm CeO₂ NPs a 1:1 conjugation ratio would theoretically be sufficient, but an excess of protein was employed to ensure complete surface coverage.

To test the aggregation kinetics of CeO₂ NPs in media, 100 µL of CeO₂ NP suspension were added to 900 µL of the corresponding medium, ensuring that the NP volume did not exceed 10% of the total volume to avoid altering medium composition. For conjugated NPs, samples were pre-incubated for 24h, as mentioned; importantly, the final protein/serum concentration was adjusted to the intended experimental value (typically 10% FBS for most cell culture media, or 4% RSA in the *in vivo* study), and this contribution was considered when preparing the nanoparticle-containing media. Because UV-vis monitoring is not suitable in this context (the strong albumin absorbance peak around 280 nm overlaps with the CeO₂ NP signal region), hydrodynamic size was instead followed by DLS. In this approach, the size distribution by intensity of the samples is compared with

that of albumin/FBS-only controls and with a positive aggregation control consisting of intentionally aggregated CeO₂ NPs in 1X PBS, allowing the aggregation state of the CeO₂ NPs@albumin conjugates to be monitored over time.

CellROX™ Deep Red assay for intracellular ROS quantification

HTR-8/SVneo cells were seeded at a density of 5×10^4 cells/well in 12-well plates and allowed to adhere for 24h. The following day, cells were pre-incubated with 100 or 200 µg/mL CeO₂ NPs, and in parallel, culture medium for the hypoxia group was placed in the hypoxia workstation to allow deoxygenation. On the next day, the culture medium was replaced to remove non-internalized NPs, and cells were subsequently maintained for 48h either in fresh normoxic medium (normoxia group) or in preconditioned hypoxic medium within the hypoxia workstation InvivoO₂® 300 (hypoxia group) at 0.5% O₂. For ROS detection, cells were incubated with CellROX™ Deep Red Reagent (CellROX™ Deep Red, C10491, Thermo Fisher Scientific, Invitrogen) at 500 nM for 60 min at 37 °C protected from light, and then washed three times with HBSS 1X (Hank's Balanced Salt Solution, Gibco, Thermo Fisher Scientific) to remove excess probe.

Subsequently, cells were stained with SYTOX Blue Dead Cell Stain (1 µM, 15 min), gently scraped, and collected into flow cytometry tubes for analysis; as assay controls, N-acetyl-L-cysteine (NAC, 200 µM) was used as an antioxidant and tert-butyl hydroperoxide (TBHP, 400 µM) as a ROS inducer to validate the performance of the CellROX™ assay, although these validation data were not included in the final results. Finally, samples were acquired on an LSRFortessa flow cytometer (BD Biosciences) using a 640 nm red laser with a 660/20 nm emission filter for CellROX™ Deep Red and a 405 nm violet laser with a 450/50 nm emission filter for SYTOX Blue, collecting a minimum of 10.000 events per sample. Data were analyzed using FlowJo v10 software (BD).

Sample preparation for visualization of CeO₂ NPs by TEM, HR-TEM, HAADF, and EDX in HTR-8/SVneo cells

HTR-8/SVneo cells were seeded at a density of 5×10^5 cells per well in 6-well plates and incubated for 24h to allow adhesion. The following day, CeO₂ NPs were added to the cultures and cells were incubated for an additional 24h to permit nanoparticle uptake; cells were then harvested, transferred to 1.5 mL microcentrifuge tubes, and centrifuged to obtain a compact pellet of approximately 0.02-0.05 mL, and the supernatant was carefully removed without allowing the pellet to dry. Cell pellets were fixed in a solution containing 1% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), using a volume 10-fold higher than the pellet volume, and incubated for at least 1h at RT. After fixation, samples were centrifuged, the fixative was discarded, and cells were washed with buffer before post-fixation with a reduced osmium solution (2% OsO₄ and 1.5% K₄[Fe(CN)₆]) for 1h, followed by a second osmium step with 2% OsO₄ for 1h and overnight staining with 1% uranyl acetate; between each staining step, samples were washed three times with distilled water for 3 min. Subsequently, samples were dehydrated through a graded ethanol series (30, 50, 70, 90, and 2 x 100%) for 5 min at each concentration and then infiltrated with medium TAAB 812 epoxy resin. The specimens were embedded in fresh resin and polymerized for 48h at 60 °C to obtain solid blocks suitable for ultramicrotomy; ultrathin sections of approximately 74 nm thickness (cut from areas of about 350 μ m $\times$ 350 μ m) were collected on 200-mesh Formvar/carbon-coated copper grids and post-stained with 4% uranyl acetate in 50:50 ethanol/water followed by Reynolds lead citrate to enhance contrast.

Cell ultrastructure and CeO₂ NP localization were examined using TEM, HR-TEM, HAADF imaging, and the presence of Ce⁺³ was confirmed by EDX. After fixation, resin embedding was carried out at the CiTT-UB facility (Hospital Clínic, Barcelona), and TEM, HR-TEM, HAADF and EDX imaging and Ce⁺³ detection was performed by the ICN2 (Institut Català de Nanociència i Nanotecnologia) microscopy service.

Cell viability assay (PrestoBlue™)

HTR-8/SVneo cells were seeded at a density of 5×10^3 cells per well in 96-well plates and allowed to adhere for 24h at 37 °C and 5% CO₂ under normoxic conditions. The following day, cells were exposed to 100 or 200 µg/mL CeO₂ NPs, and in parallel, culture medium for the hypoxia group was placed in the hypoxia workstation to allow deoxygenation. After 24h of NP exposure, the culture medium was replaced to remove non-internalized NPs, and cells were subsequently incubated for an additional 48h either in fresh normoxic medium (normoxia group) or in preconditioned hypoxic medium within the hypoxia InvivoO₂® 300 workstation set at 0.5% O₂ (hypoxia group). Cell viability was then assessed using the PrestoBlue™ Cell Viability Reagent (10% v/v; A13261, Invitrogen, Thermo Fisher Scientific), which is based on the reduction of resazurin (non-fluorescent, blue) to resorufin (fluorescent, purple) by metabolically active cells, leading to a color change from blue to purple and an increase in fluorescence signal. Plates were incubated with the diluted PrestoBlue™ solution for 3h at 37 °C and 5% CO₂, and fluorescence was measured using a Varioskan Lux microplate reader (excitation 560 nm, emission 590 nm); cell viability was expressed as a percentage of the untreated normoxic control after background subtraction and normalization.

Annexin V/SYTOX™ apoptosis assay under hypoxia

HTR-8/SVneo cells were seeded at a density of 5×10^4 cells/well in 12-well plates and allowed to adhere for 24h at 37 °C and 5% CO₂. The following day, cells were pre-incubated with 100 or 200 µg/mL CeO₂ NPs, and in parallel, culture medium for the hypoxia group was placed in the hypoxia workstation to allow deoxygenation, as described for the CellROX™ assay. On the next day, the medium was replaced to remove non-internalized NPs, and cells were subsequently maintained for 48h either in fresh normoxic medium or in preconditioned hypoxic medium within the hypoxia InvivoO₂® 300 workstation at 0.5% O₂.

After treatment, cells were gently washed with 1X PBS, detached by scraping, pelleted by centrifugation, and resuspended in 100 μ L of binding buffer containing 5 μ L Pacific Blue™ Annexin V (A35136, Invitrogen, Thermo Fisher Scientific) and 1 μ L SYTOX™ AADvanced™ Dead Cell Stain, following the manufacturer's instructions. The cell suspension was incubated for 30 min at RT in the dark, after which 400 μ L of additional binding buffer were added prior to acquisition. Stained cells were analyzed on an LSRFortessa™ flow cytometer (BD Biosciences) using a 405 nm violet laser with the appropriate emission filter for Pacific Blue™ Annexin V and a 488 nm or 561 nm laser with the recommended emission filter for SYTOX™ AADvanced™, acquiring at least 10.000 events per sample; data were processed with FlowJo™ v10 software to discriminate viable, early apoptotic, late apoptotic, and necrotic cell populations

RNA extraction and cDNA synthesis

HTR-8/SVneo cells were seeded at a density of 5×10^3 cells per well in 12-well plates and, after 24h, were treated with CeO₂ NPs (100 and 200 μ g/mL). Cells assigned to hypoxic conditions were cultured using pre-equilibrated medium that had been placed in a hypoxia workstation to allow deoxygenation, as described for the CellROX™ assay. On the following day, the medium was replaced to remove non-internalized NPs, and cells were subsequently maintained for 48h either in fresh normoxic medium or in preconditioned hypoxic medium within an InvivoO₂® 300 physiological cell culture workstation set to 0.5% O₂. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), following the manufacturer's protocol. Briefly, culture medium was removed, cells were washed with 1X PBS, and 350 μ L Buffer RLT supplemented with 1% β -mercaptoethanol were added per well; lysates were homogenized, mixed with an equal volume of 70% ethanol, and loaded onto RNeasy spin columns, followed by on-column DNase I treatment, sequential RW1 and RPE washes, a final dry spin, and elution of RNA in 50 μ L RNase-free water. RNA concentration and purity were measured spectrophotometrically using a NanoPhotometer NP80 (Implen), and only samples with an A260/A280 ratio between 1.9 and 2.1 were used

for downstream applications. For cDNA synthesis, 2 µg of total RNA per sample were reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a final volume of 20 µL, according to the manufacturer's instructions. The reaction mix contained RT buffer, dNTPs, random primers, MultiScribe reverse transcriptase, and RNase inhibitor, and was incubated in a thermocycler using the following program: 25 °C for 10 min, 37-42 °C for 60-120 min, 85 °C for 5 min, and hold at 4 °C. The resulting cDNA was stored at -20 °C until use in qPCR

RT-qPCR setup and amplification

RT-qPCR was performed using TaqMan® Fast Universal PCR Master Mix (2×) (Applied Biosystems) and pre-designed TaqMan® Gene Expression Assays (Thermo Fisher Scientific) in 96-well plates compatible with the LightCycler® 480 II instrument (Roche). For each reaction, 10 µL total volume were used, consisting of 5 µL TaqMan Fast Universal Master Mix (2X), 0.5 µL TaqMan® probe (20X), 3.5 µL nuclease-free water, and 1 µL cDNA (corresponding to approximately 50-100 ng RNA input), as optimized in the laboratory. Plates were sealed, briefly centrifuged at 1000 rpm for 1 min to remove bubbles, and run on the LightCycler® 480 II using a standard fast TaqMan cycling protocol (e.g. 95 °C 20 s, followed by 40 cycles of 95 °C 3 s and 60 °C 30 s), with fluorescence acquisition at the end of each extension step. Reference genes RPL37A were used for normalization, based on its stable expression under the experimental conditions. RT-qPCR data were analyzed using the LightCycler® 480 software, and relative expression levels were calculated using the $\Delta\Delta C_t$ method with the corresponding reference gene as internal control and normalized to the appropriate control condition (e.g. untreated or vehicle-treated cells).

Target (Gene)	TaqMan® Assay ID
<i>RPL37A</i>	Hs06050959_g1
<i>HIF1A</i>	Hs00153153_m1
<i>IL6</i>	Hs00985639_m1
<i>IL1B</i>	Hs01555410_m1
<i>CCL2</i>	Hs00234140_m1
<i>SLC2A1</i>	Hs00892681_m1
<i>PPARG</i>	Hs01115513_m1
<i>MMP9</i>	Hs00957562_m1
<i>MMP2</i>	Hs01548727_m1
<i>VEGFA</i>	Hs00900055_m1
<i>PGF</i>	Hs00182156_m1

Table 5.1. TaqMan® Gene Expression Assays used for qRT-PCR analysis of HTR-8/SVneo cells.

Hypoxia Green flow cytometry assay in HTR-8/SVneo cells

HTR-8/SVneo cells were seeded at a density of 5×10^4 cells per well in 12-well plates and allowed to adhere for 24h at 37 °C and 5% CO₂. On the following day, cells were exposed to hypoxic conditions (0.5% O₂) for 6, 24, or 48h within an InvivoO₂® 300 physiological cell culture workstation, using pre-equilibrated culture medium that had been placed in the hypoxia workstation to allow deoxygenation 24h before; in parallel, normoxic control cells were maintained at 18% O₂ in a standard cell culture incubator.

At the end of each hypoxic time point, cells were gently washed with 1X PBS, detached by scraping, pelleted by centrifugation, and resuspended in pre-warmed complete medium containing Hypoxia Green Reagent for Flow Cytometry (H20035, Thermo Fisher Scientific) at a final concentration of 1 µM. Cell suspensions were incubated for 3h at 37 °C in the dark under their respective oxygen conditions (normoxia or hypoxia) to allow accumulation of the fluorogenic hypoxia signal, then washed once with 1X PBS, resuspended in 500 µL

of HBSS 1X, and immediately analyzed by flow cytometry. Stained cells were acquired on an LSRFortessa™ flow cytometer (BD Biosciences) using a 488 nm blue laser and a FITC-compatible bandpass filter (e.g., 530/30) to detect Hypoxia Green fluorescence, collecting at least 10.000 events per sample; data were analyzed using FlowJo™ v10 software to quantify hypoxia-positive cells and mean fluorescence intensity under normoxic and hypoxic conditions at 6, 24, and 48h.

ELISA procedures for HIF-1 α and inflammatory mediators *in vitro* and *in vivo*

For the *in vitro* experiments, HTR-8/SVneo cells were seeded at appropriate densities in either multi-well or UniWell plates, depending on the specific assay, and allowed to adhere for 24h at 37 °C and 5% CO₂. The exact seeding density and plate format used for each experiment are described in the corresponding assay. Cells were then pre-incubated with 100 or 200 μ g/mL CeO₂ NPs, and in parallel, culture medium for the hypoxia group was placed in the InvivoO₂® 300 hypoxia workstation to allow deoxygenation, as described for the hypoxia assays. On the following day, the medium was replaced to remove non-internalized NPs, and cells were subsequently maintained for 48h either in fresh normoxic medium or in preconditioned hypoxic medium within the InvivoO₂® 300 hypoxia workstation set to 0.5% O₂.

For the *in vivo* experiments, blood was collected into lithium heparin Microvette® CB 300 LH tubes (300 μ L), centrifuged at 1.500xg for 10 min to separate plasma and serum, and stored at -80 °C until analysis. The upper transparent fraction was considered serum, while the lower red cell-rich fraction was considered plasma. ELISA measurements were performed on serum samples.

HIF-1 α levels in HTR-8/SVneo cell lysates were quantified using the Human HIF-1 α SimpleStep ELISA kit (Abcam, ab171577; Abcam, Cambridge, UK), following the manufacturer's protocol. Cells were seeded at 6.4x10⁴ cells per well in UniWell plates,

exposed to the corresponding normoxic or hypoxic treatments, and treated with CeO₂ NPs before lysis for protein extraction.

Human IL-6 and CCL2 in cell culture supernatants from HTR-8/SVneo cells seeded at 5x10⁴ cells per well in 12-well plates were measured using the Human IL-6 Quantikine ELISA kit (R&D Systems, D6050; R&D Systems, Minneapolis, MN, USA) and the Human CCL2/MCP-1 Quantikine ELISA kit (R&D Systems, DCP00; R&D Systems, Minneapolis, MN, USA), according to the manufacturers' instructions. After treatments, supernatants were collected, clarified by centrifugation, and analyzed immediately.

Serum IL-6, CCL2 and TNF- α concentrations in rat samples were determined using Rat Quantikine ELISA kits (Rat IL-6 Quantikine ELISA kit, R6000B; Mouse/Rat CCL2/JE/MCP-1 Quantikine ELISA kit, MJE00; Rat TNF- α Quantikine ELISA kit, RTA00; R&D Systems, Minneapolis, MN, USA), according to the manufacturers' instructions. Serum obtained from heparinized blood (as described above) was thawed on ice, diluted as recommended, and loaded in triplicates onto pre-coated plates for quantification of each analyte.

These assays are sandwich ELISAs, in which standards and samples are captured by immobilized antibodies, detected with enzyme-conjugated secondary antibodies, and quantified by colorimetric readout at 450 nm using a standard curve. ELISAs were read in a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific).

BCA protein assay for total protein determination and normalization in cell lysates and serum

Total protein concentration in HTR-8/SVneo cell lysates (for HIF-1 α normalization) and in serum samples from rat blood (for cytokine normalization) was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) in a microplate format, following the manufacturer's instructions. Bovine serum albumin (BSA) standards and appropriately diluted samples (150 μ L) were pipetted into a 96-well plate, and 150 μ L of freshly prepared

BCA working reagent was added to each well (sample:reagent ratio 1:1). Plates were incubated at 37 °C for 30 min at room temperature in the dark, and absorbance was then measured at 562 nm using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific). The average 562 nm absorbance of blank wells was subtracted from all standards and samples, and protein concentrations were calculated from the BSA standard curve using linear regression.

Wound healing assay under hypoxic conditions

In the wound healing assay, HTR-8/SVneo cells were seeded in 24-well plates equipped with Culture-Insert 2 Well for self-insertion, ibiTreat surface (Ibidi, Cat. No. 80209), by adding 70 µL of a cell suspension at 3×10^5 cells/mL per well and were left to adhere for 24h. After cell attachment, CeO₂ NPs were added at final concentrations of 50, 100, or 200 µg/mL, and in parallel, culture medium for the hypoxia group was placed in the InvivoO₂® 300 hypoxia workstation to allow deoxygenation. On the following day, the medium was replaced to remove non-internalized NPs, and cells were subsequently maintained for 48h either in fresh normoxic medium or in preconditioned hypoxic medium within the InvivoO₂® 300 hypoxia workstation set to 0.5% O₂. Prior to removing the inserts on day 5, culture medium without FBS was changed again (using hypoxic medium that had been pre-equilibrated for 24h) to allow a 6h serum-starvation period before initiation of the wound healing assay. Time-lapse phase-contrast images were acquired using a LSM980 laser-scanning confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with an incubation system set to 0.5% O₂, 37 °C and 5% CO₂, and imaging was continued until wound closure to enable detailed cell tracking over time. Wound closure

dynamics were quantified using the appropriate ImageJ/Fiji (v1.54) plugin, following the protocol described in the referenced article:¹

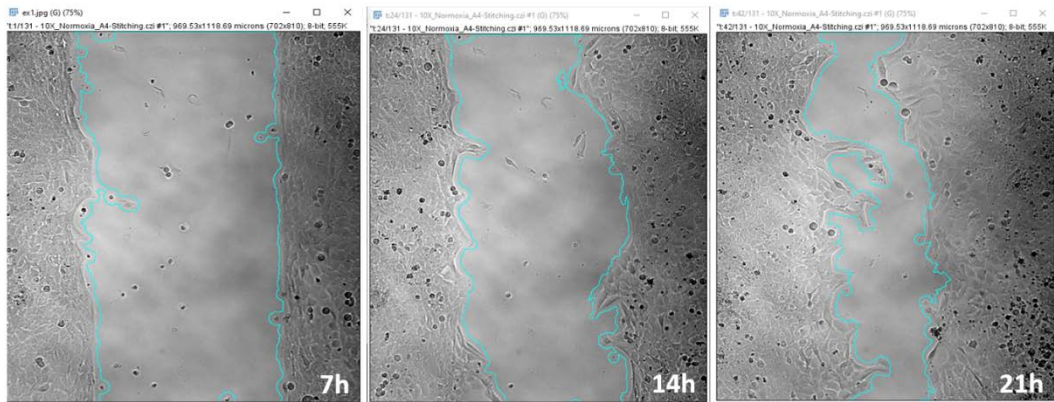


Figure 5.1. Representative example of wound area segmentation by ImageJ/Fiji (v1.54) plugin at 7, 14 and 21h (normoxia).

MitoTracker™ confocal imaging and mitochondrial network analysis workflow

For MitoTracker™ Green and MitoTracker™ Orange, Z-stacks of representative fields of view for each condition were acquired using a LSM980 laser-scanning confocal microscope equipped with the Airyscan 2 detector in multiplex SR-4Y mode, which scans four image pixel rows simultaneously while minimally compromising spatial resolution, thereby increasing overall scanning speed. Samples were imaged with an alpha Plan-Apochromat 100×/1.46 NA oil-immersion objective, and raw data were pre-processed using the automatic Airyscan Processing module. MitoTracker™ Green (Invitrogen, 500 nM working concentration) and MitoTracker™ Orange (Cat. no. M7510, Invitrogen, 500 nM working concentration) were used at their respective final concentrations in pre-warmed culture medium, and the excitation and emission configurations are summarized in **Table 5.2**.

Dye	Excitation	Emission
MitoTracker™ Green FM	488 nm, 8% (2.67%) ^a laser power	Whole emission
MitoTracker™ Orange CMTMRos	561 nm, 0.4% (0.13%) ^a laser power	Whole emission

Table 5.2. Excitation and emission configurations for the dyes in LSM980 Airyscan mode. ^aDue to laser beam shaping in multiplex Airyscan mode, the laser power reaching a single pixel is substantially lower than in conventional confocal scanning; therefore, the equivalent power values for standard confocal mode are indicated in parentheses for each excitation setting.

Subsequently, images were deconvolved using the Fast-Iterative deconvolution algorithm implemented in ZEN 3.0 software (Carl Zeiss Microscopy GmbH, Jena, Germany). To ensure a homogeneous stack size for downstream segmentation, blank (non-informative) Z-tiles were added to all image stacks to match the Z-depth of the largest stack. Stacks were then segmented using the pixel classification workflow in Ilastik,² selecting all available 2D features. Resulting probability maps were converted into binary masks that were used for mitochondrial feature extraction in Fiji/ImageJ (v1.54). The 3D Objects Counter plugin was used to quantify morphometric features of individual mitochondrial objects,³ while the Skeletonize and Analyze Skeleton plugins were applied to characterize branching of the mitochondrial network.⁴ Skeletonize reduces the binary mask to a 1-pixel-wide skeleton, and Analyze Skeleton extracts skeleton metrics such as number of branches and mean branch length. In this context, “objects” are defined as groups of pixels that are considered connected in any of the three dimensions (x, y or z); “branching elements” are skeletonized objects considered connected in three dimensions; and “branches” are defined as skeleton segments between junctions. For the analysis of fractal properties, the FracLac plugin for ImageJ was used to compute the lacunarity by the box-counting method, using default parameters.

Data generated from the image analysis was batch-processed in R (v. 4.5.0) using RStudio (v. 2025.05.0). For mitochondrial network features, branching elements were stratified into low-branching (<100 branches) and high-branching (≥100 branches) to reduce the

influence of small, artefactual networks generated by over-skeletonization of minor structures detached from the main network.

The following metrics were calculated:

- Median high branch length (>100). Median of the mean branch length of each high-branching element.
- Median object sphericity. Sphericity (degree of similarity of an object's surface to that of a sphere of equal volume) was extracted from 3D object images using the MorphoLibJ plugin in Fiji/ImageJ, and reported as mean and median sphericity per image.
- Median volume of 3D objects. Median volume of all objects in each image, expressed in μm^3 .
- Mean mitochondrial intensity per volume. For each image, the integrated fluorescence intensity of all mitochondrial objects is divided by the sum of their volumes, yielding the average signal intensity per unit mitochondrial volume (a.u./ μm^3) for each experimental condition.
- Mean lacunarity. Sum of lacunarity values of all images within a group divided by the number of images in that group.

MitoTracker™ Orange mitochondrial membrane potential assay under hypoxia

HTR-8/SVneo cells were seeded at a density of 5×10^4 cells/well in 12-well plates and allowed to adhere for 24h at 37 °C and 5% CO₂. Cells were then pre-incubated with 100 or 200 $\mu\text{g}/\text{mL}$ CeO₂ NPs, while in parallel the culture medium for the hypoxia group was pre-equilibrated in the hypoxia workstation. After 24h, the medium was replaced to remove non-internalized NPs and cells were further maintained for 48h either in fresh normoxic medium or in preconditioned hypoxic medium within the InvivoO₂® 300 workstation at 0.5% O₂. At the end of the treatment, cells were incubated with MitoTracker™ Orange CMTMRos

(Cat. no. M7510, Invitrogen; 25 nM working concentration) diluted in pre-warmed culture medium for 30 min at 37 °C in the dark to allow membrane-potential-dependent accumulation in mitochondria. Cells were then gently washed with 1X PBS, detached by scraping, pelleted, and resuspended in HBSS 1X for acquisition. Fluorescence was recorded on an LSRFortessa™ flow cytometer (BD Biosciences) using the E:YFP-A (561 nm) laser line and the appropriate emission filter for MitoTracker™ Orange, acquiring at least 10.000 events per sample; data were analyzed with FlowJo™ v10 software to quantify changes in mitochondrial membrane-potential-dependent fluorescence across normoxic and hypoxic CeO₂ NP-treated groups.

Cresyl violet staining and DPX mounting

Cresyl violet staining and DPX mounting were performed as follows: tissue sections were immersed in cresyl violet solution (e.g., Cresyl Violet acetate, Sigma-Aldrich/Merck) for 5 min, briefly rinsed in distilled water, differentiated and dehydrated in 95% ethanol for 2 min followed by 100% ethanol for 2 min, and then cleared in xylene for 1 min before coverslipping with DPX mounting medium (DPX new, non-aqueous mounting medium for microscopy, 100579, Sigma-Aldrich/Merck) and imaging at 10× magnification on a light microscope.

LPS solution preparation

LPS from *Escherichia coli* serotype O111:B4 (Sigma-Aldrich/Merck, e.g., L2630) was prepared the day before administration by dissolving the lyophilized powder in sterile 1X PBS to obtain a stock solution corresponding to 0.5 µg LPS kg⁻¹ bw for a 300 g pregnant rat in an injection volume of 0.5 mL. The exact injection volume was adjusted in situ for each animal according to its individual body weight at the time of dosing and administered with a 25G needle at the tail vein.

CeO₂ NP dosing and formulation

CeO₂ NP dosing was defined based on both the available literature and the group's previous experience, and a final working dose of 630 µg CeO₂ NPs kg⁻¹ bw in an injected volume of 0.5 mL was selected. For *in vivo* administration, RSA was first diluted in 10 mM PB and then mixed with CeO₂ NPs to obtain a 1:2 CeO₂ NPs:RSA ratio, and the colloidal stability and hydrodynamic size distribution of this CeO₂ NPs@RSA formulation were evaluated by DLS, as described in the Methods section dedicated to NP-protein conjugation and aggregation monitoring, confirming that the formulation remained stable under the conditions used for *in vivo* dosing. The formulation was administered via tail vein with a 25G needle.

Ultrasound assessment of pregnancy

Pregnancy confirmation was performed at GD11 in pregnant rats using a Chison ECO2 ultrasound system (Chison Medical Imaging Co., Ltd., Jiangsu, China; model ECO2). Animals were lightly anesthetized with Isoflurane 100% (Isoflurano 100%, Insufarma, 250 ml; INS-ISO250, Insufarma, Chile) delivered in oxygen via a precision vaporizer. Rats were positioned in the supine position on a heated pad, and the abdominal region was thoroughly clipped and shaved to minimize air interference and improve image quality. Ultrasound coupling was achieved using Transonic blue ultrasound gel (Transonic blue ultrasound gel, Telic Group, Barcelona, Spain; ref. TOG02), a salt-free, hydrosoluble diagnostic ultrasound gel that does not irritate the skin, is non-greasy, and does not damage the transducer. Gestational sacs and embryos were visualized in B-mode to confirm intrauterine pregnancy, and images were stored digitally for subsequent documentation.

Body and organ weight assessment

Body weight of the dams was recorded before mating and at selected gestational time points using an electronic veterinary weighing scale 60052 (Stoelting Europe, Dublin,

Ireland). On the day of necropsy, individual fetal body weight was measured with the same scale immediately after extraction from the uterine horns. Organ weights were obtained after careful dissection and were weighed separately on the electronic veterinary weighing scale prior to ICP-MS analysis.

Systolic blood pressure measurement

Systolic blood pressure was measured non-invasively using a Panlab LE50021 (Barcelona, Spain) non-invasive blood pressure system for rodents equipped with a rat tail-cuff. Before the experimental recordings, rats were habituated to the procedure and apparatus for two weeks to minimize stress-related artefacts. For each session, animals were placed in appropriately sized red tubes to reduce environmental visual stimuli, with the tail exteriorized for cuff placement. The tail was gently warmed using heating pads to promote vasodilation and improve signal quality. Blood pressure measurements were then obtained in conscious rats by positioning the occlusion cuff and transducer around the base of the tail and recording several consecutive readings (minimum 3), which were subsequently averaged to obtain a representative systolic blood pressure value for each time point.

Cerium Content Determination by ICP-MS

All organs, placentas, and fetuses were collected on GD19 (sacrifice and necropsy), immediately frozen at -20 °C, and stored under these conditions until analysis. Prior to ICP-MS measurement, tissues were completely mineralized in an acidic mixture containing 45% HNO₃, 5% H₂O₂, and 50% H₂O in a microwave digestion system to ensure full dissolution of the organic matrix and CeO₂ NPs, thus converting the NPs into soluble ionic cerium species. Digestions were carried out in an Ethos™ Easy microwave digestion system (Ethos Easy, Milestone Srl, Sorisole, Italy) under a 200 °C digestion cycle for 120 min. Subsequently, tissue cerium ion content was determined by ICP-MS (7900 ICP-MS, Agilent) at the Chemical Analysis Service of the Universitat Autònoma de Barcelona (UAB, Barcelona, Spain), where all measurements were performed using a 1000 µg/g certified cerium

standard to construct the calibration curve and calculate cerium ion concentrations by interpolation.

Haematoxylin and eosin staining and sample fixation

Tissue samples for H&E staining were fixed immediately after necropsy to minimize autolysis. Organs were first placed into individually labelled histology cassettes and then immersed in 4% neutral buffered formaldehyde (10% neutral buffered formalin), using a tissue-to-fixative volume ratio of at least 1:10, and kept in fixative for 48h at 4 °C. After fixation, tissues were gently rinsed and transferred to 1X PBS for storage at 4 °C until processing.

Histological processing and paraffin embedding were performed in an automated tissue processor (VIP-type Leica ASP300S), using graded ethanol and xylene baths for progressive dehydration and clearing, followed by paraffin infiltration at 50-70 °C in a program of approximately 9h. The processed tissues were then embedded in paraffin blocks, sectioned at 3-5 µm, mounted on glass slides, and stained with routine H&E. Paraffin embedding, staining, and histopathological evaluation were carried out at the Servei d'Anatomia Patològica from Vall d'Hebron Hospital Campus.

Urine collection and storage

Morning urine was collected from conscious rats without the use of metabolic cages in order to minimize stress and reduce the incidence of pregnancy loss. Animals were placed individually in clean plastic boxes, and the first urine of the day was collected into sterile 1.5 mL eppendorf, then immediately centrifuged at 1500xg for 10 min at 4 °C to remove cells and debris. Finally, the clarified supernatant was aliquoted and stored at -20 °C until analysis.

Bradford assay for proteinuria detection

Proteinuria content, considered to represent predominantly the total protein excreted in urine, was quantified using the Bradford colorimetric assay (Bradford reagent, Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA) adapted to a 96-well plate format. BSA standards were prepared by serial dilution in 0.9% NaCl to obtain final calibration points ranging from 0 to 360 $\mu\text{g/mL}$ in the wells. All standards and the blank (0.9% NaCl without BSA) were loaded in triplicate, and Bradford reagent pre-equilibrated at room temperature was added to each well. After a 10 min incubation at room temperature, absorbance was measured at 595 nm using a Varioskan LUX multimode plate reader. Absorbance values were corrected by subtracting the blank (BSA=0) and plotted as $\log_{10}$ [BSA concentration] versus absorbance to fit a fourth-order polynomial calibration curve. Urinary protein concentrations were obtained by non-linear regression, considering the log transformation and the corresponding sample dilution factor.

Creatinine determination in urine

Urinary creatinine was quantified using a picric acid-based colorimetric assay (Jaffé-type reaction) adapted to a 96-well plate format, using picric acid solution and creatinine, 98% (Thermo Scientific Chemicals, Waltham, MA, USA; cat. no. 228940500). A creatinine stock solution of 0.2 mg/mL was prepared in 0.9% NaCl and used to generate a calibration curve covering 0-200 $\mu\text{g/mL}$ by mixing defined volumes of the stock with 0.9% NaCl directly in the wells; each standard concentration and the blank were loaded in triplicate. Urine samples were diluted 1:5 in 0.9% NaCl and dispensed into individual wells in parallel with the standards.

First, 0.75 M NaOH was added to all wells using a multichannel pipette, followed immediately by the addition of picric acid solution (43.65 mM), ensuring identical timing for standards and samples. After a 10 min incubation at RT, absorbance was measured at 492 nm using the Varioskan LUX plate reader, and a linear regression was fitted to the

standard curve to obtain sample creatinine concentrations, which were then multiplied by 5 to correct for the initial dilution. Urinary creatinine reflects the concentration/dilution of the urine and was therefore used to normalize urinary protein measurements, expressing proteinuria relative to creatinine content.

Proteinuria calculation

Proteinuria was expressed as the ratio of urinary total protein to creatinine, using the Bradford-derived total protein concentration (predominantly albumin) normalized to the corresponding creatinine concentration for each sample.

Euthanasia and necropsy

Rats were euthanized by inhalation of carbon dioxide (CO₂) in a dedicated rodent chamber, in accordance with international guidelines for the care and use of laboratory animals and procedures approved by the institutional ethics committee. CO₂ concentration was increased in a controlled manner until complete loss of consciousness and respiratory arrest were achieved, thereby minimizing stress and discomfort, and death was confirmed by the absence of reflexes and cardiorespiratory arrest before proceeding to necropsy. Following confirmation of death, a midline laparotomy and thoracotomy were performed under sterile conditions to expose the thoracic and abdominal organs. Organs, placentas and fetuses were weighed and collected in eppendorf tubes either for storage at -20 °C for subsequent ICP-MS analysis or fixed in 4% paraformaldehyde for subsequent paraffin embedding and H&E staining.

5.2 References

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6.1 Scalable and reproducible synthesis of biocompatible CeO₂ nanoparticles (chapter 2)

I. The synthesis process developed is highly robust and reproducible, producing monodisperse 3 nm (HR-TEM) CeO₂ NPs with a stable fluorite structure and minimal batch-to-batch variability. Multimodal characterization including: UV-Vis absorption at 283 nm, DLS monodispersity, TEM/HRTEM uniform morphology and crystallinity, XRD fluorite-phase identification, confirms the internal consistency of the material and meets essential requirements for future industrialization.

- a) Maintaining appropriate molar ratios between all reactants is critical to achieve controlled growth, preserve NP monodispersity and colloidal stability, and ensure the reproducibility of the synthesis.
- b) CeCl₃ and Ce (NO₃)₃ precursors generate NPs of equivalent quality, although CeCl₃ is preferable due to its more favorable environmental and safety profile, and better alignment with GMP-oriented production.
- c) Sodium citrate is essential for colloidal stabilization. The Ce:SC molar ratio in forming Ce-citrate complexes strongly influences the NPs size and monodispersity. In contrast, the complexation time and order of mixing are robust parameters: variations in complexation time or mixing sequence do not significantly affect the final outcome.
- d) Oxygen availability, and pH control, are critical for NPs formation. Oxidation of Ce³⁺ to Ce⁴⁺ is essential for the CeO₂ formation, with dissolved oxygen acting as the oxidant by accepting the electrons released from Ce³⁺. Furthermore, the strongly basic pH provided by TMA-OH is crucial for cerium to precipitate out the solution into CeO₂ NPs form.
- e) Thermal treatment improves NP stability by dehydrating the precipitate and stabilizing nanoparticle surface chemistry over time, without altering particle size or crystallinity, thereby providing highly reproducible materials.

II. CeO₂ NPs show strong antioxidant activity, efficiently reducing ABTS•⁺ and decomposing H₂O₂ via a mechanism analogous to natural catalase. This activity is governed by the intrinsic catalytic properties associated with redox switching between Ce³⁺ and Ce⁴⁺, and by the oxygen vacancy content, permitting tunability of the catalytic response for different applications.

III. Optical properties, including UV-Vis spectra and the corresponding extracted band-gap values, are highly sensitive to changes in defect chemistry. Processes such as thermal treatment and H₂O₂ exposure modify the Ce³⁺/Ce⁴⁺ redox balance and the oxygen-vacancy concentration, demonstrating a strong coupling between surface chemistry, defect structure, and optical behavior. This sensitivity enables the use of optical measurements as an effective probe to monitor these defect-chemistry changes.

IV. The stability of the nanoceria, their controlled 3 nm size, high reproducibility and robustness, together with their sustained antioxidant and catalase-like activity, position these CeO₂ NPs as promising therapeutic agents for biomedical applications involving oxidative stress and inflammation.

6.2 Cytoprotective effect of CeO₂ NPs on extravillous trophoblasts in a severe hypoxia preeclampsia-like model (chapter 3)

I. The *in vitro* hypoxia model was validated as a robust and reproducible system. The 0.5% O₂ / 48h protocol reliably induced significant intracellular hypoxia to HTR-8/SVneo trophoblasts, stabilized HIF-1 α protein, and activated inflammatory, metabolic, migratory, and angiogenic pathways characteristic of trophoblastic dysfunction under preeclampsia severe hypoxia.

II. CeO₂ NPs were confirmed to be biocompatible within a defined concentration window. CeO₂ NPs at 50-200 μ g/mL remained colloidally stable and non-toxic under both normoxia and hypoxia, supporting their safe application in trophoblast cultures and hypoxic cellular environments.

III. Within the biocompatible validated concentration window (50-200 μ g/mL), 200 μ g/mL emerged as the most effective dose.

IV. Owing to their reactive oxygen species (ROS)-scavenging properties, CeO₂ NPs proved to indirectly mitigate multiple aspects of severe hypoxia-induced damage in HTR-8/SVneo trophoblasts:

- a) CeO₂ NPs markedly reduced *IL6*, *IL1B* and *CCL2* expression, demonstrating a potent anti-inflammatory effect.
- b) CeO₂ NPs reduced the hypoxia-induced overexpression of *SLC2A1* arising from the metabolic shift toward anaerobic glycolysis. This decrease in *SLC2A1* is associated with a partial restoration of metabolic homeostasis.

- c) C. CeO₂ NPs modulated matrix metalloproteinase expression and restored controlled trophoblast migration while reducing *MMP2* and *MMP9* expression, normalizing the aberrantly accelerated migration observed under hypoxia.
 - d) D. CeO₂ NPs attenuated hypoxia-induced dysregulation of angiogenic signaling by diminishing *VEGFA* and *PGF* overexpression, thereby contributing to the correction of the angiogenic imbalance triggered by severe hypoxia.
- V. CeO₂ NPs partial preserve mitochondrial structure and function under severe hypoxia.
- a) Severe hypoxia induced mitochondrial fragmentation, increased sphericity, and a marked reduction in network complexity, all indicative of mitochondrial damage.
 - b) CeO₂ NPs modulated mitochondrial dynamics, promoting mitochondrial hyperfusion and improved network integrity as an adaptive response to sustained harmful hypoxia.
 - c) Mitochondrial membrane potential ($\Delta\Psi_m$) was preserved in CeO₂ NP-treated cells, indicating that mitochondria retained their bioenergetic capacity and maintained their functional integrity under severe hypoxia.

6.3 Biodistribution and protective effects of CeO₂ NPs in an LPS-induced pre-eclampsia model: Preliminary pilot study (chapter 4)

I. The intravenous 0.5 $\mu\text{g}\cdot\text{kg}^{-1}$ bw low-dose LPS protocol, administered either on GD14 or GD15 in pregnant rats, successfully mimicked several aspects of a PE-like phenotype, including maternal sickness behavior, elevated SBP, an altered cytokine profile, reduced maternal and fetal weights, and kidney alterations, including histopathological changes and proteinuria, consistent with PE-related systemic damage.

II. LPS was administered, either i.p or i.v in early gestation (GD5), doses ranging from 0.5 to 10 $\mu\text{g}\cdot\text{kg}^{-1}$ bw consistently resulted in pregnancy loss.

III. Only low dose of 0.5 $\mu\text{g}\cdot\text{kg}^{-1}$ bw,(i.v) administered at late gestation (GD15) allowed pregnancies to progress until necropsy, higher doses also resulted in miscarriage.

IV. The ultra-low-dose regimen successfully mimicked several systemic features of PE, but did not fully reproduce placental impairment. Therefore, a dose of 0.5 $\mu\text{g}\cdot\text{kg}^{-1}$ bw would more closely resemble a late-onset preeclampsia model, as late PE does not always present with overt placental damage.

V. CeO₂ NPs demonstrated protective effects on maternal well-being, leading to an improvement in behavioral and physical signs in LPS-treated pregnant rats. Both administration strategies improved maternal status, being the prophylactic strategy more effective.

VI. Exposure to ultra low-dose LPS resulted in a clear reduction in fetal growth, consistent with an FGR-like phenotype, a hallmark of PE. Both CeO₂ NPs administration strategies partially counteracted this effect.

VII. Administration of CeO₂ NPs, likely due to their capacity to modulate redox imbalance through ROS-neutralizing activity and consequently attenuate inflammation, resulted in a

marked reduction of circulating pro-inflammatory cytokines, particularly CCL-2 and IL-6, while TNF- α levels remained unchanged. Thus, nanoceria, particularly the prophylactic administration, show to partially dampen the acute inflammatory response triggered by LPS.

VIII. CeO₂ NPs exhibited a characteristic biodistribution profile, widely reported in the literature, with the liver as the primary site of accumulation, retaining approximately 80% of the injected dose, followed by the spleen.

IX. Analysis of placental and fetal tissues revealed a higher placental accumulation of CeO₂ NPs in the treatment scheme compared with prophylaxis, likely reflecting increased inflammation and tissue permeability at the time of NPs administration, while fetal accumulation remained negligible in both conditions. This represents a particularly relevant finding, as available data on nanoceria biodistribution in the materno-fetal compartment are scarce and often contradictory, and our results support the concept of preferential placental retention with minimal direct fetal exposure.

X. The LPS ultra-low-dose regime induced renal involvement, characterized by tubular lumen dilatation and glomerular swelling with reduced Bowman's space, together with a tendency toward increased proteinuria. CeO₂ NPs, in both prophylactic and treatment regimens, partially preserved renal architecture and attenuated proteinuria, supporting a modest renoprotective effect in this PE-like model.

XI. Further, placentas did not show overt histological alterations by H&E but exhibited macroscopic darkening compatible with vascular congestion and inflammation, a pattern that was also observed in the intestines. CeO₂ NPs attenuated these macroscopic inflammatory changes, suggesting a partial protective effect on maternal vascular and tissue inflammatory status.

6.4 Thesis general conclusions

- I. A reproducible method was established to synthesize monodisperse 3 nm CeO₂ NPs with a fluorite structure, validated by comprehensive physicochemical characterization.
- II. CeO₂ NPs reproducibility critically depends on Ce:citrate ratio, pH control, oxygen availability as a key factor.
- III. CeO₂ NPs exhibit antioxidant and catalase-like activity, governed by the Ce³⁺/Ce⁴⁺ balance and oxygen vacancies, enabling tunable redox behavior.
- IV. UV-Vis/band-gap features are sensitive to redox state and defect chemistry, providing a practical probe to monitor treatment-induced changes.
- V. The severe hypoxia cell model (0.5% O₂, 48h) reliably reproduced inflammatory, metabolic, migratory, and angiogenic alterations consistent with trophoblastic dysfunction in PE.
- VI. Within the biocompatible range (50-200 µg/mL), CeO₂ NPs, particularly at 200 µg/mL, attenuated hypoxia-induced responses by reducing pro-inflammatory mediators, moderating the glycolytic shift, and improving migration and angiogenic markers.
- VII. At the mitochondrial level, CeO₂ NPs partially preserved network integrity and membrane potential ($\Delta\Psi_m$) under hypoxic stress.
- VIII. In the ultra-low-dose 0.5 µg·kg⁻¹ bw LPS *in vivo* pilot, the model induced PE-like systemic alterations but did not fully reproduce placental impairment, resembling a late-onset preeclampsia-like phenotype characterized predominantly by maternal

systemic involvement rather than overt placental pathology. In this context, CeO₂ NPs afforded partial maternal-fetal protection, evidenced by improved general physical and behavioral status, reduced inflammatory cytokines, renoprotection, and attenuation of fetal growth restriction, with prophylactic administration proving more effective than treatment dosing.

- IX. Biodistribution analyses showed predominant accumulation in the liver (and spleen), variable placental retention, and no fetal exposure, supporting a favorable maternal-fetal safety profile. Placental uptake was scheme-dependent, being higher in the treatment regimen than in prophylaxis, likely influenced by the heightened inflammatory state and increased placental permeability at the time of dosing.

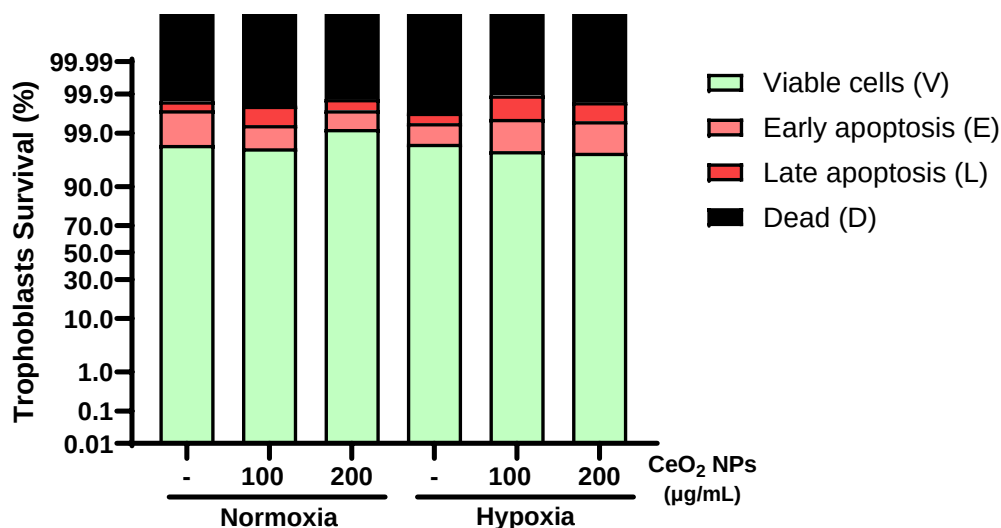
Additional graph of the 0.5% O₂ hypoxia (48h) PE-like model

Figure A1. Apoptosis and cell death in HTR-8/SVneo trophoblasts following CeO₂ NPs exposure. Cells were treated with CeO₂ NPs (0, 100, or 200 µg/mL) for 48h under normoxic (18% O₂) or hypoxic (0.5% O₂) conditions. A stacked bar graph summarizes the percentage distribution of cell populations across treatment conditions, including viable (V), early apoptotic (E), late apoptotic (L), and dead (D) cells, as indicated in the legend. Apoptosis assessed by flow cytometry using the Pacific Blue™ Annexin V / Sytox™ AADvanced™ Apoptosis Kit and analyzed with FlowJo. Annexin V Pacific Blue was acquired in the Pacific Blue channel (V450-A) with the violet laser (405 nm), and Sytox™ AADvanced™ was acquired in the YG610-A channel with the yellow-green laser (561 nm). stacked bar graph summarizes the percentage of each population across conditions and doses; legend indicates V (viable), E (early apoptosis), L (late apoptosis), and D (dead). Data are mean ± SD (n = 3). One-way ANOVA with Tukey's post hoc test; $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

Graphs of the 0.5% O₂ hypoxia (24h) - 0.5% O₂ reoxygenation (6h) PE-like model

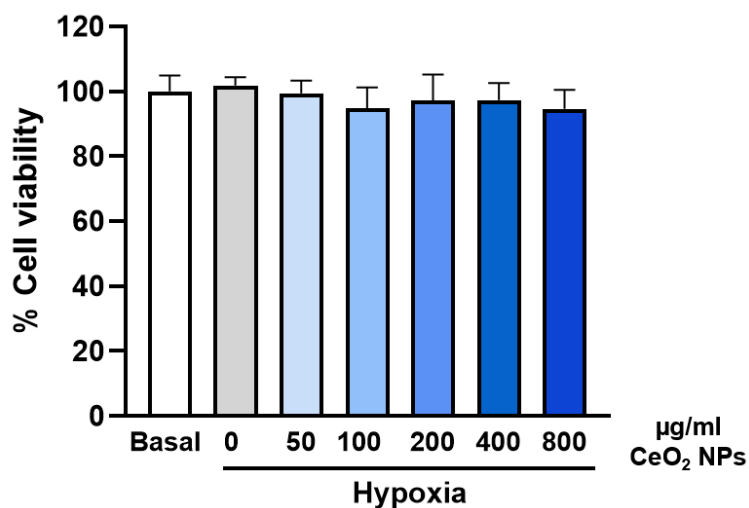


Figure A2. Cell survival of HTR-8/SVneo trophoblasts pre-exposed to 50, 100, 200, 400, and 800 µg/mL CeO₂ NPs under 0.5% O₂ (hypoxia) for 24h, followed by 6h hours of reoxygenation at 18% O₂. Assay assessed by PrestoBlue™ assay. Data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

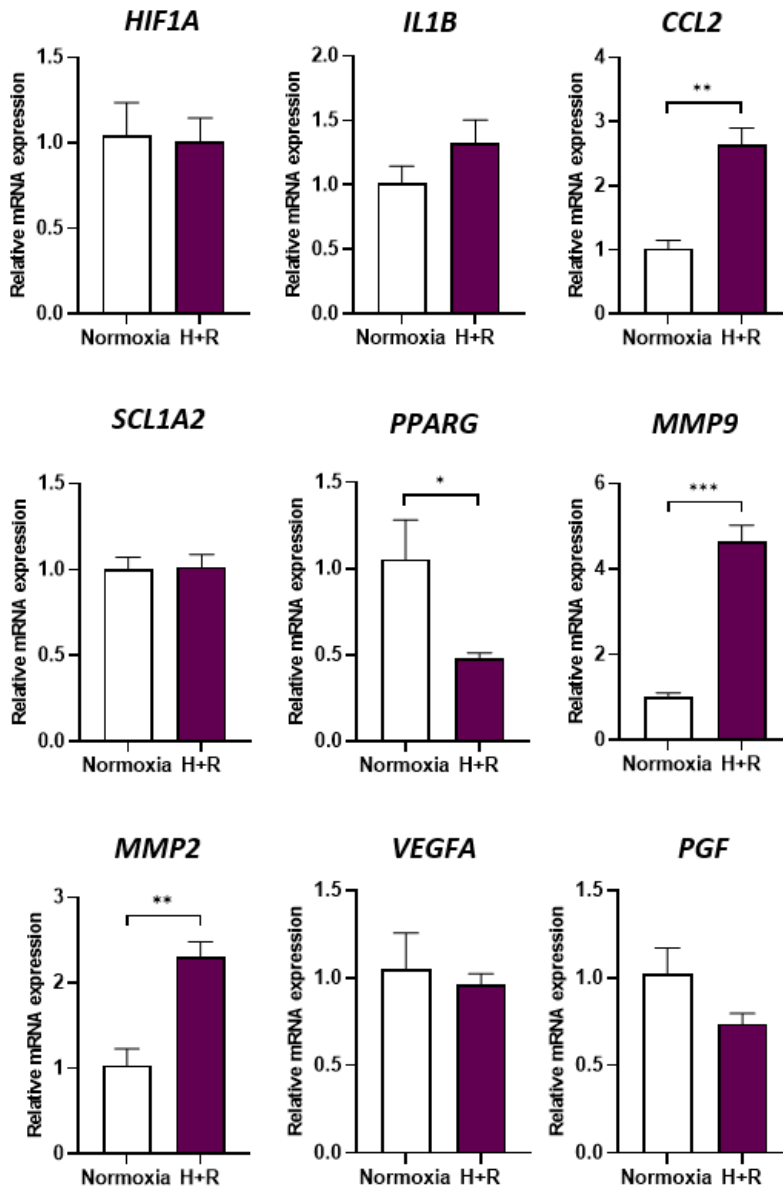


Figure A3. Effect of hypoxia-reoxygenation on gene expression in HTR-8/SVneo trophoblasts. HTR-8/SVneo cells were cultured under normoxia (Basal, 18% O₂) or subjected to hypoxia (0.5% O₂) for 24h followed by a reoxygenation period under Normoxia (18% O₂) for 6h (H+R). Relative mRNA expression levels of *HIF1A*, *IL1B*, *CCL2*, *SCL1A2*, *MMP9*, *MMP2*, *PPARG*, *VEGFA* and *PGF* were measured by qRT-PCR and normalized to the housekeeping gene *RPL37A*. Results are presented as fold change relative to the basal. Data are shown as mean $\pm$ SD (n = 3). Statistical significance was assessed using an unpaired two-tailed Student's t-test. p $\leq$ 0.05 (*), p $\leq$ 0.01 (**), p $\leq$ 0.001 (***), p $\leq$ 0.0001 (****).

Graphs of the LPS (24h) PE-like model

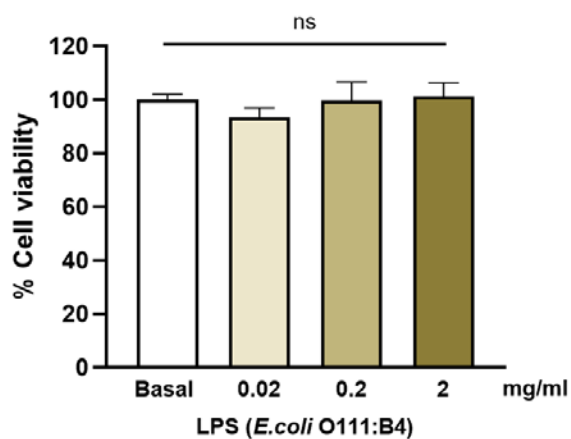


Figure A4. Cell viability of HTR-8/SVneo trophoblasts after exposure to LPS from *E. coli* O111:B4 for 24h. Cells were exposed to increasing concentrations of LPS (0, 0.02, 0.2, and 2 mg/mL) for 24h. Cell viability was assessed using the PrestoBlue™ assay. Data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

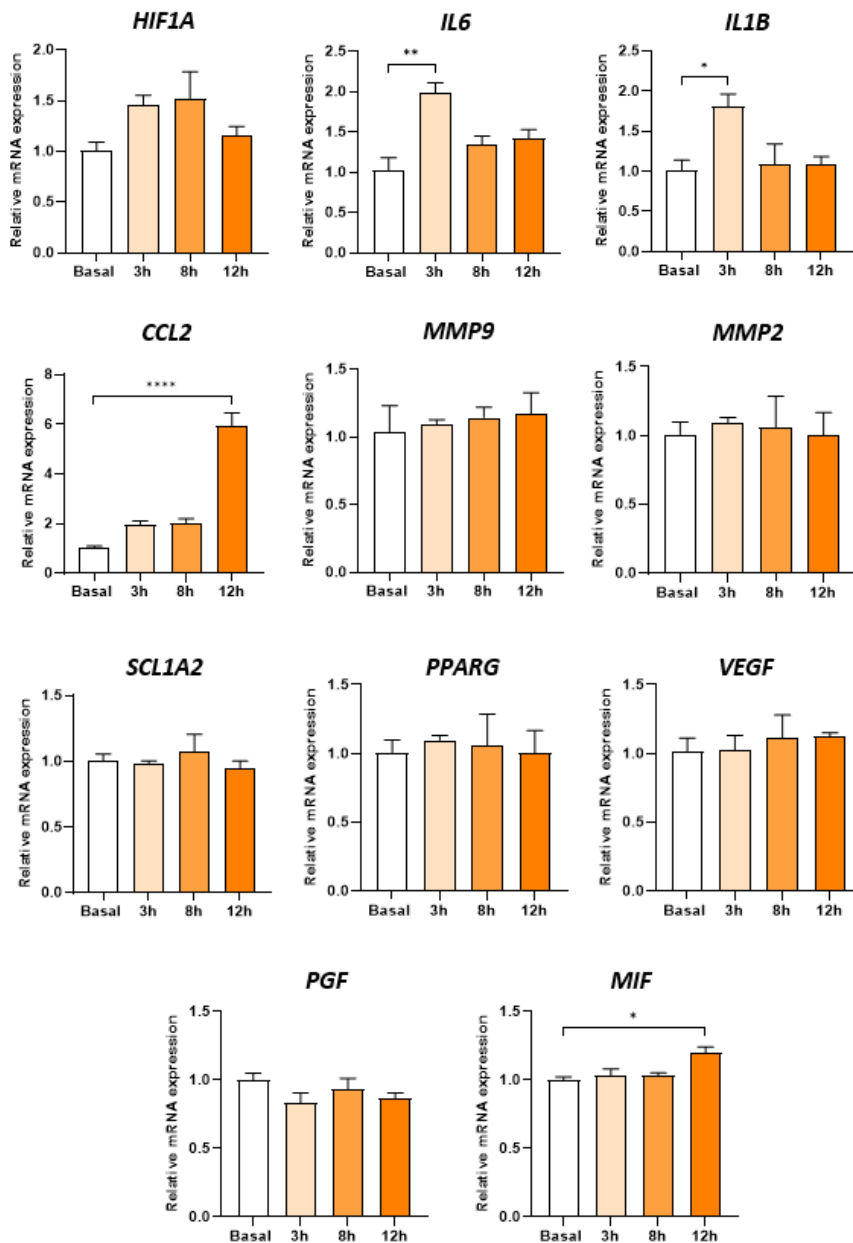


Figure
A5.
Time-

dependent effect (3 h, 8 h, 12 h) of *E. coli* O111:B4 LPS (200 µg/mL) on gene expression in HTR-8/SVneo trophoblasts. HTR-8/SVneo cells were stimulated with 200 µg/mL of LPS from *Escherichia coli* O111:B4 for 3h, 8h, and 12h, and compared to unstimulated basal. Relative mRNA expression levels of *HIF1A*, *IL6*, *IL1B*, *CCL2*, *MMP9*, *MMP2*, *SCL1A2*, *PPARG*, *VEGFA*, *PGF* and *MIF* were measured by qRT-PCR. Gene expression was normalized to the housekeeping gene *RPL37A* and is shown as fold change relative to the basal. Data represent mean $\pm$ SD (n = 3). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

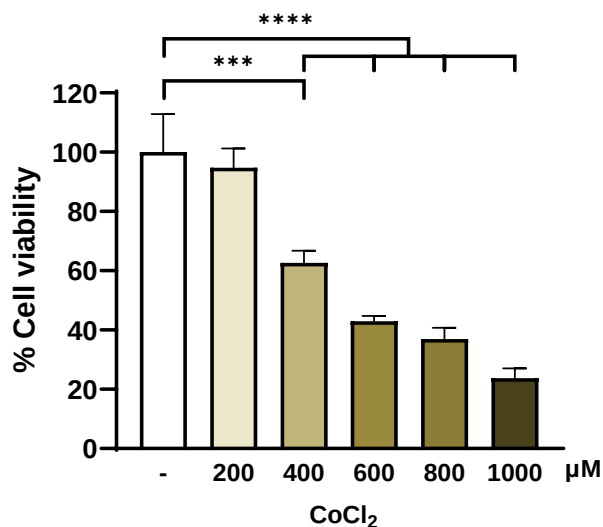
Graphs of the CoCl_2 PE-like model

Figure A6. Cell viability of HTR-8/SVneo trophoblasts after exposure to CoCl_2 for 24h. Cells were treated with increasing concentrations of CoCl_2 (200, 400, 600, 800, and 1000 μM). Cell viability was assessed using the PrestoBlue™ assay. Data are expressed as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

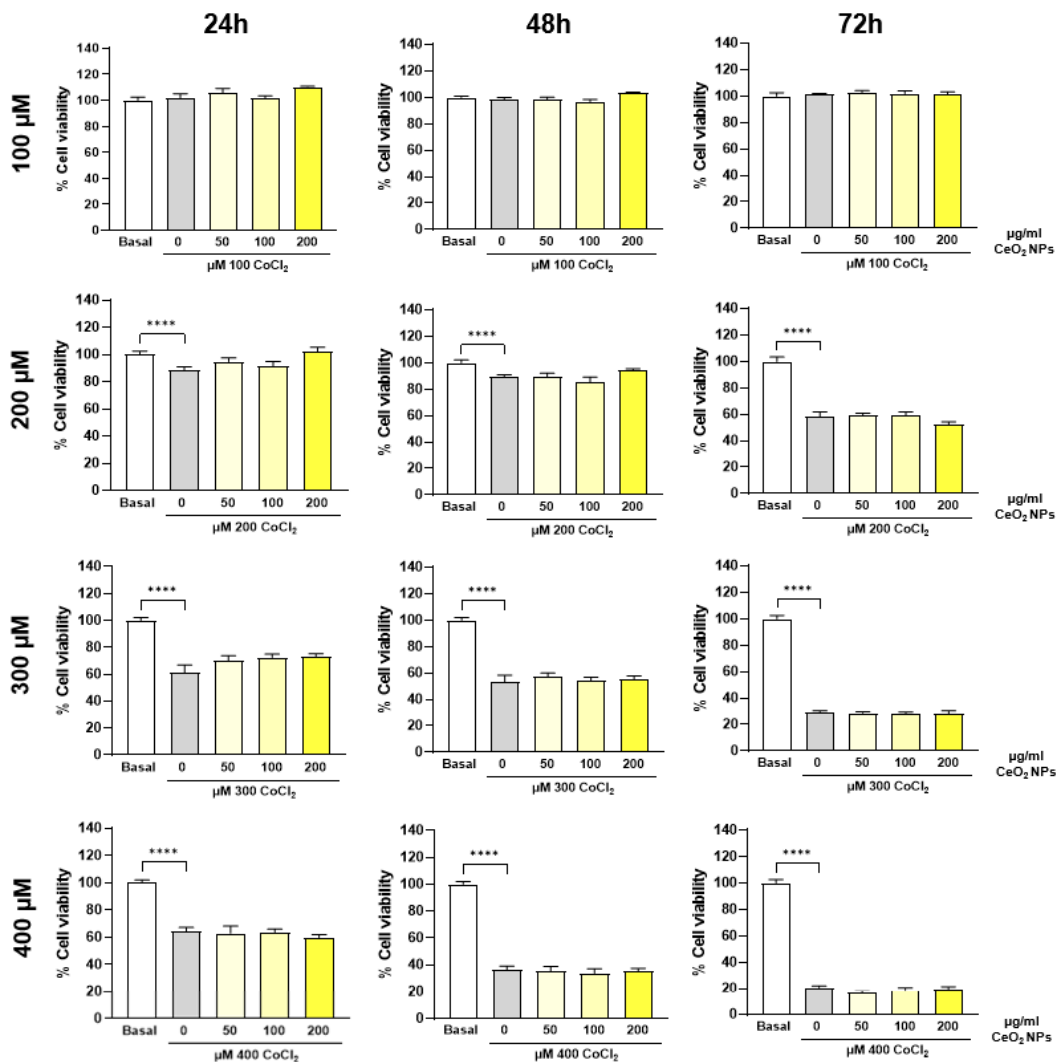


Figure A7. Effect of CeO₂ NPs against CoCl₂-induced cytotoxicity in HTR-8/SVneo trophoblasts. Cell viability pre-exposed to 0, 50, 100, and 200 µg/mL CeO₂ NPs, followed by exposure to various concentrations of CoCl₂ (100, 200, 300, and 400 µM) for 24, 48, and 72h. Cell viability was assessed using the PrestoBlue™ assay. Data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

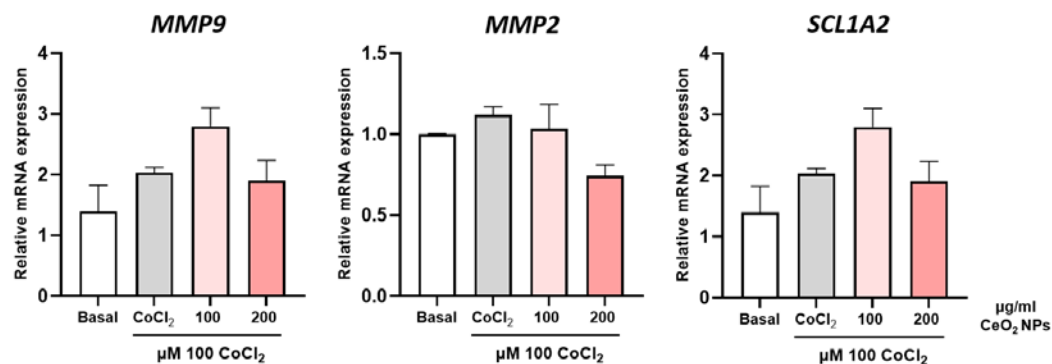


Figure A8. Effect of CoCl_2 on gene expression in HTR-8/SVneo trophoblasts. HTR-8/SVneo cells were treated with CoCl_2 , 100 μM in the presence or absence of CeO_2 NPs (0, 100, and 200 $\mu\text{g}/\text{mL}$) and compared with untreated basal controls. Relative mRNA expression levels of *MMP9*, *MMP2*, and *SLC1A2* were quantified by qRT-PCR. Gene expression was normalized to the housekeeping gene *RPL37A* and expressed as fold change relative to the basal condition. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

Articles not included in this thesis

Ernst, L. E., Mondragón, L., **Ramis, J.**, Gustà, M. F., Yudina, T., Casals, E., Bastús, N. G., Fernández-Varo, G., Casals, G., Jiménez, W. & Puentes, V. Exploring the Long-Term Tissue Accumulation and Excretion of 3 nm Cerium Oxide Nanoparticles after Single Dose Administration. *Antioxidants* **12**, 765 (2023). doi.org/10.3390/antiox12030765

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Krug, A., Ernst, L. M., Mhaidly, R., **Ramis, J.**, Gusta, M. F., Bastus, N. G., Martinez-Turtos, A., Tosolini, M., Di Mascio, L., Tari, G., Boyer, L., Gaulard, P., Lemonnier, F., Ricci, J.-E., Verhoeven, E. & Puentes, V. Scavenging Reactive Oxygen Species by Cerium Oxide Nanoparticles Prevents Death in a Peripheral T Cell Lymphoma Preclinical Mouse Model. *ACS Nano* **19**, 18644–18660 (2025). doi.org/10.1021/acsnano.5c02860

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