UNIVERSITI TEKNOLOGI MARA

DOSE-DEPENDENT EFFECTS OF OVALBUMIN-INDUCED ALLERGIC ASTHMA ON SYSTEMIC INFLAMMATION, OXIDATIVE STRESS AND PREIMPLANTATION EMBRYO VIABILITY, AND THE PROTECTIVE ROLE OF TOCOTRIENOL-RICH FRACTION SUPPLEMENTATION IN MICE

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ABSTRACT

Allergic asthma is an inflammatory airway disease affecting approximately 339 million people worldwide encompassing both children and adults. Its pathogenesis involves activation of T-helper type 2 (Th2) immune responses and oxidative stress resulting from an imbalance between reactive oxygen species (ROS) and antioxidants. This imbalance further exacerbates airway inflammation and tissue injury. While the respiratory manifestations of asthma are well-characterised, its systemic repercussions on reproductive physiology and early embryonic stage remain poorly understood. Maternal asthma has been associated with adverse pregnancy outcomes including preterm birth and foetal growth restriction likely due to systemic inflammation and oxidative stress during the sensitive preimplantation period. Given that inflammatory and oxidative insults at this stage can disrupt developmental potential, morphological quality, organelles features and epigenetic regulation in embryos, maternal supplementation with natural compounds possessing both antioxidant and anti-inflammatory properties may offer protection. This study aimed to determine the protective effects of maternal tocotrienol-rich fraction supplementation (60 mg/kg body weight) on systemic inflammation and oxidative stress, pregnancy hormone levels and subsequent effects on developmental potential, morphological quality, ultrastructural features and gene expression of 2-cell embryos in a mouse model of OVA-induced allergic asthma. In Experiment I, the study established that increasing doses of ovalbumin (OVA; 50–150 µg/200 µL) elicited dose-dependent maternal inflammation and oxidative stress significantly impairing 2-cell embryo development and morphology. In Experiment II, maternal supplementation with tocotrienol-rich fraction (TRF; 60 mg/kg body weight) markedly ameliorated systemic inflammation and oxidative stress markers, and enhanced the developmental potential and morphological quality of two-cell embryos. In addition, transmission electron microscopy (TEM) revealed increased organelles and mitochondrial volumes, mitochondrial clustering and enhanced mitochondria-lipid droplets interaction resulted in peridroplet mitochondria formation suggesting improved inter-organelle communication. Moreover, gene expression profiling (Fluidigm qPCR) demonstrated that TRF downregulated oxidative stress-related genes (*Nox1*, *Sod1*, *Gpx4*), promoted mitochondrial fusion (*Mfn1*, *Mfn2*, *Opa1*), supported mitochondrial bioenergetics (*Atp5e*, *Cox4i1*) and inhibited apoptosis (*Bax*, *Bcl2*). Collectively, these findings establish that maternal TRF supplementation effectively counteracts allergic asthma–induced systemic inflammation and oxidative stress, thereby safeguarding early embryonic development. This work unveils a novel mechanistic link between maternal inflammation and oxidative stress-mitochondrial health-embryo viability while positioning TRF as a promising maternal nutraceutical to fortify embryonic resilience and improve reproductive success in inflammatory conditions.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

Symbols

α	Alpha
β	Beta
δ	Delta
γ	Gamma
°C	Celsius
%	Percentage
<	Less than
≤	Less than or equal to
>	Greater than
µg	Microgram
µL	Microliter
µm	Micrometre
E	Analysis of Variance (ANOVA)
g	Gram
G	Gauge
IU	International Unit
Kg	Kilogram
kV	Kilovolt
M	Molar

mg	Milligram
mL	Millilitre
mM	Millimolar
mm	Millimetre
mmol	Millimole
ng	Nanogram
nm	Nanometre
OD	Optical Density
pg	Picogram
rpm	Revolutions per Minute
Vv	Relative Volume

LIST OF ABBREVIATIONS

Abbreviations

ADP	Adenosine Diphosphate
AHR	Airway Hypersensitivity
ANOVA	Analysis of Variance
APCs	Antigen Presenting Cells
ASEBIR	Association for the Study of Reproductive Biology
ATP	Adenosine Triphosphate
BDMA	Benzyldimethylamine
CARE	Committee on Animal Research and Ethics
CAT	Catalase
cDNA	Complimentary Deoxyribonucleic Acid
Ct	Cycle Threshold
d.p.c	Days Post Coitum
DCs	Dendritic Cells
DDSA	Dodecenyl Succinic Anhydride
DNA	Deoxyribonucleic Acid
DNMT	Deoxyribonucleic Acid Methyltransferases
DOHaD	Developmental Origins of Health and Disease
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplasmic Reticulum

ETC	Electron Transport Chain
$FADH_2$	Flavin Adenine Dinucleotide (reduced form)
FRAP	Ferric-reducing Ability of Plasma
FSH	Follicle Stimulating Hormone
GOI	Genes of interest
GPx	Glutathione Peroxidase
hCG	Human Chorionic Gonadotropin
HDM	House Dust Mite
HRP	Horseradish Peroxidase
ICM	Inner Cell Mass
IFC	Integrated Fluidic Circuit
IMMB	Institute of Medical Molecular Biotechnology
i.n	Inhalation
i.p	Intraperitoneal
Ig	Immunoglobulin
IL	Interleukin
IMS	Intermembrane Space
LABAs	Long-acting Beta-agonists
LACU	Laboratory Animal Care Unit
LAFAM	Laboratory Animal Facility and Management
LD	Lipid Droplet
LH	Luteinising Hormone

LPS	Lipopolysaccharide
MDA	Malondialdehyde
MIM	Inner Mitochondrial Membrane
MNA	Methyl Nadic Anhydride
MOM	Outer Mitochondrial Membrane
MOMP	Mitochondrial Outer Membrane Permeabilization
MPOB	Malaysian Palm Oil Board
MZT	Maternal-to-zygotic Transition
n.i	Intranasal instillation
NADH	Nicotinamide Adenine Dinucleotide
NADP+	Nicotinamide Adenine Dinucleotide Phosphate Ion
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NPBs	Nucleolus Precursor Bodies
OVA	Ovalbumin
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PMSG	Pregnant Mare Serum Gonadotropin
PVS	Perivitelline Space
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Stress
ROS	Reactive Oxygen Species

RQ	Relative Quantification
SABAs	Short-acting Beta-agonists
SD	Standard Deviation
SEM	Standard Error of Mean
TCA	Tricarboxylic Acid
TCP	Tocopherol
TCT	Tocotrienol
TMB	3,3',5,5'-tetramethylbenzidine
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP nick-end Labeling
TE	Trophectoderm
TEM	Transmission Electron Microscopy
Th2	T-helper type 2
TRF	Tocotrienol-rich Fraction
UiTM	Universiti Teknologi MARA
WHO	World Health Organisation
ZGA	Zygotic Genome Activation
8-OHdG	8-hydroxy-2'-deoxyguanosine

LIST OF NOMENCLATURES

Nomenclatures

$Al(OH)_3$	Aluminium Hydroxide
Atp5e	ATP Synthase F1 Subunit Epsilon
BALB/c	Bagg's Albino
Bax	BCL2 Associated X, Apoptosis Regulator
Bcl2	B-cell Lukemia/lymphoma 2
Casp3	Caspase-3
CO_2	Carbon Dioxide
Cox4i1	Cytochrome c Oxidase, Subunit 4I1
E_2	Oestrogen/ Oestradiol
$FeSO_4$	Ferrous Sulfate
$FeSO_4 \cdot 7H_2O$	Ferrous Sulfate Heptahydrate
Fe^{3+}-TPTZ	Ferric-tripyridyltriazine
Fe^{2+}-TPTZ	Ferrous-tripyridyltriazine
Gpx1	Glutathione Peroxidase 1
Gpx4	Glutathione Peroxidase 4
H^+	Hydrogen Ion
H_2O_2	Hydrogen Peroxide
Mfn	Mitofusin
NaOH	Sodium Hydroxide

NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B cells
NFATc1	Nuclear Factor of Activated T cells 1
Nox1	NADPH Oxidase 1
Nrf2	Nuclear Factor E2-Related Factor 2
O_2	Oxygen
$O_2•-$	Superoxide Anion
OH^-	Hydroxyl Radical
Opa1	OPA1 mitochondrial dynamin like GTPase
P_4	Progesterone
PGC-1α	Peroxisome Proliferator–activated Receptor Gamma Coactivator-1 Alpha
Ppargc1a	Peroxisome Proliferator-activated Receptor Gamma Coactivator 1-alpha
Sod1	Superoxide Dismutase 1
Sod2	Superoxide Dismutase 2
Tfam	Transcription Factor A, Mitochondrial
TNF	Tumor Necrosis Factor

CHAPTER 1
INTRODUCTION

1.1 Background

Allergic asthma is an inflammatory disease of the airways that is triggered by allergens. The World Health Organization (WHO) estimates that 262 million people worldwide have been affected by asthma in the past five years and the prevalence is projected to rise to 339 million in the near future. Asthma can affect individuals of all ages, from childhood to adulthood. Notably, it remains the most prevalent chronic respiratory disease among children. Allergic asthma is characterised by inflammation of the airways which leads to structural remodelling, thickening and narrowing of the airway walls and overproduction of mucus (J. Cui et al., 2019). These changes contribute to airway obstruction and lead to typical asthma symptoms such as shortness of breath, chest tightness, wheezing and coughing (H. Wang et al., 2020). Over time, these symptoms can result in chronic morbidity and in severe cases may lead to death. In Malaysia, the mortality rate due to asthma is 1.29% (Ministry of Heatlh Malaysia, 2018).

The pathogenesis of allergic asthma is complex and is influenced by numerous factors including allergens, age, genetic predisposition and environmental influences. In most cases of allergic asthma, the T-helper (Th) type 2 immune response is activated in the airways. This response occurs in two phases: sensitisation and challenge. During sensitisation, antigen-presenting cells (APCs) process allergens and present them to naïve Th2 cells which then secrete cytokines such as interleukin (IL)-4, IL-5 and IL-13. These cytokines stimulate the B cells to produce immunoglobulin (Ig)-E (Habib et al., 2022). In the challenge phase, IgE binds to the same allergen upon re-exposure and triggers the release of inflammatory mediators such as histamine, leukotrienes and ILs which contribute to airway inflammation and clinical symptoms (Habib et al., 2022).

In addition to immune mechanisms, oxidative stress which occurs when the balance between oxidants and antioxidants is disrupted has been shown to play a key role in the pathophysiology of allergic asthma (V. Mishra et al., 2018). In oxidative stress, excessive oxidants or reactive oxygen species (ROS) overwhelm the antioxidant defence system resulting in damage to cellular components (Lushchak & Storey, 2021).

Several studies have established a link between inflammation and oxidative stress in asthma. They show that inflammatory cells recruited to the airways produce ROS that exacerbate tissue damage and worsen the severity of the disease (J. Qu et al., 2017).

Although many studies have focused on airway inflammation and oxidative stress in asthma, the systemic effects of this disease on other physiological systems such as reproductive system are still poorly understood. For example, maternal asthma at early pregnancy stage has been associated with unfavourable pregnancy outcomes at later stage such as intrauterine growth restriction, congenital anomalies and preterm birth (Bonham et al., 2018; Fazel et al., 2018; Pali-Schöll et al., 2017). This study further investigates how maternal inflammatory responses in asthma can affect communication between mother and embryo in the early stage of pregnancy.

The preimplantation period is the earliest stage of pregnancy characterized by high sensitivity to maternal physiological and environmental factors. After fertilisation, zygote undergoes cleavage division to form a two-cell embryo that is heavily dependent on maternal transcripts and organelles. This is followed by zygotic genome activation (ZGA), a crucial event for further development (Abe et al., 2018; Bruce & Mihajlovic, 2017). At this stage, the embryo is particularly susceptible to external influences such as maternal physiological stress. For example, exposure to cadmium during the preimplantation period has been shown to increase ROS, impair mitochondrial structure and disrupt epigenetic processes such as deoxyribonucleic acid (DNA) methylation, thereby reducing embryo viability (Zhu et al., 2018, 2021).

At cellular level, organelles integrity is essential for embryo viability. The Mitochondria are one of the important organelles for early embryonic development as they play major role in providing energy and supporting essential molecular functions. Therefore, preservation of mitochondrial integrity is essential for optimal energy generation. Mitochondrial activity is regulated by fusion genes (*Mfn1*, *Mfn2*, *Opa1*) which maintain mitochondrial integrity, and adenosine triphosphate (ATP) synthesis genes (*Atp5e, Cox4i1*) that regulates electron transport chain (ETC) and ATP synthase complex. Disruption of these genes reduces ATP availability leading to abnormal or arrested development due to apoptosis (Arribat et al., 2019; Ojaimi et al., 2022; Xian & Liou, 2021). However, the impact of maternal asthma–induced inflammation and oxidative stress on mitochondrial function in preimplantation embryos remains poorly understood highlighting the need for further investigation.

While existing asthma treatments primarily focus on relieving airway

obstruction and inflammation, they do not adequately address the systemic consequences of the disease particularly those affecting reproductive health (Papi et al., 2020). Commonly prescribed medications may not fully prevent oxidative damage where in some cases it may pose potential risks to pregnancy outcomes when used long-term or at high doses (Namazy et al., 2013). The persistent oxidative stress and inflammatory responses associated with maternal asthma can extend beyond the respiratory system potentially disrupting reproductive function and early embryonic development. This limitation highlights a crucial gap in current therapeutic strategies as controlling airway symptoms alone may not prevent the adverse reproductive outcomes linked to maternal asthma. Therefore, investigating safer and biologically active alternatives such as natural antioxidants with both anti-inflammatory and antioxidant properties is essential to support maternal well-being and protect preimplantation embryos.

Tocotrienol-rich fraction (TRF) is a naturally derive vitamin E extracted from sources such as palm oil, rice bran and annatto possesses strong anti-inflammatory and antioxidant properties that have been demonstrated in respiratory and reproductive models. A study by Zainal et al., (2019) had proven the role of TRF as anti-inflammatory agent by improving airway structure and reducing airway inflammation in allergic asthma rat model. In addition, TRF had also be proven to be beneficial in improving embryonic development by attenuating nicotine-induced oxidative stress in mice (Hamirah et al., 2019). Despite the known benefits, the specific effects on embryos under maternal allergic asthma conditions are still poorly defined.

Hence, this study investigates the potential protective effects of TRF on preimplantation embryos exposed to maternal stress induced by allergic asthma. Specifically, the study examines maternal systemic inflammation, oxidative stress and pregnancy hormone levels as well as embryonic developmental potential, morphological quality, ultrastructural feature and gene expression in 2-cell stage embryos to elucidate the mechanisms underlying TRF-mediated improvement in embryo viability.

1.2 Problem Statement

Allergic asthma is a chronic inflammatory airway disease characterised by exaggerated Th2 immune responses. Clinical and experimental evidence indicates that

poorly controlled maternal asthma is associated with adverse pregnancy outcomes including impaired foetal development. Although evidence remains limited, asthma is suggested to exert systemic effects beyond the lungs, altering circulating cytokines, redox balance and metabolic homeostasis which may influence the maternal reproductive environment during early pregnancy. The preimplantation stage represents a critical window of early embryonic development that is highly sensitive to maternal physiological conditions. However, still limited studies focused their investigation on the systemic effects during preimplantation stage. Consequently, the relationship between asthma-induced systemic stress and preimplantation embryo viability remains inadequately defined. Currently, asthma therapies primarily target airway inflammation but do not directly address systemic stress, and concerns regarding pharmacological exposure during pregnancy highlight the need for safer adjunctive strategies. TRF, a natural potent antioxidant and anti-inflammatory compound, has demonstrated protective effects in various disease models. Nevertheless, no study has examined the dose-dependent systemic effects of ovalbumin (OVA)-induced allergic asthma on preimplantation embryo viability or evaluated the protective role of maternal TRF supplementation in this context. Therefore, this study aimed to investigate the systemic effects of maternal allergic asthma on early embryo viability and elucidate the protective role of TRF supplementation in mitigating asthma-induced stress on maternal systemic health and preimplantation embryo viability.

1.3 Research Questions

This study addressed the following research questions related to the protective effects of maternal TRF supplementation in ovalbumin (OVA)-induced allergic asthma:

1. What is the optimal dose of OVA required to induce systemic inflammation and oxidative stress that subsequently impair murine 2-cell embryos?
2. What are the effects of maternal TRF supplementation on systemic inflammation, oxidative stress and pregnancy hormone levels in OVA-induced allergic asthma mice?
3. How does maternal TRF supplementation affect the development and morphological quality of 2-cell embryos in OVA-induced allergic asthma mice?

4. What are the effects of maternal TRF supplementation on the ultrastructural features of 2-cell embryos in OVA-induced allergic asthma mice?
5. How does maternal TRF supplementation influence the expression of oxidative stress, mitochondrial fusion, mitochondrial bioenergetics and apoptotic genes of 2-cell embryos in OVA-induced allergic asthma mice?

1.4 Research Objectives

1.4.1 General Objective

To determine the protective effects of maternal TRF supplementation (60 mg/kg body weight) on systemic inflammation, oxidative stress, pregnancy hormone levels and subsequent effects on developmental potential, morphological quality, ultrastructural features and gene expression of 2-cell embryos in a mouse model of OVA-induced allergic asthma.

1.4.2 Specific Objectives

1. To determine the optimal OVA dosage (50, 100 and 150 µg/200µL) required to induce systemic inflammation and oxidative stress that negatively affect developmental potential and morphological quality of murine 2-cell embryos.
2. To evaluate the protective effects of maternal TRF supplementation (60 mg/kg body weight) on systemic inflammation, oxidative stress and pregnancy hormones in OVA-induced allergic asthma mice using enzyme-linked immunosorbent assay (ELISA).
3. To investigate the protective effects of maternal TRF supplementation (60 mg/kg body weight) on developmental potential and morphological quality of 2-cell embryos in OVA-induced allergic asthma mice using microscopic analysis following ASEBIR morphological scoring system.
4. To determine the protective effects of maternal TRF supplementation (60 mg/kg body weight) on ultrastructural features of 2-cell embryos in OVA-induced allergic asthma mice using transmission electron microscopy (TEM).
5. To identify the protective effects of maternal TRF supplementation (60 mg/kg body weight) on the expression of oxidative stress (*Nox1, Sod1, Gpx4*), mitochondrial

fusion (*Mfn1, Mfn2, Opa1*), mitochondrial bioenergetics (*Atp5e, Cox4il*), and apoptotic (*Bax, Bcl2*) genes of 2-cell embryos in OVA-induced allergic asthma mice using Fluidigm quantitative polymerase chain reaction (qPCR).

1.5 Research Hypotheses

1. Different doses of OVA induce different levels of systemic inflammation and oxidative stress that impair the development and morphology of murine 2-cell embryos.
2. Maternal TRF supplementation at 60 mg/kg body weight reduces systemic inflammatory cytokines and oxidative stress markers and restores pregnancy hormone levels in OVA-induced allergic asthma mice.
3. Maternal TRF supplementation at 60 mg/kg body weight improves the developmental potential and morphological quality of 2-cell embryos exposed to maternal allergic asthma.
4. Maternal TRF supplementation at 60 mg/kg body weight preserves the ultrastructural features of 2-cell embryos from asthmatic mice as observed under the TEM.
5. Maternal TRF supplementation at 60 mg/kg body weight modulates the expression of oxidative stress, mitochondrial fusion, mitochondrial bioenergetics and apoptotic genes in 2-cell embryos from OVA-induced asthmatic mothers.

1.6 Scope of Study

This study investigates the effects of maternal allergic asthma on the systemic physiology and subsequent effects on 2-cell embryo viability using a mouse model. The effects of different doses of OVA on systemic inflammation, oxidative stress and embryonic outcomes will be investigated to establish a reliable asthma model. The study will then assess the protective role of maternal TRF supplementation by analysing maternal biomarkers as well as the developmental potential, morphological quality, ultrastructural features and gene expression of the 2-cell embryos exposed to maternal allergic asthma. Parameters will be analysed using ELISA, light and transmission electron microscopy and Fluidigm qPCR to provide a comprehensive analysis of the potential protective effects of TRF during the preimplantation period.

CHAPTER 2
LITERATURE REVIEW

2.1 Allergic Asthma

2.1.1 Definition of Allergic Asthma

Asthma is defined as chronic inflammatory disorder of the airways characterized by symptoms such as wheezing, shortness of breath, coughing and chest tightness. Although the exact aetiology remains unclear, it is well established that both genetic susceptibility and environmental factors contribute to the development and progression of the disease (Gohal et al., 2024). There are different types of asthma which are allergic asthma, non-allergic asthma, occupational asthma and adult-onset asthma. Among the types, allergic asthma is the most common as it occurs in more than half of asthma patients (Clifton et al., 2016).

2.1.2 Prevalence of Allergic Asthma

The prevalence of asthma is influenced by a complex interplay between genetic predisposition and environmental exposures which together influence the susceptibility and disease progression of an individual. Globally, asthma already affects approximately 339 million people and this number is expected to increase by another 100 million (World Health Organisation, 2018). In Malaysia, updated data on asthma prevalence at the national level is currently not available. However, it is estimated that the prevalence in children is 8.9 – 13.0 % (Ahad & Ming Khoo, 2017; Pearce et al., 2007) while in adults is 6.3 % (Chan et al., 2015).

Globally, asthma ranks as the 16th leading cause of years lived with disability and the 28th leading cause of burden of disease (Bruyne & Nathan, 2018). Asthma not only causes morbidity but is also responsible for mortality. Asthma-related mortality rate is higher in low- to middle-income countries than in high-income countries due to poor asthma management and environmental factors (Dharmage et al., 2019). In Malaysia, the mortality rate due to asthma was 1.29 % making it the 27th leading cause of death (Ministry of Heatlh Malaysia, 2018).

2.1.3 Triggers and Symptoms of Allergic Asthma

Allergic asthma can be triggered when the airways are exposed to harmless substances known as allergens. Commonly known allergens include cockroach droppings, house dust mites, moulds, animal dander and pollen. In rare cases, foods such as milk, shellfish, eggs, soya products, peanuts, gluten, tree nuts and sesame seeds can also trigger allergic asthma when consumed (Baglivo et al., 2024; Cockcroft, 2014; Nelson, 2000). Allergens are found both indoors and outdoors. Exposure to allergens, particularly through inhalation causes an asthma attack which resulted to exacerbation of allergic asthma symptoms. Therefore, every asthma patient must know their triggers in order to prevent the occurrence of an asthma attack (Baglivo et al., 2024; Nelson, 2000).

An asthma attack can occur all year round, but in temperate countries, it can worsen in spring because more pollen is produced. An asthma attack is characterised by asthmatic symptoms such as wheezing, coughing, chest tightness and shortness of breath. Airway remodelling including subepithelial fibrosis, smooth muscle hyperplasia and goblet cell metaplasia contribute to the development of the asthmatic symptoms due to airflow limitation and airways obstruction (Gohal et al., 2024; Mims, 2015).

2.1.4 Pathogenesis of Allergic Asthma

As a multifactorial disease, the pathogenesis of allergic asthma is complex. The key underlying mechanisms involve allergic inflammation mediated by T-helper (Th) 2 cells and oxidative damage. Figure 2.1 illustrates the interplay between Th2-mediated inflammation and oxidative stress in contributing to the pathogenesis of allergic asthma.

2.1.4.1 T helper-2 Inflammation

Inflammation is a defence mechanism of the body against pathogens, abrasions, chemical irritants, cellular changes or extreme temperatures. One of the tasks of inflammation is to remove microbes, toxins or foreign material from the body. In allergic asthma, inflammation is activated when an allergen enters the airways through inhalation. Antigen-presenting cells (APCs) which are also known as dendritic cells (DCs) are found in the epithelium of the airways. These cells are able to take up the

allergen and then process it into a smaller form known as peptides (Holgate, 2012). The DCs then present the allergen to naïve T cells which leads to differentiation of the naïve T cells into Th2 cells (Ren et al., 2019).

Activation of Th2 cells leads to the release of Th2 cytokines (Ayakannu et al., 2019; Ren et al., 2019; D. S. Robinson, 2010). Recent studies have investigated transcription factors such as Nuclear Factor of Activated T cells 1 (NFATc1) in the modulation of immune responses in allergic asthma highlighting the potential therapeutic targets (Z. Yang et al., 2025). Cytokines synthesised by T cells are extracellular signalling molecules that have the ability to regulate the immune response and haematopoiesis (Ayakannu et al., 2019). Several cytokines play a specific role in the pathogenesis and pathophysiology of allergic asthma including the expression of allergic asthma features.

Interleukin (IL)-4 is one of the key players which play role in stimulating B cells to release immunoglobulin (Ig)-E. Consequently, IgE production activates macrophages by binding to their receptors to eliminate allergens (D. S. Robinson, 2010). In addition, IL-4 causes goblet cell hyperplasia leading to overproduction of mucus (Ayakannu et al., 2019). On the other hand, IL-5 promotes eosinophilopoiesis in the bone marrow which further promotes the infiltration of eosinophils into the airways and therefore shows eosinophilic inflammatory features in allergic asthma (D. I. Kim et al., 2019). Once they reach the airways, the eosinophils release their leukotrienes to fight the allergens (Possa et al., 2013). In addition, IL-13 is responsible for the development of airway hypersensitivity (AHR) which causes hypersensitivity towards allergens (Finkelman et al., 2010).

Understanding the Th2 immune response has provided a rough picture of the pathogenesis and pathophysiology of allergic asthma. To summarise, activation of the Th2 immune response leads to airway obstruction due to airway remodelling which thickens the airways and narrows the airway lumen (Fehrenbach et al., 2017). In addition, overproduction of mucus in the airways can also lead to airway obstruction. Finally, the development of AHR causes the airways to become hypersensitive to allergens which promotes bronchoconstriction.

2.1.4.2 Oxidative Stress

Oxidative stress is a state of imbalance between free radicals and antioxidants. There are two forms of free radicals, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Chandimali et al., 2025). Structurally, free radicals consist of unpaired electrons that allow them to donate or accept electrons from other molecules (Chandimali et al., 2025; Pizzino et al., 2017). This makes free radicals very unstable and highly reactive. In low or moderate concentrations, ROS are useful for cell metabolism and processes such as cell proliferation, division and signalling. However, an accumulation of ROS leads to detrimental effects such as damage to lipids, proteins and nucleic acids in the cell (Birben et al., 2012).

There are endogenous and exogenous stimuli for ROS production. At the cellular level, organelles such as mitochondria, endoplasmic reticulum (ER) and peroxisomes have the ability to generate ROS (Martemucci et al., 2022; Meo et al., 2016). Disorders of body physiology such as cellular inflammation and oxygen deprivation are examples of endogenous stimuli that can trigger the production of ROS. In the case of exogenous stimuli, the penetration of foreign substances such as pollutants, cigarette smoke, heavy metals, certain medications, radiation and allergens can trigger the production of ROS (Martemucci et al., 2022; Pham-huy et al., 2008). In allergic asthma, the exposure of the airways to allergens leads to inflammation. As a result, the development of asthma symptoms leads to a cellular oxygen (O_2) deficiency. This causes more ROS are produced leading to accumulation (Koumpagioti et al., 2025).

In addition, all living cells including immune cells such as DCs, eosinophils and macrophages also produce ROS (Hosoki et al., 2014). Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase is an enzyme found in living cells and is located at the plasma membrane. This enzyme catalyses the production of ROS by reducing O_2 to a superoxide anion ($O_2^{\bullet-}$) ($NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet-}$). The $O_2^{\bullet-}$ is then dismutased by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). The H_2O_2 undergoes the Fenton reaction and then forms a hydroxyl radical (OH^-) (Kayama et al., 2015; Koumpagioti et al., 2025). All these processes lead to generation of free radicals ($O_2^{\bullet-}$, H_2O_2 and OH^-).

Therefore, when immune cells are activated, too many ROS are produced leading to accumulation. Activation of the Th2 immune response in allergic asthma

leads to infiltration of DCs, eosinophils and macrophages. The accumulation of these immune cells therefore leads to increased ROS production particularly of $O_2^{\bullet-}$, H_2O_2 and OH^- (Hosoki et al., 2014; Koumpagioti et al., 2025). In this state, high levels of ROS exacerbate the pathophysiology of allergic asthma and cause damage to macromolecules such as lipids, proteins and deoxyribonucleic acid (DNA). Figure 2.1 illustrates the role of oxidative stress in the pathogenesis of allergic asthma. Understanding oxidative stress in asthma is crucial as it can have systemic effects on other organs including reproductive tissues. This is the basis for the study of oxidative mechanisms interaction between mother and embryo.

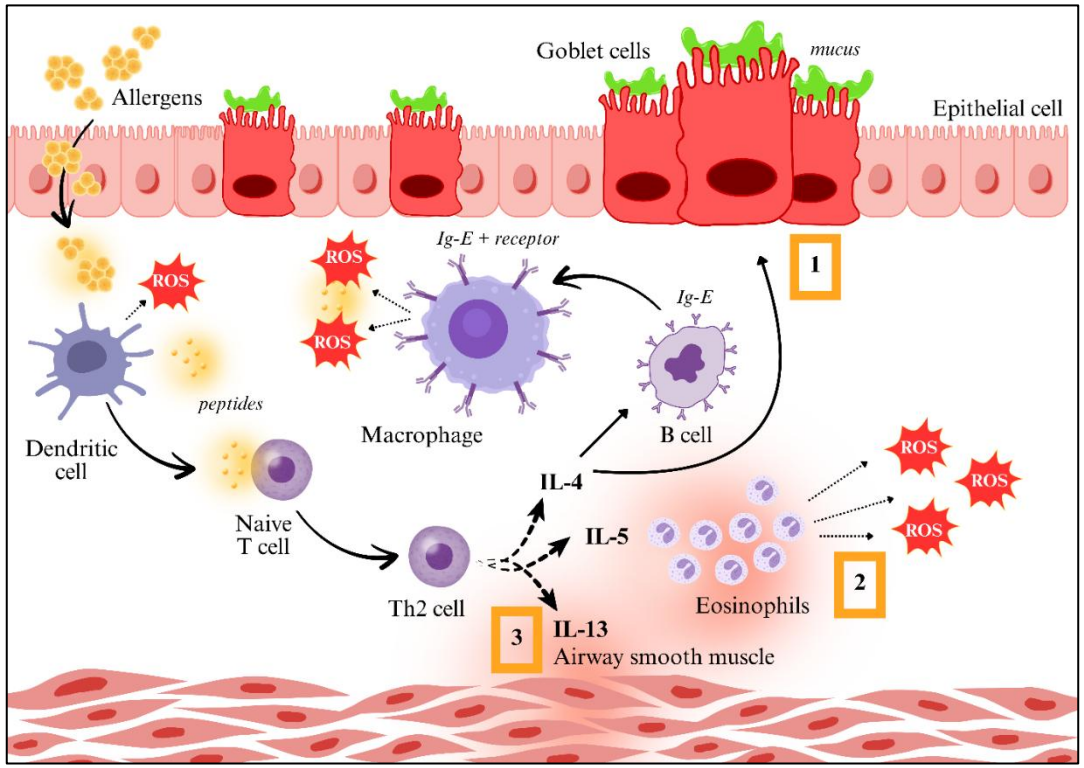


Figure 2.1 Pathogenesis of Allergic Asthma Involving Th2-Mediated Inflammation and Oxidative Stress

This figure explains the key mechanisms underlying the pathogenesis of allergic asthma. Exposure to inhaled allergens triggers the activation of dendritic cells (DCs), which initiate the differentiation of naïve T cell into T-helper (Th) 2 cell. The Th2 cell release cytokines such as IL-4, IL-5 and IL-13, leading to exacerbation of allergic asthma features; [1] mucus overproduction due to goblet cell hyperplasia, [2] eosinophilic inflammation and [3] airway hyperresponsiveness. Concurrently, activated immune cells, including DCs, eosinophils and macrophage generate excessive reactive oxygen species which contribute to oxidative damage to airway tissues. The combined effects of inflammation and oxidative stress lead to airway remodelling and obstruction, which worsen asthmatic symptoms.

2.1.5 Allergic Asthma in Women of Reproductive Age

Allergic asthma is a common chronic respiratory disease that affects a significant proportion of women of reproductive age between the age of 18 and 49. This phase of life is characterised by complex hormonal, physiological and immunological changes. These changes not only influence the severity of asthma but can also affect reproductive processes such as menstruation, conception and early embryonic development.

Hormonal fluctuations during the menstrual cycle particularly changes in oestrogen and progesterone levels can modulate systemic inflammation and immune responses (Balzano et al., 2001; Sciarra et al., 2023). These fluctuations can exacerbate asthma symptoms in some women. Although there is little direct evidence of a link between menstrual periods and the severity of allergic asthma, some studies suggest that asthma symptoms worsen around menstruation in a subset of women. This suggests a possible interaction between hormonal and immunological mechanisms that should be investigated further.

During pregnancy, approximately one-third of asthmatic women report worsening symptoms particularly in the second trimester (Murphy et al., 2019). Although clinical findings vary, recent evidence suggests that maternal asthma may influence the course of pregnancy from the earliest stages. The preimplantation period which includes the transition from fertilisation to blastocyst formation is particularly sensitive to changes in maternal physiology. Maternal inflammation and oxidative stress during this period have been shown to impair early embryonic development, disrupt cell lineage distribution and reduce implantation success (Feuer & Rinaudo, 2012; Terada-Ikeda et al., 2020).

In the later stages of pregnancy, maternal asthma can contribute to complications by impairing oxygen exchange and increasing systemic inflammation. Structural changes in the airways such as wall thickening and excessive mucus production can reduce the mother's oxygen saturation and impair oxygen supply to the foetus. These effects are associated with adverse outcomes such as prematurity and low birth weight (Bonham et al., 2018; Namazy et al., 2013). However, the specific mechanisms linking asthma severity to perinatal outcomes are not yet fully understood particularly in cases where allergic inflammation is present.

These findings emphasise the importance of investigating how maternal allergic asthma affects the early stages of development. Understanding this relationship is particularly important in the preimplantation phase which can be influenced by maternal immune status and oxidative stress. This study aims to fill this knowledge gap by investigating these systemic effects in a mouse model of allergic asthma.

2.1.6 Treatments of Allergic Asthma

Current knowledge on the pathogenesis and pathophysiology of asthma has established the treatment of asthma. Currently, treatments for asthma are categorised into 1) control agents and 2) relievers (Papi et al., 2020; Rabe & Schmidt, 2001; Seluk et al., 2025). The list of medications used to treat asthma is summarised in Table 2.1. Although medications are currently available to treat asthma, the treatments only aim to improve asthmatic symptoms and are not as effective in preventing asthma attacks (Papi et al., 2020). Despite their therapeutic efficacy in the airways, these drugs often lack systemic effects and may not alleviate the underlying inflammation or oxidative stress relevant to other body systems and physiologies including embryonic development.

Table 2.1
Summary of Common Asthma Medications and Their Functions

Drug Category	**Drug Type**	**Function**
Controllers (Long-term management)	Inhaled corticosteroids	Reduce inflammation of the airways and prevent exacerbations
	Inhaled long-acting beta-agonists (LABAs)	Relax the smooth muscles of the airways for prolonged bronchodilation
	Leukotriene receptor antagonists	Block leukotriene signalling pathways to reduce inflammation and bronchoconstriction
	Omalizumab (anti-IgE monoclonal antibody)	Prevents IgE-mediated mast cell activation and allergic responses

	Cromolyn sodium	Stabilises the mast cells to prevent the release of histamine and other mediators
	Theophylline	Relaxes the smooth muscles of the respiratory tract and improves the contractility of the diaphragm
Relievers (Rapid symptom relief)	Short-acting beta-agonists (SABAs)	Provides rapid bronchodilation in asthma attacks
	Anticholinergics	Reduces bronchoconstriction and mucus secretion by blocking muscarinic receptors
	Combination therapies (SABA + anticholinergic)	Provides rapid relief through the combination of bronchodilator and expectorant effects

This table elucidates the commonly used asthma medications. It is categorised into two main groups: Controllers for long-term treatment of the disease and Relievers for rapid relief of symptoms. Each type of medication is listed with its primary function in the treatment of airway inflammation, bronchial constriction and mucus secretion. Although these medications are effective in relieving lung symptoms, they may not be able to adequately address systemic inflammation or oxidative stress.

2.1.7 Allergic Asthma Experimental Model

Animal models are widely used to study the underlying mechanisms, pathophysiology and treatment options for allergic asthma. While clinical trials are critical for evaluating patient outcomes and therapeutic response, animal models provide controlled conditions for exploring disease mechanisms, validating biomarkers and testing novel interventions prior to clinical implementation.

A number of allergens have been used to induce experimental asthma in laboratory animals including house dust mite (HDM), ragweed, cotton dust and ovalbumin (OVA) (Kianmehr et al., 2016). Among these, OVA is widely regarded as a major model allergen due to its ability to provoke strong IgE-mediated immune responses in sensitised hosts. Structurally, OVA is the predominant protein in egg white accounting for approximately 54 % of the total protein content. Its antigenic epitopes bind to IgE antibodies on mast cells triggering degranulation and the subsequent release of histamine and other inflammatory mediators (Qiao et al., 2024).

Consequently, OVA serve as a well-established experimental allergen particularly for the induction of allergic asthma in rodents. In murine models, OVA sensitisation and challenge reliably reproduce key pathological features of human allergic asthma including eosinophilic airway inflammation, airway hyperresponsiveness (AHR), mucus hypersecretion and elevated serum IgE levels (Casaro et al., 2019). To effectively model these immunopathological features, appropriate mouse strains are essential. Among the available strains, BALB/c mice are most frequently employed in allergic asthma studies due to their inherent predisposition toward a strong T helper 2 (Th2)-biased immune response. This heightened Th2 sensitivity enhances IgE production and eosinophilic inflammation following OVA exposure, making BALB/c mice particularly suitable for investigating allergic airway inflammation (Boyce & Austen, 2005; Nials & Uddin, 2008; Schulte et al., 2008).

The standard protocol for developing an OVA-induced allergic asthma model consists of two main phases: 1) sensitisation and 2) challenge. During sensitisation, OVA is administered intraperitoneally (i.p) with alum as adjuvant (Paik et al., 2014). The first phase of OVA sensitisation was conducted to prime the immune system and simulate the initial allergen exposure phase of allergic asthma. This phase stimulated B-cell activation, increased serum IgE levels, and promoted recruitment of eosinophils and other inflammatory cells, thereby establishing systemic immunological memory (Jiao et al., 2024). However, excessively high OVA doses may induce immune tolerance and suppress Th2 responses while low doses may be insufficient to prime the immune system.

On the other hand, the OVA challenge phase is designed to induce a local allergic reaction in the airways, typically through repeated intranasal exposures during the third week (Paik et al., 2014). Upon exposure, OVA bind to IgE on mast cells and basophils in the airway mucosa triggering rapid degranulation and the release of histamine, leukotrienes, and cytokines within minutes. This immediate immune response produces the characteristic of early allergic asthma symptoms. Following this, Th2-mediated inflammation recruit more eosinophils and lymphocytes to the airway while cytokines such as IL-13 drive mucus hypersecretion and goblet cell hyperplasia. Together, these changes reproduce the key features of an asthma exacerbation such as AHR and airway remodelling (Jiao et al., 2024).

Although the pulmonary manifestations of OVA-induced allergic asthma including inflammation, airway remodelling and oxidative stress had been extensively

studied, less attention has been paid to the systemic consequences. In particular, little is known about how maternal OVA-induced allergic asthma affects other physiological systems such as the reproductive axis and embryonic development.

Given the increasing interest in the Developmental Origins of Health and Disease (DOHaD) paradigm, it is important to investigate how systemic inflammation and oxidative stress in allergic asthma may impair preimplantation embryo viability. To date, no studies had investigated this relationship. Therefore, a systematic investigation is needed to characterise the effects of OVA-induced maternal allergic asthma on the earliest stages of embryonic development. This study aims to fill this gap by using a mouse model to investigate how OVA-induced maternal inflammation and oxidative stress affect the preimplantation embryo viability and whether these detrimental effects can be mitigated by supplementation with a tocotrienol-rich fraction (TRF).

Given the growing interest in the systemic consequences of maternal allergic asthma particularly the potential impact on early reproductive events, the next section explains the preimplantation stage of embryonic development. This critical window is highly sensitive to maternal physiological signals such as inflammation and oxidative stress.

2.2 Preimplantation Embryos

The preimplantation stage refers to the period of embryonic development that occurs after fertilization but before the embryo attaches to the uterine wall. This stage encompasses a series of critical events including cleavage divisions, morula formation, blastocyst development and zona pellucida hatching (Bissiere et al., 2023). In contrast, the implantation stage commences once the blastocyst has completely hatched from the zona pellucida and begins the sequential processes of attachment, adhesion, and invasion into the maternal endometrium (S.-M. Kim & Kim, 2017).

Successful progression through these early developmental stages is critically dependent on appropriate maternal reproductive physiology. In murine models, reproductive competence is tightly regulated by the oestrous cycle which governs ovulation and uterine receptivity followed by gestational adaptations that create a supportive endocrine, immune and metabolic environment for embryonic development (Murray et al., 2010). Therefore, an understanding of the murine oestrous cycle and

gestational period is essential to provide a physiological foundation in investigating preimplantation embryos which constitutes the primary focus of this study.

2.2.1 Oestrus Cycle

In reproductive studies, female rodents such as mice and rats are widely used due to their well-characterised oestrous cycles and ease of handling. The onset of reproductive maturity occurs at approximately 26 days of age, marked by vaginal opening (Quignon, 2023). The oestrous cycle represents the reproductive cycle in rodents and is comparable to the human menstrual cycle which comprises coordinated ovarian and uterine changes (Ajayi & Akhigbe, 2020).

In mice, the oestrous cycle consists of four distinct phases: proestrus, oestrus, metestrus, and dioestrus, and typically spans 4–5 days (Auta & Hassan, 2016). Various methods have been used to assess the oestrous cycle based on physiological and anatomical changes including visual inspection, vaginal cytology, histology, vaginal impedance, and urine biochemistry. Among these, visual assessment is widely preferred as it is non-invasive, simple, cost-effective, quick and minimally stressful (Ajayi & Akhigbe, 2020).

Because the oestrous cycle varies among mice, superovulation is essential in reproductive studies. This protocol enhances ovulation, increases the yield of mature oocytes, and ensures adequate numbers for fertilisation and subsequent analysis (C. Luo et al., 2011). Subsequently, attention shifts to the gestational period, during which maternal physiological conditions may influence implantation and early embryonic development.

2.2.2 Gestational Period

A clear understanding of murine gestational period is essential in reproductive studies. The murine gestational period spans approximately 19–21 days and involves tightly regulated endocrine, immunological and physiological adaptations that support embryonic and foetal development (Murray et al., 2010). Early gestation (0–4 day post-coitum, d.p.c) encompasses fertilisation and sequential cleavage divisions leading to morula and blastocyst formation (Bissiere et al., 2023). During this period, progesterone secreted by the corpus luteum prepares the endometrium for implantation and maintains

uterine receptivity (H. Luo et al., 2025). Implantation occurs around 4–5 d.p.c and involves blastocyst apposition, adhesion, trophoblast invasion, and decidualisation of uterine stromal cells, processes that are coordinated by ovarian steroid hormones and local cytokine signalling (Yoshinaga, 2013).

Mid-gestation (6–15 d.p.c) is characterised by placental development, angiogenesis and organogenesis accompanied by increased metabolic demand and physiological oxidative stress (Elmore et al., 2022; Panja & Paria, 2021). These processes require precise regulation of redox balance to maintain normal embryonic and placental development. Late gestation (16–21 d.p.c) involves rapid foetal growth and a progressive shift toward a pro-inflammatory environment that facilitates parturition through functional progesterone withdrawal and increased prostaglandin production (Mccarthy et al., 2018).

Importantly, the preimplantation period represents a highly sensitive developmental window. The preimplantation embryo depends largely on maternally derived transcripts, mitochondrial function, and tightly regulated metabolic pathways to sustain cleavage and lineage specification (S. Xu & Egli, 2024). Perturbations in maternal endocrine homeostasis, systemic inflammation or oxidative stress during this early stage may impair embryonic competence, morphology, and subsequent implantation potential. Therefore, understanding the physiological events occurring during early murine gestation provides a critical framework for investigating factors that influence preimplantation embryo viability.

2.2.3 Preimplantation Embryonic Development

Preimplantation period which extends from fertilisation to implantation is a critical window in human and mammalian development. It undergoes several metabolic processes, protein synthesis, energy consumption and amino acid uptake. After fertilisation, the zygote undergoes a series of cleavage divisions to develop into a 2-, 4- or 8-cell embryo (Bissiere et al., 2023). At this stage, the number of blastomeres and cell size can be used to assess the viability of the embryo. The 2-cell stage particularly in mice, marks the peak of zygotic genome activation (ZGA) which is a critical event that ensures autonomous developmental progression (S. Xu & Egli, 2024). Subsequently, the cleavage embryo undergoes compaction to become morula. The morula then undergoes cell differentiation resulted in the formation of two distinct cells:

inner cell mass (ICM) and trophectoderm (TE). At the same time, the polarisation of the morula causes fluid to penetrate forming the blastocoel cavity (Kaneko, 2016; Mohebi & Ghafouri-Fard, 2019; White et al., 2016). Figure 2.2 shows the sequential stages of murine preimplantation embryonic development.

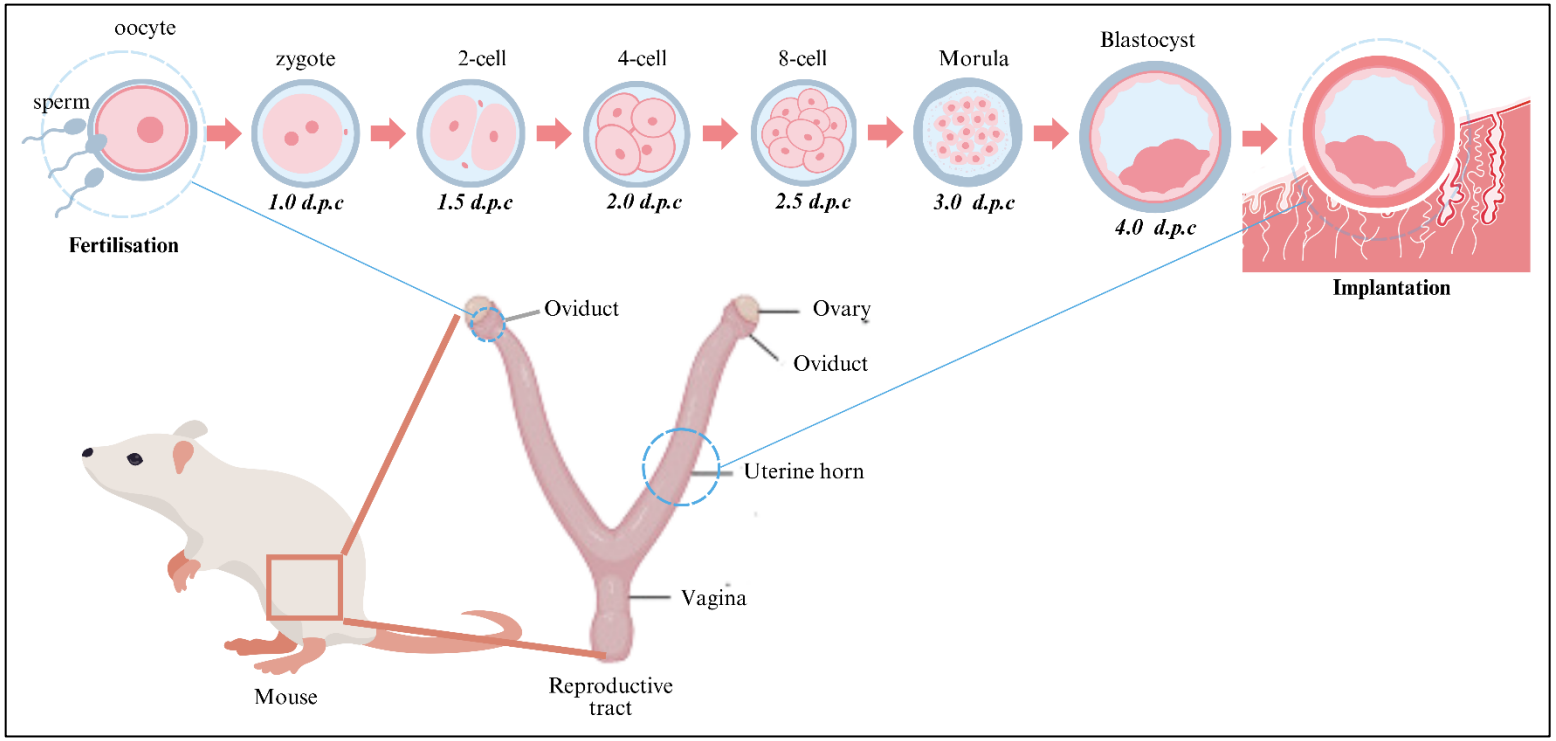


Figure 2.2 Sequential Stages of Murine Preimplantation Embryonic Development

This figure illustrates the sequential stages of preimplantation embryonic development in the mouse, beginning with fertilisation and ending with implantation. Following fertilisation of the oocyte by sperm in the ampulla region of the Fallopian tube, the zygote undergoes a series of cleavage divisions to form the 2-cell (1.5 days post coitum, d.p.c), 4-cell (2.0 d.p.c), 8-cell (2.5 d.p.c), and morula (3.0 d.p.c) stages. As development progresses, the embryo continues to move through the Fallopian tube toward the uterine cavity. By approximately 4.0 d.p.c, the embryo reaches the blastocyst stage, characterised by the formation of a fluid-filled cavity (blastocoel), an inner cell mass, and a surrounding trophectoderm layer. The blastocyst subsequently attaches to and implants into the uterine lining, initiating the next stage of embryonic development.

2.2.4 Morphogenesis and Molecular Signalling

The preimplantation period is characterised by strictly regulated morphogenetic events and molecular signalling cascades that control embryonic development. These include cleavage divisions, cellular polarisation, compaction and the onset of embryonic genome activity. The precise coordination of these events is essential for developmental competence and successful implantation which represents good embryo viability.

2.2.4.1 Mitotic Cleavage Division

After fertilisation, the zygote undergoes a series of mitotic divisions that give rise to blastomeres which become progressively smaller with each division. These division processes are crucial for the development of the embryonic architecture and are used as indicators of the quality of the embryo. The embryo develops sequentially from the 2-cell to 4-cell and then to the 8-cell stage before compacting into a morula (Bruce & Mihajlovic, 2017; Saiz et al., 2018).

Normal cleavage is characterised by synchronous division in which the blastomeres divide at similar intervals, typically within 18 to 20 hours. In contrast, asynchronous or abnormal division patterns such as 3-cell, 5-cell or uneven- sized blastomeres are associated with reduced developmental potential and may indicate underlying chromosomal or metabolic defects (S. G. Kim et al., 2018). In addition, mitotic errors during early cleavage can lead to aneuploidy, impaired compaction or impaired blastocyst formation (Martin et al., 2024; McCollin et al., 2020; T. Yang et al., 2023).

2.2.4.2 Maternal-to-Zygotic Transition (MZT)

The maternal-to-zygotic transition (MZT) is a crucial molecular event during which control of development shifts from maternally deposited ribonucleic acids (RNAs) and proteins to the embryonic genome (R. Xu et al., 2025). This transition involves two coordinated processes: the degradation of maternal transcripts and the activation of zygotic gene expression. In mice, the main activation of the ZGA occurs at the 2-cell stage and allows the embryo to direct its own development (S. Xu & Egli, 2024).

Disruptions during MZT can severely impair developmental outcomes. For example, acrylamide-induced oxidative stress has been shown to impair ZGA, leading to 2-cell arrest and embryonic failure (S. Le Wu et al., 2023). As this stage depends on precise gene activation and transcript clearance, it is highly susceptible to maternal environmental influences, including inflammation and oxidative stress that are the key mechanisms of allergic asthma pathophysiology.

2.2.5 Energy Metabolism

The most important energy sources for embryos are carbohydrates and lipids. In the early stage of preimplantation, the embryo mainly uses pyruvate and lactate as an energy source while glucose is used in the later stage (Lipinska et al., 2023). Energy metabolism in the early embryo is mainly based on oxidative phosphorylation (OXPHOS) and to a lesser extent on glycolysis. Both OXPHOS and glycolysis take place in the mitochondria, a site where energy is generated in the form of adenosine triphosphate (ATP) (Keane & Ealy, 2024). Therefore, mitochondrial bioenergetics is important to regulate energy metabolism to enhance embryo viability for successful preimplantation development. Figure 2.3 illustrates the metabolic transition during preimplantation embryonic development of mouse embryo.

2.2.5.1 Oxidative Phosphorylation (OXPHOS)

The OXPHOS in early embryos represents a vital mitochondrial process responsible for generating the majority of cellular ATP required for early development. The process occurs within the inner mitochondrial membrane where electron transport chain (ETC) complexes transfer electrons derived from NADH and $FADH_2$ to molecular O_2 (Acin-Perez et al., 2023; Keane & Ealy, 2024). This electron transfer establishes a proton gradient across the inner membrane and the return flow of protons through ATP synthase (Complex V) drives the phosphorylation of adenosine diphosphate (ADP) to generate ATP, thereby providing the energy necessary for continued embryonic growth and division (Keane & Ealy, 2024).

Therefore, mitochondrial health plays crucial roles in determining the efficiency and activity of OXPHOS in cells including early embryos. The number of mitochondria directly influences the total OXPHOS capacity as a greater mitochondrial population

provides more ETC complexes and ATP synthase enzymes for ATP generation (Čunátová et al., 2024). Conversely, a reduction in mitochondrial number limits ATP generation, thereby compromising energy-dependent processes such as cleavage, biosynthesis, and early embryonic development.

In addition, mitochondrial morphology also modulates OXPHOS efficiency through alterations in the structure of the inner mitochondrial membrane particularly the cristae where ETC complexes are located. Elongated, fused mitochondria with well-developed cristae exhibit enhanced OXPHOS activity due to the increased membrane surface area and optimized organization of ETC super-complexes whereas fragmented mitochondria are often associated with reduced respiratory efficiency and metabolic dysfunction (Čunátová et al., 2024; Yao et al., 2019).

Accumulation of ROS has been reported to damage mitochondria such that impaired OXPHOS leads to low ATP generation (Millet et al., 2024; Pellegrini & Scorrano, 2007; Pohl et al., 2025). Consequently, any mitochondrial dysfunction during the preimplantation stage may impair ATP production and jeopardise essential biosynthetic processes that are crucial for successful embryo cleavage and compaction.

2.2.5.2 Glycolysis

Glycolysis is a metabolic pathway that breaks down glucose into pyruvate generating ATP which critical for early development. In the earliest stages of development, glucose utilization through glycolysis is minimal or even inhibitory (Chi et al., 2020). As development progresses, particularly after compaction, glucose metabolism and glycolytic activity increase to accommodate the heightened energy demands and biosynthetic needs of the morula and blastocyst stages (Chi et al., 2020). This metabolic shift toward glycolysis not only provides ribose sugars for nucleic acid synthesis but also generates intermediates for lipid and amino acid biosynthesis, thereby supporting rapid cell proliferation and a gradual transition toward anaerobic energy metabolism (Chi et al., 2020; S. H. Lee & Rinaudo, 2024).

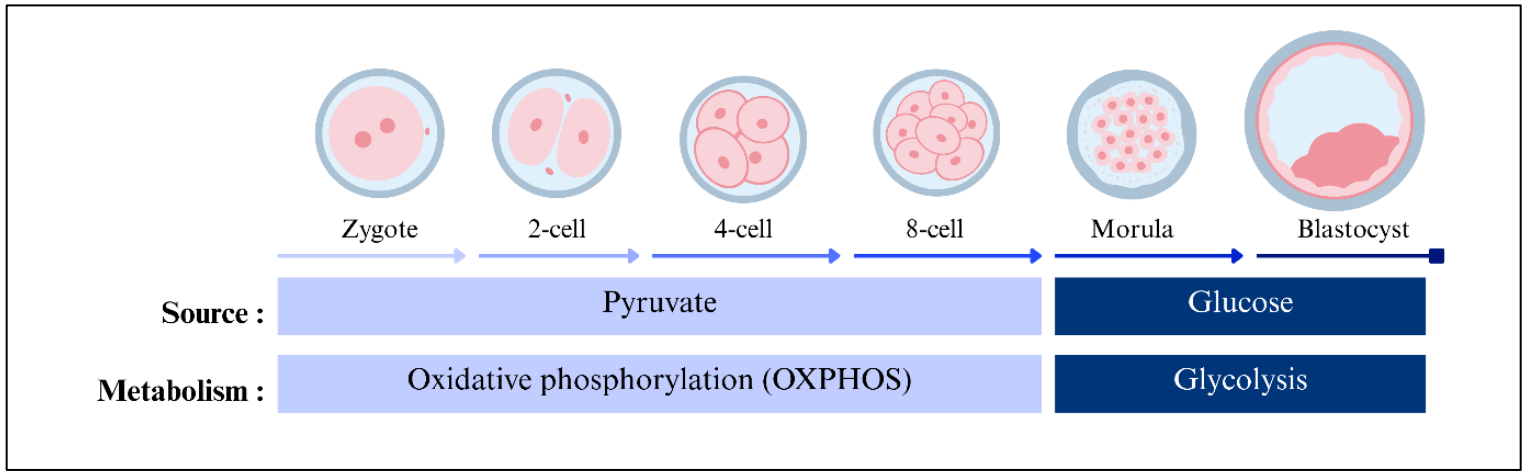


Figure 2.3 Metabolic Transition During Preimplantation Embryonic Development of Mouse Embryos
This figure illustrates the shift in energy substrate utilization and metabolic pathways during the preimplantation stages of mouse embryonic development. In the early stages, from zygote to 8-cell, the embryo primarily relies on pyruvate and oxidative phosphorylation (OXPHOS) within mitochondria as the main sources of energy. During this period, glucose metabolism is minimal, and excessive glycolytic activity may even be inhibitory to normal development. As the embryo progresses to the morula and blastocyst stages, there is a metabolic transition towards glucose utilization and glycolysis. This shift supports the increased biosynthetic and energetic demands required for compaction, blastocoel formation, and preparation for implantation.

2.2.6 Roles of Organelles

The preimplantation embryo contains the typical eukaryotic cell organelles essential for early embryonic development such as nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes and cytoskeletal components. These organelles collectively play vital roles in maintaining metabolic activity, intracellular signalling, and degradation pathways that support the embryo viability for successful developmental progress.

2.2.6.1 Nucleus

The primary function of nucleus is to control gene expression and DNA replication, which both are fundamental to establishing the embryonic developmental program (Dasso & Fontoura, 2018). Specifically, the nucleus in a 2-cell embryo plays a central role in genetic regulation and early developmental processes. Within the nucleus, nucleolus precursor bodies (NPBs) are prominent structures that not only serve as sites for ribosome biogenesis but also function as regulatory centres for chromatin organization (B. Fu et al., 2024). This organization is critical for maintaining totipotency and facilitating the transition from totipotency to pluripotency. Furthermore, the nucleolus responsible for activating genes essential for ZGA and subsequent embryonic progression by regulating the expression of key transcription factors such as *Dux* (H. Yu et al., 2021).

2.2.6.2 Mitochondria

The mitochondrion is an organelle with a double membrane consisting of the outer mitochondrial membrane (MOM) and the inner mitochondrial membrane (MIM), which are separated by a space, the intermembrane space (IMS). Both membranes are important for the mitochondria to fulfil their functions and maintain their normal morphology (Keane & Ealy, 2024; R. X. Santos et al., 2013; Song et al., 2024). Figure 2.4 illustrates the double membrane structure of mitochondrion.

Morphologically, mitochondrial cristae are formed in the MIM, a structure in which the ETC is located. The main role of embedded ETC in the cristae is to generate

ATP (Busch, 2020; Cogliati et al., 2016; Zick et al., 2009). The ETC generates ATP thru OXPHOS which involves two major processes; 1) electron transfer and 2) ATP synthesis. The electron transfer occurs at the I - V complexes whereas ATP synthesis occurs at the V complex (Caron & Bertolin, 2024; Joubert & Puff, 2021). Therefore, impairment of MIM will certainly lead to low ATP generation.

On the other hand, both MOM and MIM are also involved in mitochondrial fusion activity. Fusion activity which is regulated by the mitofusin (*Mfn*)*-1*, *-2* and *Opa1* genes leads to the formation of tubular or elongated mitochondria which is important for optimising mitochondrial function and maintaining mitochondrial integrity (Ojaimi et al., 2022; Xian & Liou, 2021). Figure 2.4 illustrates mitochondrial fusion activity focusing on MOM then MIM fusion. An interesting feature of the early embryo is that all processes of preimplantation development rely on the mitochondria that are already present at the time of ovulation (S. Li & Winuthayanon, 2017). Nonetheless, the later the stage of preimplantation embryos, the lower the number of mitochondria in the blastomeres as mitochondrial biogenesis only begins at the blastocyst stage (He et al., 2023; P. Mishra & Chan, 2014).

Currently, ultrastructural analysis using transmission electron microscopy (TEM) provides crucial insights into mitochondrial health and integrity during early development. TEM enables high-resolution visualisation of key mitochondrial features such as cristae architecture, matrix density and outer membrane continuity which are essential indicators of mitochondrial function (Halvaei et al., 2016; P. Qu et al., 2020; Sathananthan et al., 1999).

In embryology, TEM has been used to assess mitochondrial swelling, vacuolation and cristae destruction which are hallmarks of oxidative stress-induced damage (Y. Chen et al., 2025; Komatsu et al., 2014). These ultrastructural features are particularly important in preimplantation embryos exposed to maternal physiological stress. Since mitochondrial biogenesis is absent in the early stages of embryonic development, such damage can have profound effects on ATP generation which further deteriorate embryo viability. Thus, TEM remains a powerful tool for assessing the impact of maternal conditions like allergic asthma on mitochondrial structure including other organelles to gain more insights on embryo viability.

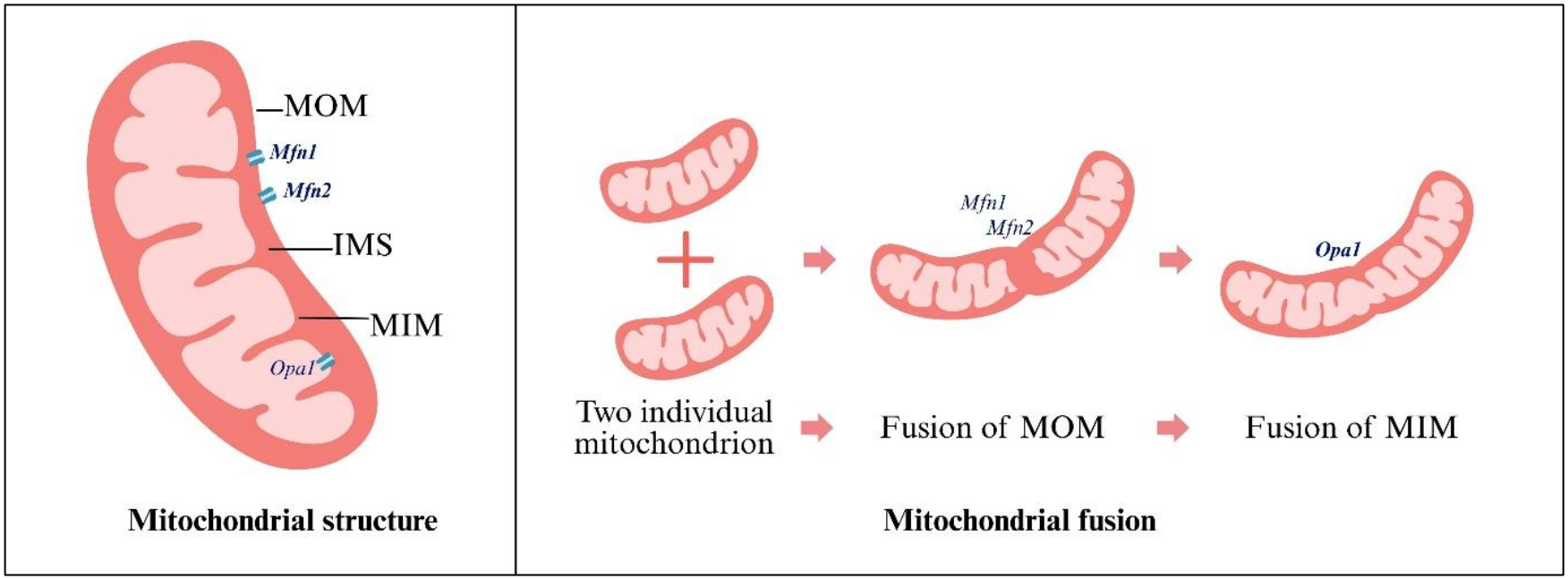


Figure 2.4 Mitochondrial Structure and Fusion Activity

This figure illustrates the double-membrane structure of a mitochondrion, consisting of the mitochondrial outer membrane (MOM) and the mitochondrial inner membrane (MIM), with the intermembrane space (IMS) located between them. It also depicts the process of mitochondrial fusion, which begins with the fusion of the MOM mediated by Mfn1 and Mfn2 genes, followed by the fusion of the MIM regulated by Opa1 gene. The completion of these events results in the formation of elongated or clustered mitochondria, reflecting enhanced mitochondrial connectivity and integrity.

2.2.6.3 Lipid Droplets

The lipid droplet (LD) is a specialised cellular organelle with a neutral lipid core covered by a monolayer phospholipid membrane and associated proteins (T. Li et al., 2023). The LD differs from other membrane-bound organelles in that it contains (1) a hydrophobic core as opposed to an aqueous phase lumen in other membrane-bound organelles, (2) a single-layer phospholipid membrane as opposed to a bilayer or double bilayer membrane in other organelles, (3) its own protein equipment referred to as LD-resident proteins (T. Li et al., 2023).

The functions of LD are lipid storage, lipid transport, lipid synthesis and lipid hydrolysis, a series of functions that go far beyond their image as a static oil reservoir (L. Cui & Liu, 2020; Zadoorian et al., 2023). In addition to their involvement in energy metabolism, LD also have the task of alleviating cellular stress and nutrient deprivation in the embryo (T. Li et al., 2023). Although the number of publications on the role of LD in preimplantation embryo is increasing, the data is still limited and needs further investigation.

2.2.6.4 Peridroplet Mitochondria

Peridroplet mitochondria are a specialized subset of mitochondria tethered to LDs in early embryos. They play crucial roles in coordinating lipid metabolism and energy homeostasis by facilitating localized fatty acid oxidation and ATP generation (L. Cui & Liu, 2020; Veliova et al., 2020).

During early embryogenesis, peridroplet mitochondria are formed through the fusion of mitochondria to LDs at specialized membrane contact sites. This association is characterized by mitochondrial cristae oriented perpendicularly to the lipid interface (Benador et al., 2018). The formation and stabilization of these contacts are mediated by mitochondrial fusion proteins such as *Mfn2*, which facilitate the structural and functional coupling between mitochondria and LDs (C. Zhang et al., 2024).

2.2.6.5 Cellular Degradation Machinery

Early embryos rely on the autophagy pathways to degrade maternal proteins, regulate developmental signalling, recycle cellular components and maintain protein

homeostasis (Satouh & Sato, 2023; Toralova et al., 2020). These processes are essential for the transition from maternal to zygotic genome control and for supporting subsequent stages of embryonic development.

At the 2-cell stage, autophagy–lysosome activity is markedly enhanced to support the metabolic and structural demands of early embryogenesis. During this phase, autophagy is induced leading to the formation and expansion of autophagosomes that sequester cytoplasmic cargo including maternal proteins and damaged organelles. These autophagosomes subsequently fuse with lysosomes to form autolysosomes where the engulfed materials are degraded and recycled (Kudriaeva et al., 2020; Parzych & Klionsky, 2014).

Under stress conditions such as oxidative stress or nutrient deprivation, autophagy is upregulated to eliminate damaged organelles and misfolded proteins, thereby preventing toxic accumulation and promoting embryonic survival under adverse environments (Rossin et al., 2025; Singh et al., 2024). However, when stress becomes excessive or prolonged, this delicate regulatory balance can be disrupted leading to impaired autophagic flux and the activation of apoptotic pathways when cellular damage exceeds the repair capacity. These processes are closely linked through the regulation of mitochondrial outer membrane permeabilization (MOMP) (H. J. Wu et al., 2015).

Under severe stress, pro-apoptotic proteins such as *Bax* is released due to *Bcl-2* inhibition resulting in the loss of mitochondrial membrane potential and subsequent activation of the intrinsic apoptotic pathway (El-Khattouti et al., 2013). In the context of maternal asthma, elevated oxidative and inflammatory stress may similarly dysregulate autophagy in preimplantation embryos compromising their developmental potential by activating apoptotic pathways.

2.2.6.6 Microvilli and Perivitelline Space

Microvilli are finger-like plasma membrane protrusions that increase the cell surface area (Orbach & Su, 2020). At 2-cell stage, microvilli enhance the plasma membrane surface area to facilitate cellular interactions, molecular exchange and polarity establishment essential for subsequent cleavage and embryonic development (White et al., 2016). The perivitelline space (PVS) is the gap between the plasma membrane of the blastomere and the surrounding zona pellucida (Okaji et al., 2020).

Studies had shown that cellular fragments and apoptotic bodies can be extruded from blastomeres into the PVS as a mechanism to preserve embryo viability by removing abnormal or damaged cellular components. This extrusion process is closely associated with programmed cell death and autophagic clearance mechanisms whereby autophagy indirectly contributes to maintaining the composition and cleanliness of the PVS through intracellular degradation and the elimination of unwanted material (Rossin et al., 2025; Singh et al., 2024).

In preimplantation embryos, abnormal PVS characteristic such as increased debris accumulation is often correlated with reduced developmental competence which potentially reflecting impaired autophagic activity (Hassa et al., 2014). Conversely, efficient autophagy supports optimal embryonic development by minimizing cytoplasmic damage and regulating the extrusion of cellular fragments into the PVS, thereby maintaining a stable and favourable microenvironment during critical early developmental stages.

2.2.7 Impairment of Preimplantation Embryos

The preimplantation period is a very sensitive time window in embryonic development. At this stage, embryos undergo rapid cell division, organelle remodelling and epigenetic reprogramming while being highly dependent on maternal transcripts and metabolic support. Due to limited antioxidant defences and the lack of zygotic gene control in the early stages, preimplantation embryos are particularly sensitive to maternal insults such as inflammation and oxidative stress.

2.2.7.1 Effects of Maternal Inflammation

Maternal inflammation has been shown to negatively affect early embryonic development particularly when induced during the zygote or cleavage phase. Systemic inflammation such as that induced by lipopolysaccharide (LPS), can disrupt embryo morphogenesis and cell lineage distribution. Williams et al., (2011) reported that maternal exposure to LPS reduced the number of ICM cells and altered the ratio of ICM to TE in developing blastocysts. Similarly, Fabian et al., (2010) demonstrated increased apoptosis in blastocysts when inflammation was induced during cleavage as evidenced

by Terminal Deoxynucleotidyl Transferase dUTP nick-end Labeling (TUNEL)-positive nuclei.

These results highlight how an inflammatory maternal environment can affect crucial developmental checkpoints. However, there are few studies that specifically address allergic-type inflammation as it occurs in asthma. Allergic asthma involves the recruitment of Th2-type immune cells and the production of cytokines that can overcome biological barriers and affect the uterine environment. Given this mechanism, maternal allergic inflammation may pose a unique risk to embryo viability but this is still an under-researched area that merits further investigation.

2.2.7.2 Effects of Maternal Oxidative Stress

Oxidative stress is another key factor affecting the quality of the embryo during preimplantation development. Elevated levels of ROS including $O_2^{\bullet -}$, H_2O_2 and OH- can damage DNA, proteins and lipids. Such damage often triggers apoptosis, disrupts mitochondrial function and impairs cell division.

Studies have shown that exposure to pro-oxidant agents such as ginsenoside (Rg1) lead to excessive ROS accumulation resulting in apoptosis and reduced blastocyst formation in mouse embryos (Y. Lee et al., 2019). Similarly, nicotine-induced oxidative stress has been associated with developmental arrest at the morula stage and impaired cytoskeletal integrity (Kamsani et al., 2012). Oxidative stress has also been associated with congenital malformations in diabetic rat models (Dennery, 2007).

While the deleterious effects of oxidative stress on embryos are well established, little is known about how maternal allergic asthma which combines both inflammation and oxidative stress can affect preimplantation embryo viability. The systemic oxidative stress associated with asthma may affect not only maternal airway tissues but also distant systems including the reproductive organs and intrauterine environment. Understanding these system-wide impact is critical for the development of interventions that protect early embryo viability in affected mothers.

2.2.7.3 Epigenetic Disruption by Maternal Stress

The preimplantation embryo undergoes extensive epigenetic reprogramming including DNA methylation, histone modifications and regulation of non-coding RNA.

These epigenetic mechanisms are critical for ZGA, lineage specification and embryo viability (R. Xu et al., 2025). Maternal physiological stress including inflammation and oxidative stress as in allergic asthma can disrupt this delicate epigenetic landscape.

Oxidative stress alters the function of DNA methyltransferases (DNMTs) and histone-modifying enzymes leading to aberrant gene expression and impaired embryonic development (Zhu et al., 2021). For example, maternal exposure to cadmium, a known pro-oxidant, leads to DNMT hypomethylation and mitochondrial dysfunction in mouse preimplantation embryos, affecting embryo quality and implantation potential (Zhu et al., 2021). Similarly, Wu et al., (2023) demonstrated that acrylamide-induced oxidative stress suppresses key transcription factors required for ZGA leading to 2-cell arrest.

The combination of inflammatory cytokines and ROS in maternal allergic asthma can therefore influence the epigenetic programming of the embryo, even if there is no direct physical contact between the embryo and maternal immune cells. These results underline the importance of investigating not only morphological changes but also molecular and epigenetic perturbations when assessing the effects of maternal stress on early embryos.

2.2.8 Gene Expression Regulation in 2-cell Embryos

During the preimplantation period, the regulation of gene expression is of fundamental importance for successful embryonic development. In mice, the largest wave of ZGA occurs at the 2-cell stage and marks the transition from maternal to embryonic control of development. At this critical stage, transcriptional activity controls processes such as cell division, mitochondrial function, apoptosis regulation and redox balance (S. Xu & Egli, 2024).

Several gene families are particularly relevant in the context of maternal oxidative and inflammatory stress. These include antioxidant genes such as *Sod2* and *Gpx1* which attenuate ROS, mitochondrial regulators such as *Ppargc1a* and *Tfam,* and apoptosis-related genes such as *Bcl2, Bax* and *Casp3* (Manti et al., 2016; Moraes-Souza et al., 2021; C. Zhang et al., 2023). Dysregulation of these genes can affect cleavage, impair mitochondrial function and lead to developmental arrest or abnormal blastocyst formation.

Maternal allergic asthma leads to systemic oxidative stress and increased cytokines that can affect these regulatory pathways. Early studies had shown that embryos exposed to maternal inflammation exhibited altered expression of stress response and apoptotic genes (Fabian et al., 2010; Williams et al., 2011). Gene expression analysis using high-throughput microfluidic Real Time (RT)-PCR platforms such as Fluidigm enables multiplex quantification of these markers in low-throughput samples such as 2-cell embryos and provides a precise overview of molecular changes under stress conditions.

By assessing the expression profiles of oxidative stress, mitochondrial fusion, mitochondrial bioenergetics and apoptotic genes, this study aims to elucidate how maternal TRF supplementation modulates the embryonic response to allergic inflammation and provides insights into protection and repair mechanisms at the molecular level.

2.2.9 Antioxidants as a Safe Alternative to Mitigate Inflammation and Oxidative Stress

Conventional treatments for allergic asthma including corticosteroids and β-agonists, primarily target airway symptoms but often fall short when it comes to systemic oxidative stress and inflammation which are two key factors responsible for reproductive impairment. In addition, the safety of many pharmacological agents during pregnancy remains a concern as their effects on early embryonic development and foetal health are not fully known (Bonham et al., 2018; Namazy et al., 2013). This emphasises the need for alternative interventions that are both effective and safe for use in a reproductive context.

Antioxidants offer a promising solution as they are able to restore redox balance, reduce levels of inflammatory cytokines and protect cellular structures from oxidative damage. Naturally occurring antioxidants such as flavonoids, polyphenols and vitamin E compounds have been shown to modulate immune responses and mitigate ROS-induced damage in both the respiratory and reproductive systems (Birben et al., 2012; V. Mishra et al., 2018). These compounds act not only by scavenging free radicals but also by regulating signalling pathways involved in inflammation, apoptosis and mitochondrial function.

Among natural antioxidants, tocotrienols (TCTs), members of the vitamin E family have received increasing attention due to their enhanced bioactivity compared to tocopherols (TCPs). Their ability to integrate into lipid membranes, interrupt lipid peroxidation and suppress redox-sensitive transcription factors such as NF-κB make them ideal candidates for the protection of tissues at risk of oxidative damage. As natural antioxidant derived from palm oil or annatto, TCTs are safe, well tolerated and possess both antioxidant and anti-inflammatory properties.

The use of tocotrienol-rich fraction (TRF) as a maternal supplement could therefore provide dual protection by reducing systemic stress and preserving the integrity of the early embryo. In the next section, the structure, bioavailability and biological effects of TRF are discussed in detail focussing on their importance for maternal health and embryo viability in allergic asthma murine model.

2.3 Tocotrienol

Vitamin E is a fat-soluble antioxidant that plays a crucial role in maintaining cellular homeostasis and protecting biological membranes from oxidative damage. It consists of two different groups of compounds: TCPs and TCTs, each comprising four isoforms known as alpha (α), beta (β), gamma (γ) and delta (δ) (Kowalska, 2024). While both groups have antioxidant properties, there is growing evidence that the TCTs particularly the gamma (γ) and delta (δ) isoforms have increased biological activity in certain areas such as inflammation, oxidative stress and cancer prevention.

2.3.1 Introduction to Tocotrienol

The importance of vitamin E was first recognised in 1922 when it was shown to be essential for fertility and maintaining pregnancy in rats (Evans & Bishop, 1922). Over the decades, research has focussed primarily on TCPs particularly α-TCP as it is abundant in the diet and is preferentially transported by the α-TCP transfer protein. However, since the early 2000s, interest has shifted to TCTs due to their unique structural properties and increasing evidence of their enhanced therapeutic potential (Kowalska, 2024; Sen et al., 2007).

Despite the increasing number of publications, the role of TCTs in health and disease remains less well understood than that of TCPs. Further studies are needed,

especially in areas related to reproductive health and early embryonic development under conditions of oxidative or inflammatory stress.

2.3.2 Chemical Structure of Tocotrienol

TCTs differ from TCPs primarily in the structure of their isoprenoid side chain as illustrated in Figure 2.5. TCTs possess three double bonds in the hydrocarbon tail giving the molecule an unsaturated and more flexible structure whereas TCPs have a fully saturated phytol tail. Both molecules share a chromanol ring with hydroxyl groups responsible for their antioxidant activity (Wong & Radhakrishnan, 2012).

The unsaturated side chain of TCTs allows them to penetrate more efficiently into lipid bilayers and move rapidly within cell membranes (Wong & Radhakrishnan, 2012). This structural feature enhances their capacity to interact with lipid radicals and terminate lipid peroxidation chain reactions more effectively than TCPs. Consequently, TCTs exhibit superior antioxidant potential in protecting cellular and mitochondrial membranes from oxidative damage thereby maintaining membrane integrity and fluidity (Wong & Radhakrishnan, 2012).

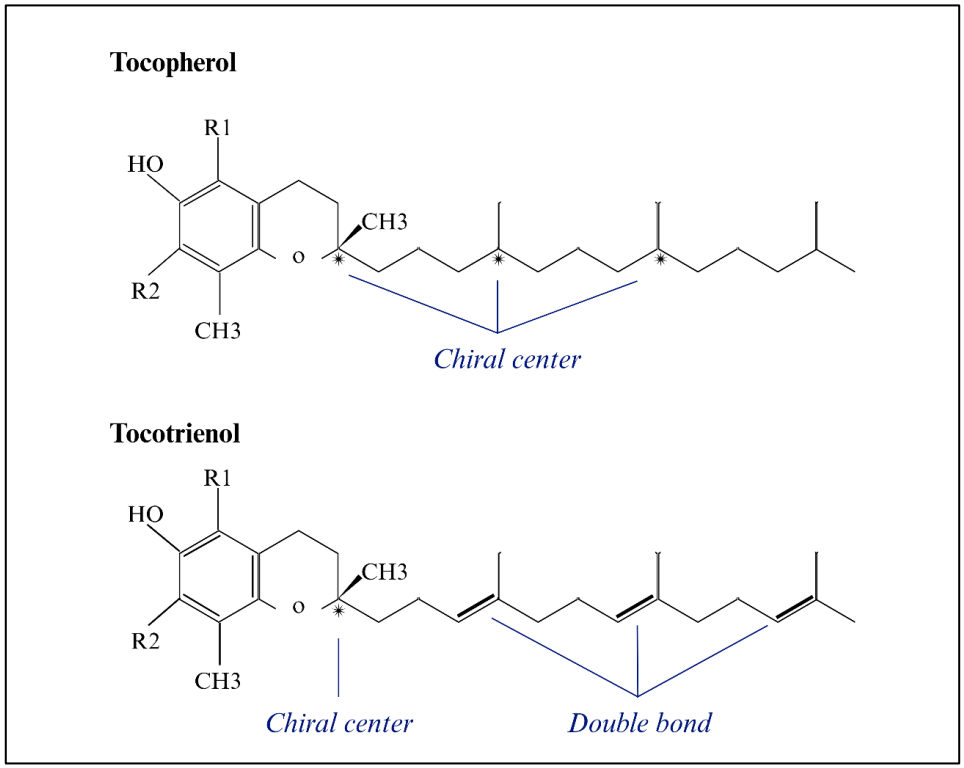


Figure 2.5 Structural Comparison Between Tocopherol and Tocotrienol

This figure shows the structural difference between tocotrienol and tocopherol. Tocotrienol has an unsaturated isoprenoid side chain with three double bonds and one chiral centre, while tocopherol has a saturated phytol tail with three chiral centres. The unsaturated tail of tocotrienol enhances its membrane mobility and antioxidant activity.

2.3.3 Natural Sources of Tocotrienol

TCTs are found in small amounts in various foods including rice bran, barley, rye, wheat germ and fruits such as cranberries and plums. However, the richest sources are annatto seeds and palm oil, both of which contain high amounts of γ- and δ-TCTs (Kabir et al., 2017; Shahidi & De Camargo, 2016). Therefore, TCTs are typically administered as part of a TRF, which contains a higher concentration of TCTs compared to TCPs primarily derived from rich natural sources such as annatto seeds and palm oil.

2.3.4 Bioavailability of Orally Administered Tocotrienol

After ingestion, the TCTs are absorbed in the intestine and incorporated into chylomicrons which are then transported through the lymphatic system. These chylomicrons then enter the bloodstream and distribute the TCTs to various tissues which is facilitated by enzymatic hydrolysis by lipoprotein lipase (Kabir et al., 2017; Kowalska, 2024). These processes are illustrated in Figure 2.6.

Despite their efficient absorption, TCTs have a relatively short half-life in the bloodstream. They usually reach maximum plasma concentrations within three to four hours and are excreted from the bloodstream within eight hours (Mahipal et al., 2016). The bioavailability of TCTs is influenced by gastrointestinal conditions. Studies had shown that simultaneous ingestion with food increased absorption (Hayes et al., 1993; Yap et al., 2010). In the laboratory especially in rodent models, an oral gavage is often used to ensure consistent absorption and therapeutic effect.

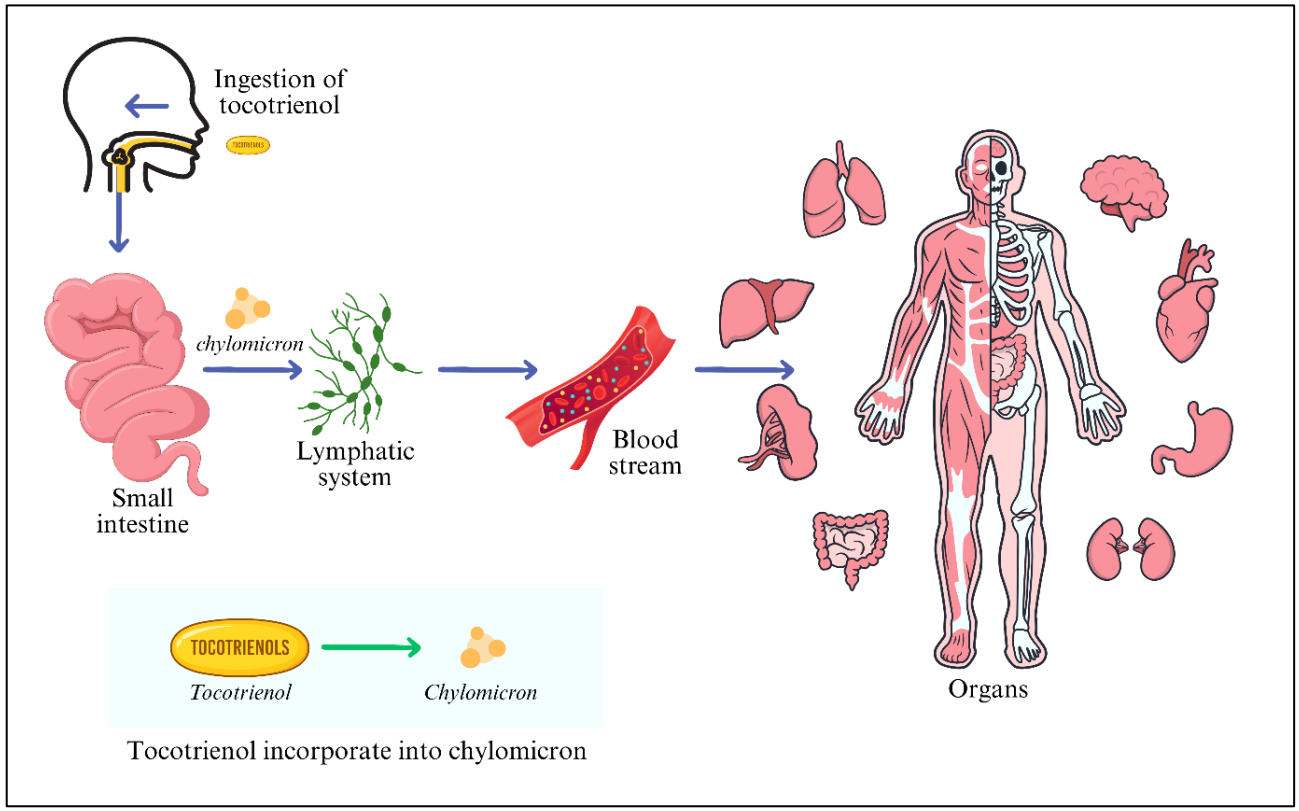


Figure 2.6 Absorption and Systemic Transport of Orally Administered Tocotrienol

This figure explains the absorption of orally administered tocotrienol that first being absorbed in the intestine, then incorporated into chylomicrons, and transported via the lymphatic system into the bloodstream which further transported to the various organ tissues.

2.3.5 Tocotrienol-rich Fraction

The TRF is a natural vitamin E extract that contains high concentrations of TCTs and smaller amounts of TCPs. The TRF is usually extracted from palm oil or annatto and had been widely studied for its strong antioxidant, anti-inflammatory and cytoprotective properties. Compared to α-TCP, the TCTs contained in TRF have shown higher bioactivity in various disease models and cellular stress (Ghani et al., 2019).

While TRF has been extensively studied in metabolic (Al-Baiaty et al., 2021; Phang et al., 2023), cardiovascular (Rafique et al., 2024) and neurological diseases (Ismail et al., 2020), its role in reproductive biology especially during early embryonic development has not yet been sufficiently explored. Due to its combined antioxidant and immunomodulatory properties, TRF may be beneficial in combating maternal stress factors such as systemic inflammation and oxidative stress associated with allergic asthma.

2.3.5.1 Preparation of Tocotrienol-rich Fraction for Laboratory Use

TRF was extracted from palm oil by-products specifically palm fatty acid distillate (PFAD) through a series of saponification, solvent partitioning and purification steps to concentrate TCTs for experimental use. The extraction process was designed to remove free fatty acids and recover the unsaponifiable fraction which contains vitamin E compounds particularly TCTs. Typically, this method yields a fraction containing approximately 20–30 % of total vitamin E compounds (TCPs and TCTs) of which around 80 % comprise TCTs that is suitable for laboratory supplementation studies (Abdah et al., 2025).

Briefly, PFAD was subjected to saponification by heating to 40 °C and mixing with hexane at a 3:1 ratio followed by the addition of calcium hydroxide [$Ca(OH)_2$] at a 1:1 (w/w) ratio. The mixture was stirred at 30°C for 30 minutes to facilitate the formation of calcium soaps from fatty acids. After centrifugation, the supernatant was separated and the residue washed with hexane. The solvent was subsequently evaporated to obtain the unsaponifiable fraction of vitamin E compounds (Gore & Bhagwat, 2022).

Further purification was performed by redissolving the unsaponifiable fraction in a hexane–water mixture and chilling the solution at 5 °C for 72 hours to promote sterol crystallisation. The crystallised sterols were removed by filtration and the solvent was evaporated to yield the final TRF. The composition of the extracted TRF was confirmed using high-performance liquid chromatography (HPLC) prior to its use in subsequent *in vivo* experiments (Gore & Bhagwat, 2022).

Extracted TRF can be dissolved in various lipid-based vehicles to facilitate oral delivery in experimental models as its lipophilic nature requires an appropriate carrier for optimal solubility and bioavailability. Commonly used vehicles include edible oils and other neutral lipid matrices that ensure stability and efficient absorption (Soontornchatchawate et al., 2021). Among these, palm olein stripped of TCTs and TCPs is frequently employed as a vehicle in rodent studies (Abdah et al., 2025). The removal of naturally occurring vitamin E isomers prevents background antioxidant interference, thereby allowing clearer interpretation of the specific biological effects attributed to TRF supplementation.

2.3.5.2 Role of Tocotrienol-rich Fraction as Antioxidant

The TRF has strong antioxidant properties via several mechanisms. It strengthens endogenous antioxidant defences by upregulating enzymes such as SOD, catalase (CAT) and glutathione peroxidase (GPx) (Chin et al., 2011). Additionally, TRF reduces lipid peroxidation, as evidenced by lower levels of malondialdehyde (MDA) and protein carbonyl in oxidative stress models (Pang et al., 2021).

In addition to enhancing antioxidant enzymes, TRF directly scavenge ROS. The chromanol ring of γ- and δ-TCTs donates hydrogen atoms to neutralise free radicals and effectively end the chain reaction of lipid peroxidation (Pizzino et al., 2017; Trela-makowej et al., 2022).

The TRF also modulates redox-sensitive signalling pathways. It inhibits the activation of NF-κB and activator protein-1 which are transcription factors that regulate genes involved in oxidative stress and inflammation (Lu et al., 2022; C. Yang & Jiang, 2019). While these mechanisms have been demonstrated in models of diabetes and neurodegeneration, their relevance to maternal reproductive physiology and embryo protection is only just beginning to be explored.

2.3.5.3 Role of Tocotrienol-rich Fraction as Anti-inflammatory Agent

The TRF has shown a significant anti-inflammatory effect in both *in vitro* and *in vivo* models. It reduces the expression of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 and suppresses the recruitment of inflammatory cells such as eosinophils and mast cells (Katengua-thamahane et al., 2014; H. Lee & Lim, 2018). These actions help to maintain tissue integrity and reduce systemic immune activation.

Although research on TRF in airway disease models is still limited, a study by Zainal et al., (2019) showed that TRF supplementation in an OVA-induced asthma model significantly reduced airway inflammation, serum IgE level and inflammatory cytokines. These results support the potential use of TRF in the modulation of immune responses in allergic asthma.

As maternal asthma is associated with systemic inflammation, the immunomodulatory properties of TRF could extend beyond the lungs to protect reproductive tissues and early embryos. However, more research is needed to determine its efficacy in mother-to-embryo signalling under inflammatory conditions.

2.3.5.4 Role of Tocotrienol-rich Fraction in Preimplantation Embryo Development

Preimplantation phase of embryonic development includes rapid cell division, metabolic transitions, remodelling of organelles and epigenetic reprogramming. These processes make embryos highly sensitive to environmental stressors particularly oxidative stress and inflammation, both of which are increased in maternal allergic asthma.

Although there are few direct studies related to TRF and preimplantation embryos, new evidence suggests potential protective effects of TRF. Sarbandi et al., (2018) reported that maternal TRF supplementation increased the number of normal 2-cell embryos while Lee et al., (2015) showed that γ-TCT reduced ROS levels and apoptosis, thereby improving blastocyst formation in stress-exposed embryos.

In addition, TRF supplementation was shown to preserve cytoskeletal integrity in models of nicotine-induced oxidative stress (Hamirah et al., 2017). This suggests a broader role for TRF in maintaining cellular architecture under maternal stress. However, studies on organelles such as mitochondria remain scarce. In a study on neuronal cells, α-TCT was reported to prevent mitochondrial dysfunction and preserve

membrane potential (Park et al., 2020) suggesting a similar protective mechanism in embryos.

In summary, TRF is a promising non-pharmacological intervention to protect embryo development during maternal inflammatory and oxidative stress. The current study aims to provide a deeper insight into the potential of TRF to improve embryo viability in a mouse model of allergic asthma.

2.3.5.5 Current Evidence on Tocotrienols in Allergic Asthma Experimental Model

Although TCTs possess potent antioxidant and anti-inflammatory properties as describe in previous subsections, only few studies have investigated their protective role in allergic asthma.

Among these, Peh et al., (2015) had reported decrease inflammatory cells in BALF, suppression of Th2- and Th17-related cytokines, downregulation of oxidative enzymes and enhancement of endogenous antioxidant defences. These findings elucidate that γ-tocotrienol significantly reduced airway inflammation and oxidative stress in HDM-induced asthma model. In addition, Zainal et al., (2019) found that palm oil-derived TRF lowered systemic inflammatory mediators and circulating IgE in an OVA-induced model. The specific outcomes of the findings are summarised in Table 2.2.

Overall, while TCTs and TRF show promising pulmonary and partial systemic benefits in allergic asthma, current evidence is limited. Notably, no study has investigated their effects on preimplantation embryos in the context of allergic asthma, leaving a critical gap in understanding the maternal and developmental potential of TCT supplementation. These gaps highlight the need for further studies exploring systemic, reproductive and mechanistic effects to fully elucidate the therapeutic value of tocotrienols in allergic asthma.

Table 2.2
Current Evidence on Tocotrienols in Allergic Asthma Experimental Model

Tocotrienol Supplementation	**Experimental Model**	**Specific Outcomes**
30, 100 and 250 mg/kg γ-tocotrienol supplemented orally (Peh et al., 2015)	HDM-induced asthma mice	- γ-tocotrienol ↓ total cells number, eosinophils and neutrophils in BALF - γ-tocotrienol ↓ infiltration of inflammatory cells in lung - γ-tocotrienol ↓ serum total (ng/ml) and HDM-specific IgE - γ-tocotrienol ↓ IL-4 (pg/ml), IL-5 (pg/ml), IL-10 (pg/ml), IL-17 (pg/ml), RANTES (pg/ml) and G-CSF (pg/ml) - γ-tocotrienol ↓ BALF RONS (%) - γ-tocotrienol ↓ BALF 8-isorostane (pg/ml), BALF 8-OHdG (pg/ml) and lung 3-NT (nM/mg) - γ-tocotrienol ↑ lung SOD (U/mg), CAT (nmol/min/mg) and GPx (nmol/min/mg) activity - γ-tocotrienol ↓ lung mRNA IL-3, IL-17 and IL-33 - γ-tocotrienol ↓ lung mRNA expression of iNOS, NOX1, NOX2, NOX3, NOX4 and HO-1
30 mg/kg palm oil-TRF supplemented orally (Zainal et al., 2019)	OVA-induced asthma rats	- TRF ↓ eosinophils, neutrophils and lymphocytes in blood - TRF ↓ serum IL-1β (pg/mL), IL-6 (pg/mL), TNF-α (pg/mL), plasma CRP (pg/mL), plasma IgE (ng/mL) and serum LTB-4 (pg/mL) levels - TRF ↑ serum levels of IL-4 (pg/mL), serum IL-13 (pg/mL) and serum LTB-5 (pg/mL) levels

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Introduction

This chapter describes the methodology used to investigate the protective effects of maternal tocotrienol-rich fraction (TRF) on systemic inflammation, oxidative stress, pregnancy hormone levels, and the development, morphology, ultrastructure and gene expression profiles of preimplantation embryos in a murine model of ovalbumin (OVA)-induced allergic asthma. The experimental approach was divided into two consecutive experiments: the first to establish an appropriate asthma model by determining the optimal OVA dose (Experiment I) and the second to evaluate the protective effects of TRF supplementation (Experiment II).

3.2 Place of Study

This study was conducted at several designated research facilities within Universiti Teknologi MARA (UiTM), Selangor and University of Malaya (UM), Kuala Lumpur:

i. Animal care and sample collection were carried out at the Laboratory Animal Care Unit (LACU), Faculty of Medicine, UiTM, Sungai Buloh Campus.
ii. Embryo processing and sample preparation were carried out in the Embryo Preparation Laboratory, Ultramicrotome Room, RNA Laboratory, DNA Laboratory and Protein Laboratory of the Institute of Medical Molecular Biotechnology (IMMB), Faculty of Medicine, UiTM, Sungai Buloh Campus.
iii. Electron microscopy and imaging analysis were performed in the imaging room of the Faculty of Pharmacy, and the ultramicrotome and TEM room of the Faculty of Health Sciences, UiTM, Puncak Alam Campus.
iv. Gene expression analysis was performed at the High Impact Research Centre, UM.

3.3 Ethics Approval

Animal handling and all experimental procedures were carried out in accordance with institutional and international guidelines for the care and use of laboratory animals. Ethics approval was obtained from the Committee on Animal Research and Ethics (UiTM CARE) of Universiti Teknologi MARA. The study was conducted under the approved protocol (reference number UiTM CARE: 318/2020) dated 14th August 2020.

3.4 Experimental Animal

Healthy female BALB/c mice aged 4 to 5 weeks (weighing 20–25 g) were purchased from the Laboratory Animal Facility and Management (LAFAM), UiTM Puncak Alam Campus. Upon arrival, the animals were kept in quarantine for seven days under controlled conditions (ambient temperature of 27 °C, 12-hour light-dark cycle and access to standard rodent chow and water *ad libitum*). All mice were examined prior to the start of the study and only those showing no signs of disease such as excessive porphyrin discharge, hair loss, reduced activity or respiratory distress were included in the study. Animals showing any abnormal health indicators were excluded from the study.

3.5 Chemicals, Reagents and Equipment

All chemicals, reagents, and laboratory equipment used in this study were of analytical or molecular biology grade to ensure high purity, reliability, and accuracy of experimental outcomes. These materials were obtained exclusively from reputable and certified suppliers to maintain consistency and minimize variability throughout the experimental procedures. The specific types, brands, manufacturers and sources of all chemicals, reagents and laboratory equipment are detailed in Appendix A (Table A1: Chemicals and Reagents) and Appendix B (Table B1: Laboratory Equipment). Therefore, the manufacturer and brand names are not mentioned within the main text. Inclusion of these details ensures transparency and supports the reproducibility of the experimental procedures described in this chapter enabling future researchers to replicate the study conditions accurately.

3.6 Sample Size Calculation

This study included two types of biological samples: 1) maternal serum and 2) two-cell stage embryos. The sample size for the maternal serum analysis was determined using "Resource Equation" which is a crude method based on the law of diminishing returns as described by Charan & Kantharia (2013). In contrast, the sample size for two-cell embryos was established through embryo pooling to ensure sufficient samples for analysis.

For maternal serum, crude method was chosen because there were no comparable previous studies that could have been used to estimate the effect size or standard deviation (SD) required for a formal power analysis. The crude method estimates the degree of freedom for a one-way analysis of variance (ANOVA) labelled as "E" in the following formula:

$$E = \text{total number of animals} - \text{total number of groups} \quad (3.1)$$

An E value between 10 and 20 is considered sufficient to detect statistically significant differences. An E value above 20 is considered excessive while a value below 10 may increase the risk of Type II error (false negative). This study consisted of two experiments. In both Experiment I and II, there were five experimental groups and five animals per group were first decided to be used. Hence, following the formula, the "total number of animals" was $5 \times 5 = 25$ whereas the "total number of groups" was 5:

$$E = 25 - 5 = 20 \quad (3.2)$$

To account for possible attrition due to induction of allergic asthma and repeated handling, a 20% buffer was included using the following adjustment:

Corrected sample size = n / (1 – attrition rate)

Corrected sample size = 5 / (1 – 20%) = 6.25 - *rounded to 6 animals per group*

The sample size calculation and E-values for both experiments are summarised in Table 3.1. This calculation justified the use of six mice per group (n = 6) for all serum analyses in both experiments.

Table 3.1
Sample Size Calculation

Experiment I	**Experiment II**
$E = (5 \times 5) - 5$	$E = (5 \times 5) - 5$
$= 20$	$= 20$
Corrected sample size	*Corrected sample size*
$= 5/ (1 - [20/100]$	$= 5/ (1 - [20/100]$
$= 6.25$	$= 6.25$

This table shows the sample size calculation for Experiment I and II. The sample size was calculated using the crude method based on the law of diminishing returns. With 5 groups and 5 animals per group, the value of E was calculated as 20, which is within the acceptable range (10–20). To account for possible data loss (20%), the corrected sample size was calculated so that at least 6 animals per group should be used in both experiments.

For embryo analysis, the sample size was based on embryo pooling which allowed a fixed number of embryos per group (N = 110) to be used for downstream applications which were 1) *in vitro* culture for developmental and microscopic analysis, 2) ultrastructural analysis and 3) gene expression. Each group provided approximately 110 pooled two-cell embryos which were divided as follows following further analysis methods:

i. 50 embryos: *in vitro* culture for developmental and microscopic analysis (*n=1; 10 pooled embryos*)
ii. 15 embryos: ultrastructural analysis using TEM (*n=1; 5 pooled embryos*)
iii. 45 embryos: gene expression profiling using Fluidigm qPCR (*n=1; 15 pooled embryos*)

3.7 Overview of Study

This study comprised two consecutive experiments. Experiment I aimed to determine the optimal dose of OVA to induce maternal allergic asthma that adversely affects preimplantation embryo viability. Experiment II investigated the effects of maternal TRF supplementation at the optimal OVA dose on maternal serum biomarkers and embryo viability focussing on developmental potential, morphological quality, ultrastructural features and gene expression. A visual summary of the study framework can be found in Figure 3.1.

In Experiment I, three different OVA doses were compared based on their effects on maternal inflammation, oxidative stress and embryonic outcomes In Experiment II, OVA-sensitised mice were administered a dose of TRF (60 mg/kg body weight) or a vehicle control (palm olein stripped of vitamin E) to investigate the potential protective effects of TRF. Parameters evaluated included maternal serum biomarkers (inflammation, oxidative stress, pregnancy hormones) and embryonic outcomes (developmental potential, morphological quality, ultrastructural features and gene expression).

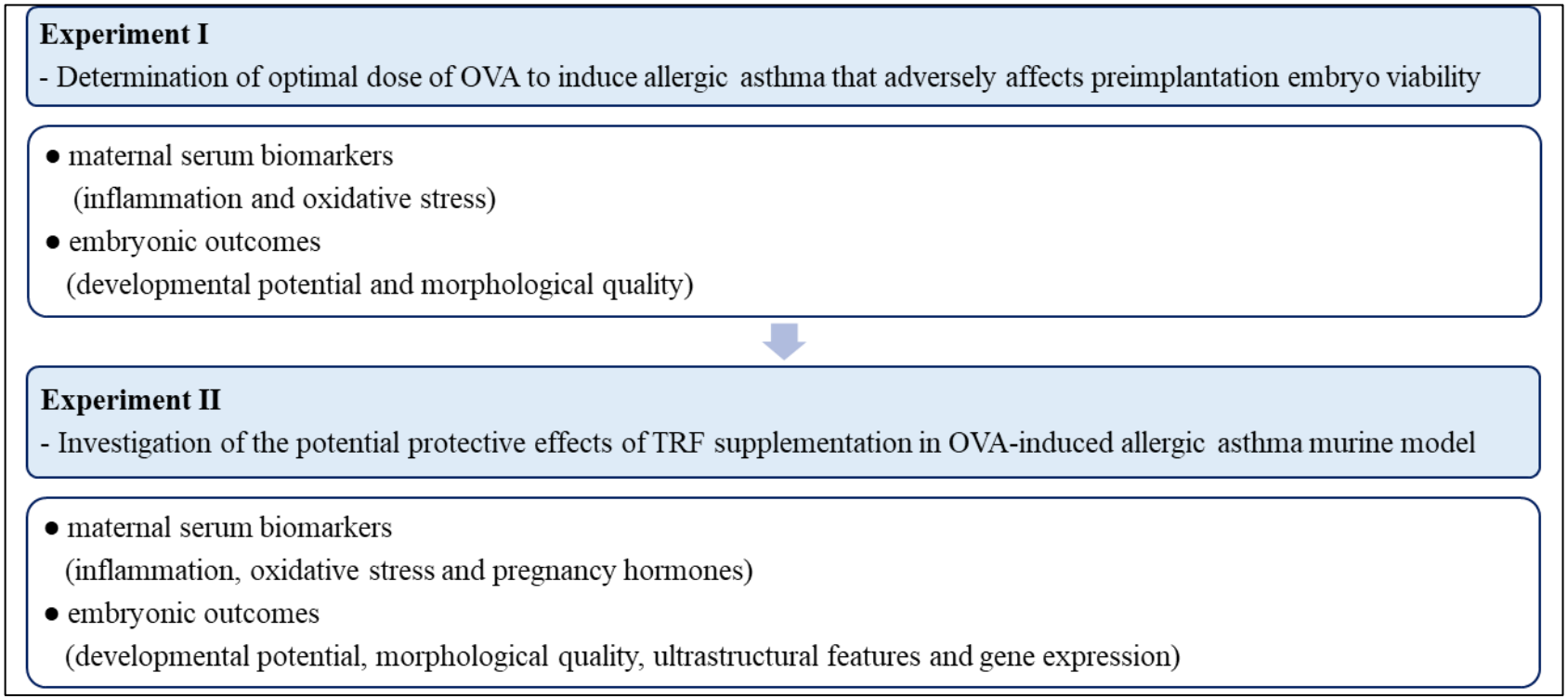


Figure 3.1 Overview of Experimental Workflow

This figure shows the overview of experimental workflow which consist two-phase study design. Experiment I was conducted to determine the optimal dose of ovalbumin (OVA) required to induce systemic inflammation and oxidative stress that impairs preimplantation embryonic development. Experiment II investigated the protective effects of a maternal tocotrienol-rich fraction (TRF) supplementation on maternal physiological status and embryonic outcomes using the optimal dose of OVA determined in Experiment I.

3.7.1 Experiment I: Determination of the Optimal Ovalbumin Dose

The aim of Experiment 1 was to determine the optimal dose of OVA required to induce allergic asthma in female mice characterised by increased systemic inflammation and oxidative stress, and to evaluate the effects on the developmental potential and morphological quality of 2-cell embryos. Three different doses of OVA were tested to determine which produced the most significant physiological and developmental changes. A total of 30 female BALB/c mice (aged 4–5 weeks, weighing 20–25 g) were randomly divided into five groups (n = 6 per group) as summarised in Table 3.2.

Allergic asthma induction was established according to the protocol described by Paik et al., (2014). In their study, a sensitisation dose of 100 µg /200 µL OVA was used, which produced clear evidence of airway inflammation. However, as this study aimed to investigate systemic effects, both a lower dose (50 µg/200 µL OVA) and a higher dose (150 µg/200 µL OVA) in between 100 µg /200 µL OVA were evaluated. This approach is adopted as systemic inflammatory and oxidative stress responses may occur even at doses lower or higher than the standard sensitisation dose, and testing a range allows determination of dose-dependent systemic effects for the OVA dose study.

Based on these selected doses, sensitisation was performed by intraperitoneal (i.p.) injection on Day 0 and 14 with the respective OVA concentrations or PBS. The mice in the SHAM group received no treatment. On Days 21, 22 and 23, all sensitised groups received intranasal (i.n.) OVA challenge (75 µg/50 µL) while the PBS groups received PBS and no treatment for SHAM group.

Next, all groups including SHAM underwent superovulation and mating protocols following Luo et al., (2011). On Day 25 (1.5 days post coitum), mice were euthanised and blood samples were collected for serum analysis using Enzyme-linked immunosorbent assay (ELISA) analysis. The 2-cell stage embryos were flushed from the oviducts then underwent *in vitro* culture for microscopic analysis.

The optimal OVA dose was determined based on three parameters: 1) increased levels of maternal inflammatory markers, 2) increased levels of maternal oxidative stress markers and 3) reduced embryo developmental progress and morphological quality. The experimental layout and procedures timeline for this phase are shown in Figures 3.2 and 3.3.

Table 3.2
Experimental Groups and Treatments in Experiment I

	Groups	Treatment Description
1.	SHAM	Untreated control
2.	PBS	Sensitised with phosphate buffered saline (PBS)
3.	OVA1	Sensitised with 50 µg/200 µL OVA
4.	OVA2	Sensitised with 100 µg/200 µL OVA
5.	OVA3	Sensitised with 150 µg/200 µL OVA

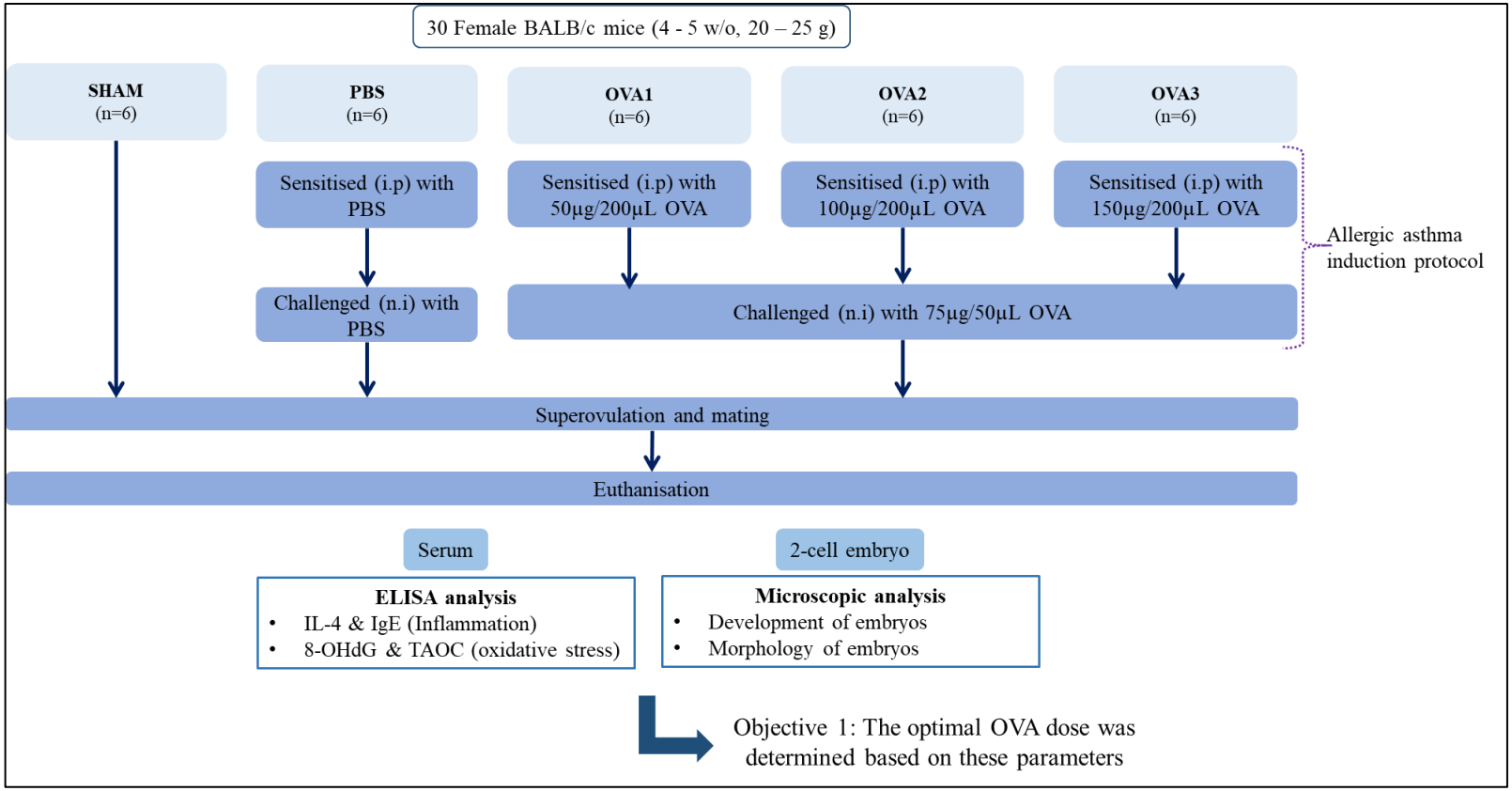


Figure 3.2 Experimental Design for Experiment I

This figure represents the procedures performed in Experiment I to determine the optimal dose of ovalbumin (OVA) to induce allergic asthma that adversely affects preimplantation embryo viability in female BALB/c mice. The protocol included intraperitoneal followed by intranasal challenges. Superovulation and mating were performed prior to sample collection. The outcomes assessed included maternal systemic biomarkers including embryonic development and morphology.

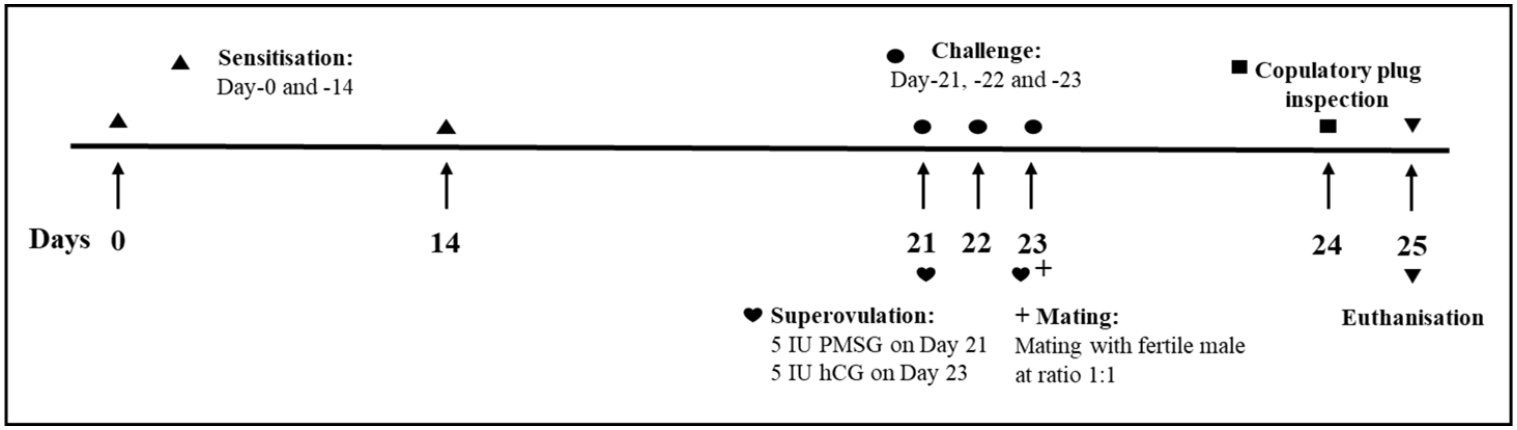


Figure 3.3 Experimental Timeline for Experiment I

This figure shows the timeline of key procedures in Experiment I, starting with OVA sensitisation (Days 0 and 14), intranasal OVA challenge (Days 21–23), hormonal stimulation for superovulation (Days 21 and 23), mating (Day 23), and sample collection at 1.5 days post coitum (Day 25). The timeline reflects the coordinated schedule for inducing allergic asthma and assessing early embryonic development.

3.7.2 Experiment II: Investigation of the Potential Protective Effects of Tocotrienol-rich Fraction supplementation

After establishing an optimal OVA dose of 150 µg/200 µL in Experiment I, Experiment II was conducted to investigate the protective effects of maternal TRF supplementation on maternal systemic responses and embryo viability in OVA-induced allergic asthma model.

Female BALB/c mice (aged 4–5 weeks, 20–25 g) were randomly divided into five groups as shown in Table 3.3: SHAM (untreated), PBS (control), OVA3 (optimal OVA dose), POVA (optimal OVA dose + palm olein supplementation) and TOVA (optimal OVA dose + TRF supplementation). In this experiment, asthmatic group supplemented with standard antioxidant or anti-inflammatory drug was not included as control because the primary objective of this study was to investigate the protective role of TRF on systemic effects and reproductive outcomes in allergic asthma. TRF has been reported to exert antioxidant and anti-inflammatory effects. Therefore, this study design focused on evaluating TRF's protective effects relative to both the asthmatic (OVA3) group and non-asthmatic control (PBS), providing clear insights into its mechanistic protective role without the need for a pharmacological comparator. Including a standard antioxidant or drug would require additional experimental groups and a separate study design which was beyond the scope of this study.

The TOVA group received oral TRF supplementation at a dose of 60 mg/kg body weight daily from Day 0 to 24 while the POVA group was given palm olein (stripped of vitamin E) as vehicle control. The 24 days supplementation was to ensure continuous systemic exposure throughout the sensitisation (Days 0 and 14) and challenge (Days 21–23) phases of the allergic asthma protocol. This duration allowed assessment of TRF effects across the complete course of immune activation and inflammatory exacerbation.

The selected TRF dose of 60 mg/kg body weight was adopted from previous *in vivo* murine studies conducted in non-asthmatic pathological model, which demonstrated its efficacy in attenuating systemic oxidative stress and preserving preimplantation embryonic development. (Kamsani et al., 2013).

In Experiment II, all groups underwent the same sensitisation and challenge protocol as described in Experiment I. Superovulation and mating were performed accordingly. Euthanasia was performed on Day 25 (1.5 days post coitum). Maternal

blood was collected for serum analyses and 2-cell embryos were harvested from the oviducts.

This experimental layout allowed for a comprehensive evaluation of maternal and embryonic outcomes under OVA-induced stress and TRF intervention. The procedure and timeline for Experiment II are shown in Figures 3.4 and 3.5 respectively.

The potential protective roles of TRF were investigated based on: 1) maternal systemic biomarkers, 2) embryonic development and morphological quality, 3) ultrastructural features and 4) gene expression. The experimental layout and procedures timeline for this phase are shown in Figures 3.4 and 3.5.

Table 3.3
Experimental Groups and Treatments in Experiment II

	Groups	**Treatment Description**
1.	SHAM	Untreated control
2.	PBS	Sensitised with PBS
3.	OVA3	Sensitised with 150µg/200 µL OVA
4.	POVA	Sensitised with 150 µg/200 µL OVA + Supplemented with palm olein
5.	TOVA	Sensitised with 150 µg/200 µL OVA + Supplemented with TRF (60 mg/kg bw)

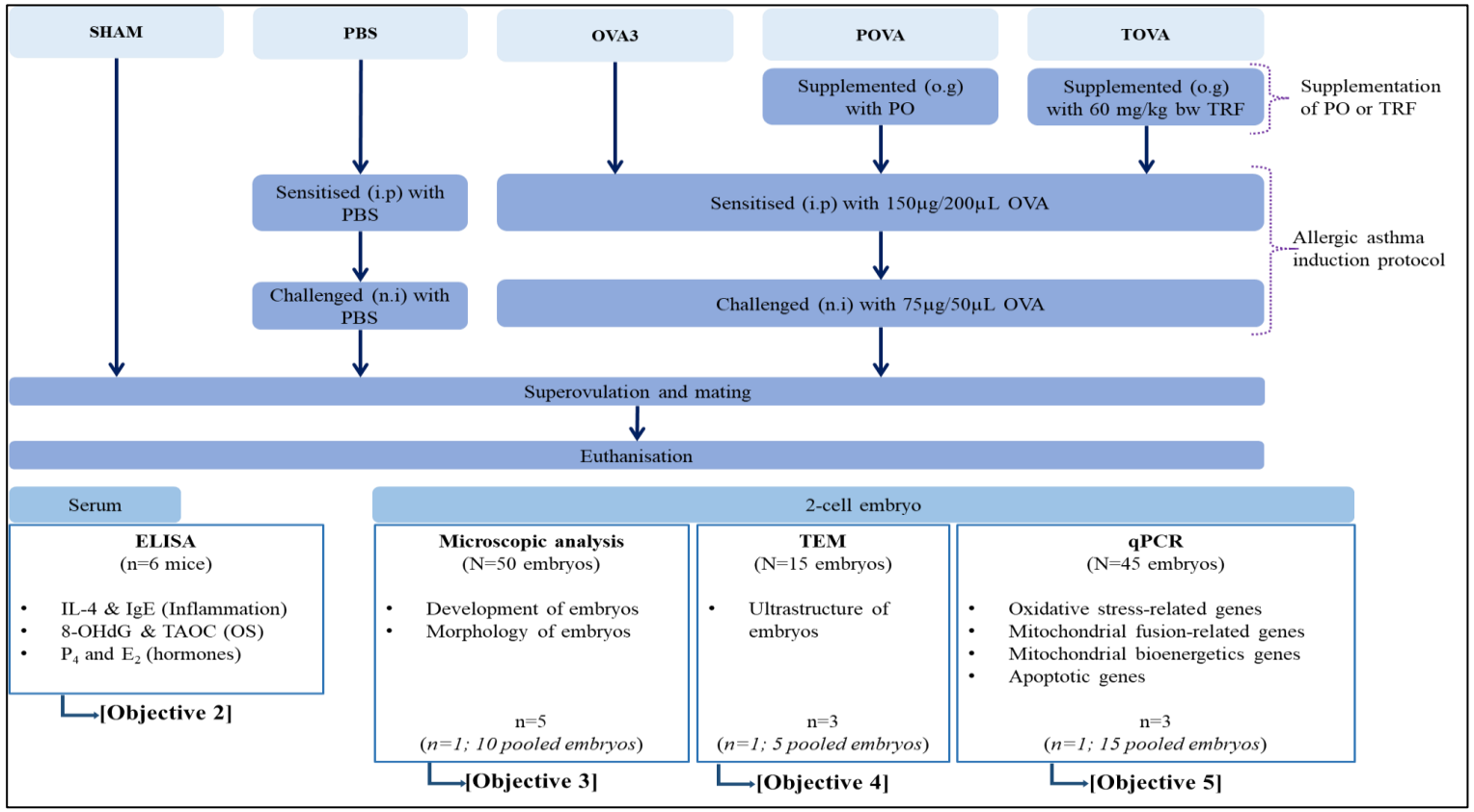


Figure 3.4 Experimental Design for Experiment II

This figure represents the procedures performed in Experiment II to investigate the protective effects of maternal TRF supplementation on maternal systemic responses and embryonic preimplantation outcomes in female BALB/c mice. The protocol included intraperitoneal followed by intranasal challenges. Superovulation and mating were performed prior to sample collection. The outcomes assessed included maternal systemic biomarkers including embryonic development, morphology, ultrastructural features and gene expression.

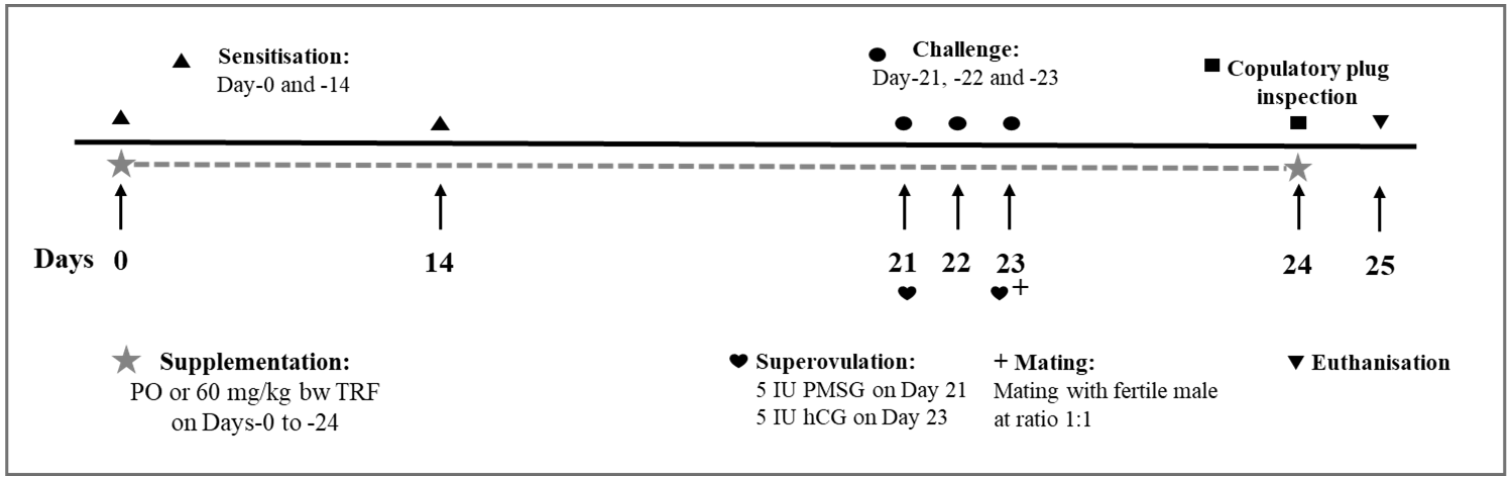


Figure 3.5 Experimental Timeline for Experiment II

This figure shows the timeline of key procedures in Experiment II, starting with administration of TRF or palm olein (Days 0–24), OVA sensitisation (Days 0 and 14), intranasal OVA challenge (Days 21–23), hormonal stimulation for superovulation (Days 21 and 23), mating (Day 23), and sample collection at 1.5 days post coitum (Day 25). The timeline reflects the coordinated schedule for inducing allergic asthma and assessing early embryonic development.

3.8 Induction of Allergic Asthma

To establish an *in vivo* model of allergic asthma, a sensitisation and challenge protocol was employed using OVA as the initiating allergen adapted from Paik et al., (2014), with modification of the OVA dosage. The OVA sensitisation supported by aluminium hydroxide [Al $(OH)_3$] as an adjuvant acted as the cause by priming the immune system to recognise OVA as a threat or inducer. Subsequent intranasal challenges served as the consequence by provoking airway inflammation and reproducing hallmark features of asthma such as immune hypersensitivity and respiratory distress. Throughout the procedure, anaesthesia and careful restraint minimised procedural stress acting as a protective defence to ensure animal welfare. Ultimately, this approach provided an integrated and reproducible model that closely mirrors human allergic asthma allowing investigation of maternal respiratory inflammation and its downstream effects. The control groups received PBS which was used as the vehicle for OVA. The summary of allergic asthma induction technique is summarised in Figure 3.6.

The systemic impact of this induced asthma was then evaluated through a series of serum biomarker assays covering maternal inflammation, oxidative stress, antioxidant defence and hormonal regulation.

3.8.1 Preparation of the Ovalbumin Sensitisation and Challenge Solutions

Three OVA concentrations (50, 100 and 150 µg in 200 µL PBS) were prepared for sensitisation. Each solution was prepared by dissolving 10, 20 or 30 mg OVA in 40 mL PBS and stored at –80 °C. Prior to injection, 1 mL of each solution was mixed with 20 mg Al $(OH)_3$ and vortexed thoroughly. The challenge solution (75 µg in 50 µL PBS) was prepared by dissolving 30 mg OVA in 20 mL PBS and stored at –80 °C until use.

3.8.2 Restraint and Anaesthesia

Mice were restrained by holding one hand at the base of the tail and using the thumb and forefinger of the other hand to stabilise the neck. This upright posture

minimised stress and allowed accurate intraperitoneal injection or intranasal administration.

Anaesthesia was achieved with a combination of ketamine (100 mg/mL) and xylazine (20 mg/mL). The xylazine stock of 100 mg/mL was first diluted at ratio 1:4 in PBS to produce 20 mg/mL dose. A working solution was prepared by mixing 9.0 mL of ketamine with 1.0 mL of diluted xylazine and 30 mL of PBS. Each mouse received 0.1 mL of this mixture per 20 g body weight by intraperitoneal injection. With proper restraint and anaesthesia technique, it ensuring both precision and welfare of animals to undergo sensitisation and challenge procedures.

3.8.3 Intraperitoneal Sensitisation Protocol

On Days 0 and 14, mice were sensitised with 200 µL of OVA–aluminium hydroxide solution via intraperitoneal injection (i.p). The injection site was located in the lower right abdominal quadrant to avoid internal organs. Control animals received PBS in equivalent volumes (200 µL). Following sensitisation, mice underwent intranasal (n.i) challenge to reproduce airway inflammation.

3.8.4 Intranasal Challenge Protocol

On Days 21, 22, and 23, anaesthetised mice received 50 µL of the OVA challenge solution intranasally (n.i) using a micropipette. The solution was administered dropwise into each nostril while the mouse was supine ensuring even delivery. Animals were closely monitored until full recovery from anaesthesia. This respiratory challenge provoked allergen-specific airway inflammation completing the induction of the allergic asthma model. Although this protocol is known to induce features consistent with acute exacerbation of allergic asthma (AEBA), this study did not specifically assess pulmonary functional impairment, as the primary objective was to evaluate systemic inflammation, oxidative stress, and reproductive outcomes. Therefore, physiological parameters such as respiratory rate, blood pressure, and oxygen saturation were not monitored. In addition, mice were anaesthetised during the intranasal challenge, which could alter cardiorespiratory measurements and confound the interpretation of such physiological assessments.

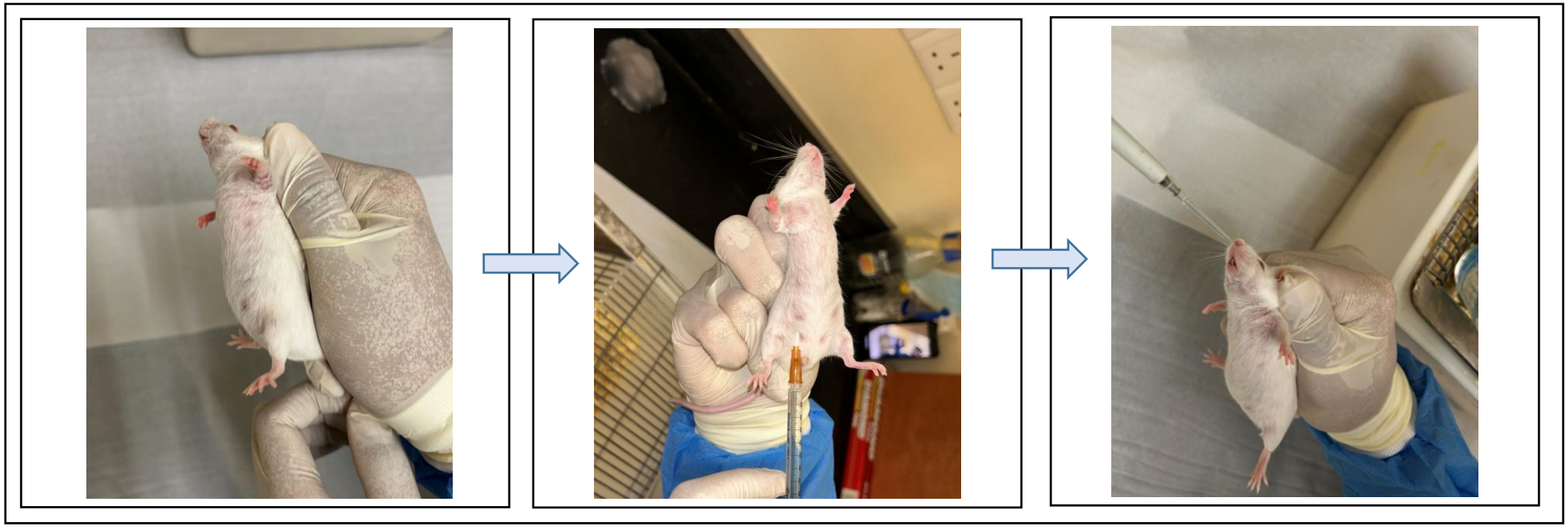


Figure 3.6 Allergic Asthma Induction Technique in Mice
This figure represents the overview of the procedures used to induce allergic asthma in BALB/c mice. Initially, proper restraint technique was used to stabilise the mouse during the procedures. Then, mouse underwent intraperitoneal (i.p.) injection of ovalbumin (OVA) mixed with aluminium hydroxide for systemic sensitisation on Days 0 and 14. Next, the mice underwent intranasal (i.n.) instillation of OVA challenge solution under anaesthesia on Days 21 to 23 to induce airway inflammation.

3.9 Superovulation and Mating

Superovulation is a hormonal strategy aimed at increasing the number of mature oocytes released during ovulation and thus improving the yield of embryos for subsequent analysis. The protocol adapted from Luo et al., (2011) uses pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The PMSG mimics the action of follicle stimulating hormone (FSH) to promote follicular growth while hCG mimics luteinising hormone (LH) to trigger final oocyte maturation and ovulation. If mating occurs after hCG administration, ovulation usually takes place within ~12 hours ensuring precise synchronisation of fertilisation and early embryonic development.

3.9.1 Preparation of Hormonal Solutions

The hormones PMSG and hCG were prepared from commercial stocks according to the manufacturer's instructions (Merck Animal Health). Final working concentrations of 5 IU per 0.1 ml were prepared and administered intraperitoneally (i.p) at the scheduled time points. Detailed preparation protocols can be found in Appendix C. These prepared solutions were then used for the timed hormone injections to induce superovulation.

3.9.2 Hormonal Injections and Timing

Superovulation was induced by two consecutive intraperitoneal injections as summarised below. These times were chosen to optimise follicular growth and ensure synchronous ovulation. After induction of ovulation, females were paired with fertile males to allow natural mating.

- Day 21: 0.1 ml of 5 IU PMSG administered between 17:00–18:00.
- Day 23: 0.1 ml of 5 IU hCG, administered 46–48 hours after PMSG, between 15:00–16:00.

3.9.3 Mating Procedure and Copulatory Plug Inspection

After hCG injection, each hormonally primed female was paired in a 1:1 ratio with a fertile male and housed overnight. Prior to mating, males were caged individually for one week by housing them singly in clean cages to stabilize their physiology, reduce stress-induced aggression and optimize fertility performance (The Jackson Laboratory, 2009). The next morning (Day 24), successful mating was confirmed by inspecting the vaginal opening for a copulatory plug, a coagulated seminal vesicle secretion (Behringer et al., 2016). The presence of a whitish plug indicated successful copulation and was designated as day 0 post coitum (d.p.c.), a standard embryological convention counting days from mating, starting at 0.5 d.p.c. on the noon the plug was detected. Plug detection marked the starting point for monitoring pregnancy and subsequent embryo retrieval (Behringer et al., 2016).

3.10 Supplementation of Tocotrienol-rich Fraction

Tocotrienol-rich fraction, a potent natural antioxidant derived from palm oil was used to investigate its protective effect against oxidative stress and inflammation induced by allergic asthma in female mice. The TRF was kindly provided by Sime Darby Research in collaboration with the Malaysian Palm Oil Board (MPOB).

The mice in the TOVA group received TRF supplementation while the mice in the POVA group received palm olein (a tocotrienol-free control). Both groups underwent the OVA-induced allergic asthma protocol as described in the previous sections. TRF or palm olein was administered once daily via oral gavage from Day 0 to 24 at a dose of 60 mg/kg body weight. The dosage was adjusted according to the final body weight of each mouse to ensure accuracy. Oral gavage was chosen as the route of administration to simulate dietary supplementation with antioxidants.

A detailed description of the preparation of the TRF and palm olein solutions including protocols for storage, dilution and handling can be found in Appendix D.

3.10.1 Oral Administration of Palm Olein and Tocotrienol-rich Fraction

Prior to oral administration, each mouse was weighed to determine the correct gavage volume. The maximum permissible volume for oral gavage in mice is 10 mL/kg.

However, a safer working volume of 5 mL/kg (equivalent to ~0.1–0.125 mL for a 20–25 g mouse) was used as recommended by Turner et al., (2011). Larger volumes may increase the risk of aspiration pneumonia due to reflux and cause rapid passage of TRF or palm olein into the duodenum.

To minimise the risk of gastrointestinal injury, an appropriate gavage needle was chosen. For 20–25 g mice, the recommended size is 20 G with a length of 1.5 inches (Institutional Animal Care and Use Committe, Washington State Universisty, 2021). The mice were first gently restrained and the head was stretched with thumb and forefinger to align the oesophagus. A gavage needle attached to a 1.0 mL syringe was carefully inserted into the oral cavity and guided into the oesophagus as shown in Figure 3.7. Once correctly positioned, the syringe plunger was slowly depressed to administer the palm olein or TRF solution. The needle was then withdrawn at the same angle as during insertion.

After administration, the mice were observed for 5–10 minutes to detect immediate signs of distress or dyspnoea. Further observations were performed 12–24 hours later to look for delayed complications such as respiratory distress or signs of perforation of the oesophagus or stomach. This supplementation protocol provided a controlled approach to evaluate the systemic effects of TRF in the allergic asthma model and formed the basis for subsequent assessment of maternal biomarkers and embryonic outcomes.

Figure 3.7 Oral Gavage Technique for Tocotrienol-rich Fraction or Palm Olein Supplementation
This figure shows the oral administration procedure for the daily administration of the tocotrienol-rich fraction (TRF) or palm olein to female mice. The dose was administered directly into the stomach using a 1 mL syringe with a stainless-steel needle. The mice were gently restrained to ensure safe and accurate administration with minimal stress

3.11 Euthanasia for Serum and Oviducts Collection

Female mice were weighed and anaesthetised using a high dose of ketamine–xylazine solution (0.2 mL per 20 g body weight, administered intraperitoneally). Once full anaesthesia was confirmed by the absence of pedal reflexes, cardiac puncture was performed using a 25 G needle attached to a 1 mL syringe to collect whole blood (Figure 3.8).

The blood was allowed to clot at room temperature for 30 minutes then centrifuged at 2000 rpm for 15 minutes at 4 °C. The resulting serum was aliquoted into sterile microcentrifuge tubes and stored at –80 °C for subsequent biochemical analyses.

After blood collection, the mice were euthanised by cervical dislocation. A midline laparotomy was performed under aseptic conditions to expose the pelvic cavity. The uterine horns which are paired tubular structures extending bilaterally from the uterine body toward the ovaries and connected to the oviducts were carefully excised. The oviducts characterised by slender coiled tubes located at the distal end of the uterine horns near the ovaries were carefully isolated (Figure 3.9). The isolated oviducts were then prepared for oviductal flushing to retrieve 2-cell embryos.

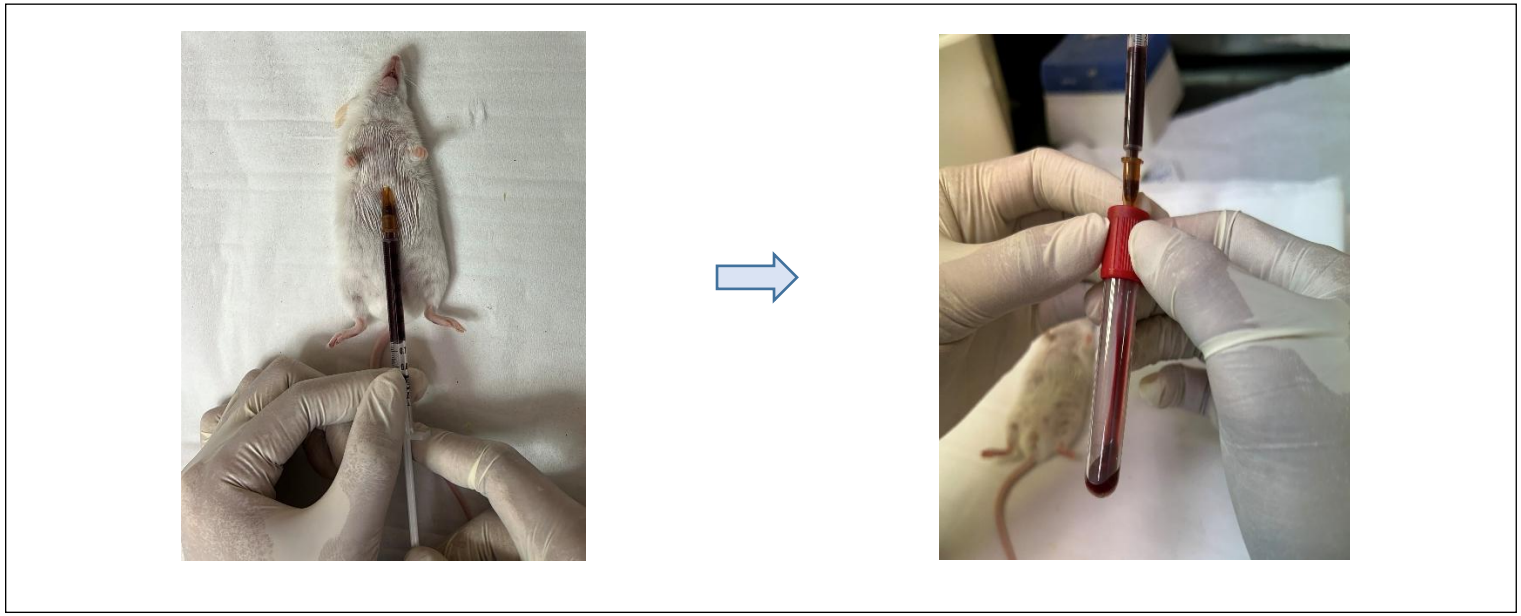


Figure 3.8 Cardiac Puncture Technique for Blood Collection

This figure shows the cardiac puncture procedure used for blood collection in female mice. Collected blood was transferred into serum collection tube. The blood was centrifuged to get serum and the serum was then stored at –80 °C for subsequent biochemical analyses

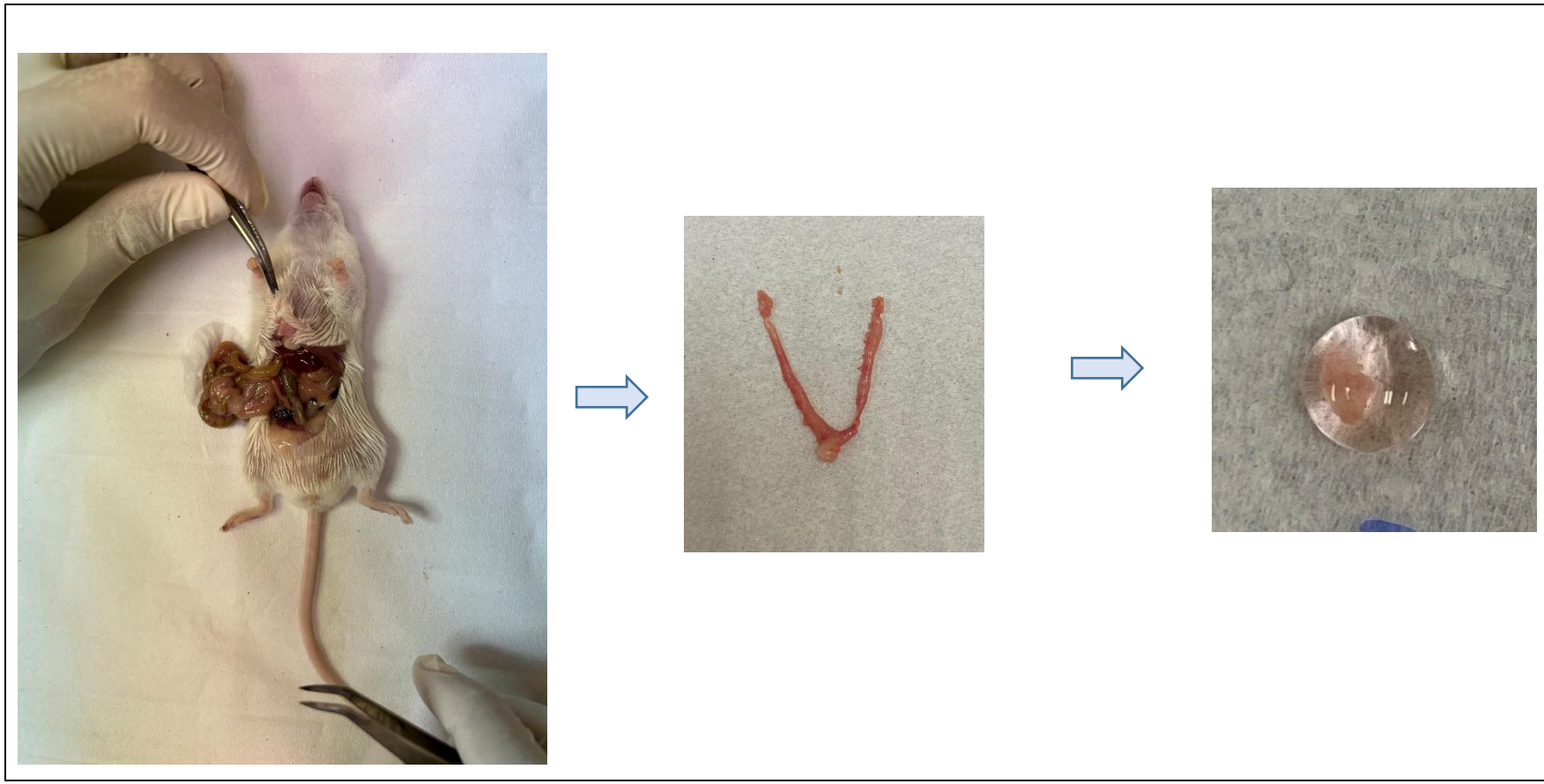


Figure 3.9 Laparotomy Technique for Oviducts Collection
This figure shows the mouse undergoing laparotomy to expose pelvic cavity. The location of uterine horns in the pelvic cavity was located and then excised. Next, the oviducts were isolated from the uterine horns.

3.12 Maternal Evaluation

Blood samples were taken on Day 25 equivalent to 1.5 days post coitum (d.p.c.) in order to investigate maternal physiological responses on maternal inflammation, oxidative stress, antioxidant capacity and hormone regulation.

3.12.1 Enzyme-linked Immunosorbent Assay (ELISA)

To provide a comprehensive picture of the maternal systemic environment, the assays were arranged to reflect the biological cascade that shapes pregnancy. Inflammation was examined first as immune activation represents an early signal that can drive downstream changes. This was followed by measurement of oxidative stress, a key consequence of persistent inflammation that leads to cellular and molecular damage. To balance this, maternal antioxidant capacity was assessed representing the body's natural defence against such oxidative insults. Finally, the assessment turned to pregnancy hormones which integrate immune, oxidative and antioxidant influences to sustain implantation and foetal development. This order reflects the interconnected nature of maternal adaptation moving from cause to consequence through defence and culminating in hormonal regulation.

3.12.1.1 Maternal Inflammation (Interleukin-4 and Immunoglobulin E)

To assess the maternal inflammatory profile, serum levels of IL-4 and IgE were measured as indicators of Th2-driven immune activity using commercial ELISA kits. The assays followed the Sandwich-ELISA principle where a 96-well plate pre-coated with specific antibodies captures the target analytes.

Standard working solutions were prepared by serial dilution from a 2000 pg/mL stock solution to obtain a range of concentrations (2000–0 pg/mL). Biotinylated Detection Antibodies and Horseradish Peroxidase (HRP) Conjugates were prepared by diluting the concentrated reagents at a 1:99 ratio, and the Wash Buffer was diluted and pre-warmed to 40 °C to prevent crystallization.

For the assay, 100 µL of standards and samples were added to the wells in duplicate and incubated at 37 °C for 90 minutes. After washing, Biotinylated Detection Antibody, HRP Conjugate and Substrate Reagent were sequentially added with

respective incubation and washing steps. Finally, Stop Solution was added and absorbance was measured at 450 nm using a microplate reader.

The results were calculated by plotting a standard curve of corrected optical density (OD) values (average OD minus the 0 pg/mL standard) against the known concentrations and the sample concentrations were determined by interpolation from this curve. Detailed reagent preparation steps, sample handling, dilution protocols and the full assay procedure are provided in Appendix E. As inflammation can initiate oxidative processes, the subsequent assay focused on oxidative stress.

3.12.1.2 Maternal Oxidative Stress (8-hydroxy-2'-deoxyguanosine)

Maternal oxidative stress status was evaluated by quantifying serum levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) a sensitive biomarker of oxidative DNA damage. The concentration of 8-OHdG was measured using a commercial 8-OHdG ELISA kit based on the Competitive-ELISA principle. In this method, the 96-well plate is pre-coated with 8-OHdG and sample or standard competes with the immobilized antigen for binding to the Biotinylated Detection Antibody.

Standard working solutions were prepared by serial dilution of a 100 ng/mL stock to obtain concentrations ranging from 100 to 0 ng/mL. Reagents such as the Biotinylated Detection Antibody and HRP Conjugate were prepared by diluting concentrated stocks at a 1:99 ratio and Wash Buffer was prepared as described in the earlier section.

For the assay, 50 µL of standard solutions and samples were added to the wells in duplicate followed by 50 µL of Biotinylated Detection Antibody. The plate was incubated at 37 °C for 45 minutes and washed three times. Then, 100 µL of HRP Conjugate was added and the plate was incubated for another 30 minutes before undergoing five rounds of washing. Subsequently, 90 µL of Substrate Reagent was added in the dark and incubated for 15 minutes followed by the addition of 50 µL Stop Solution. Absorbance values were measured at 450 nm using a microplate reader.

The data were analysed by averaging the OD readings of each standard and sample. A standard curve was plotted with corrected OD readings (y-axis) against known concentrations of 8-OHdG (x-axis) and the sample concentrations were interpolated from the resulting curve. Detailed reagent preparation steps, sample handling, dilution protocols and the full assay procedure are provided in Appendix F.

Because oxidative damage must be counterbalanced by protective mechanisms, the next assay examined maternal antioxidant capacity.

3.12.1.3 Maternal Antioxidant Capacity (Total Antioxidant Capacity)

The overall antioxidant defence was determined by measuring total antioxidant capacity (TAOC) in serum using a commercial colorimetric kit based on the ferric-reducing ability of plasma (FRAP) method. In this assay, antioxidants reduce the ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to its ferrous form (Fe^{2+}-TPTZ), producing a blue chromophore proportional to overall antioxidant power as shown in Figure 3.10 (Rubio et al., 2016). The FRAP working reagent was prepared by combining 10× Buffer, TPTZ and Substrate Solutions in a 10: 1: 1 ratio and protected from light. A 100 mM $FeSO_4 \cdot 7H_2O$ stock was serially diluted (0–2.5 mM) to generate calibration standards.

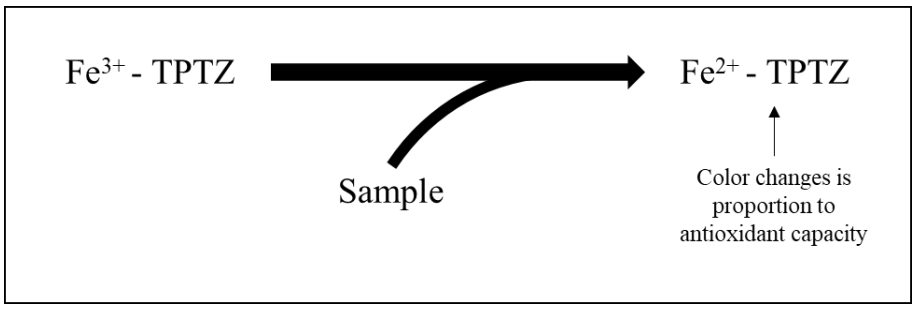


Figure 3.10 Ferric Reducing Ability of Plasma (FRAP) Method

For the assay, 5 µL of each standard or serum sample was pipetted into duplicate wells followed by 180 µL of FRAP reagent. Plates were incubated at 37 °C for 5 min and absorbance was recorded at 595 nm using microplate reader. The TAOC values were derived from a standard curve of absorbance versus $FeSO_4$ concentration; sample antioxidant capacity (mM) was calculated as:

$$TAOC\ (U/mL) = \left[\left(OD_{sample} - OD_{blank}\right) - \ std\ curve's\ intercept\right] \div \text{std curve's slope} \times Dilution\ factor$$

Detailed reagent preparation steps, sample handling, dilution protocols, and the full assay procedure are provided in Appendix G. Since oxidative stress and antioxidant balance can influence endocrine function, hormone levels were also evaluated.

3.12.1.4 Maternal Pregnancy Hormones (Progesterone and Oestrogen)

Maternal serum progesterone (P_4) and oestrogen (E_2) concentrations were quantified using commercial competitive-ELISA kits. In this format, endogenous hormone in the sample competes with plate-bound hormone for binding to a Biotinylated Detection Antibodies. Calibration curves were prepared from serial dilutions of a 20 ng/mL P_4 stock (0–20 ng/mL) and a 600 pg/mL E_2 stock (0–600 pg/mL). All ancillary reagents (Biotin-antibody, HRP Conjugate and Wash Buffer) were prepared according to the manufacturer guidelines.

For each analyte, 50 µL of standards or serum samples were added in duplicate to the pre-coated 96-well plates followed immediately by 50 µL of Biotinylated Antibodies. After 45 min incubation at 37 °C, wells were washed three times, incubated with 100 µL HRP Conjugate for 30 min, washed five times, and developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate for 15 min in the dark. The reaction was stopped with 50 µL of Stop Solution and absorbance was recorded at 450 nm using microplate reader.

Hormone concentrations were calculated by averaging duplicate OD, subtracting the zero-standard value, and interpolating from the respective standard curves (OD versus concentration). Final values were expressed in ng/mL (P_4) and pg/mL (E_2) after accounting for any sample dilution. Detailed reagent preparation steps, sample handling, dilution protocols and the full assay procedure are provided in Appendix H.

3.13 Embryo Assessment

Two-cell embryos were taken on Day 25 which was 1.5 d.p.c in order to investigate the embryonic outcomes (developmental potential and morphological quality, ultrastructural features, and gene expression). Each group provided approximately 110 pooled 2-cell embryos which were divided as follows:

i. 50 embryos: microscopic analysis
ii. 15 embryos: transmission electron microscopy
iii. 45 embryos: fluidigm qPCR (*n = 1; 15 pooled embryos*)

3.13.1 Microscopic Analysis

The microscopic analysis works involved several procedures starting with oviductal flushing → embryo handling → embryo collection → embryo culture → developmental progress and morphological quality assessment.

3.13.1.1 Oviductal Flushing, Embryo Handling and Collection

Oviducts were flushed with 0.5 mL of pre-warmed M2 medium using a sterile 32G needle attached to a 1 mL syringe filled with M2 medium was inserted into the infundibulum to flush the embryos together with the cellular debris. To ensure clean collection, the embryos were washed with 10 drops of fresh M2 medium in series using a micropipette. All procedures were conducted on a heated stage to maintain physiological temperature and minimize stress to the embryos.

All procedures were carried out under aseptic conditions in a laminar flow hood to ensure the sterility and viability of the embryos. Figure 3.11 shows the overview of oviductal flushing technique until embryonic assessment.

3.13.1.2 Embryo Culture

The M16 medium was selected for the embryo culture as it provides essential energy sources such as pyruvate and lactate. One day before culture (Day 24 or 0.0 d.p.c), four 100-µl drops of M16 medium were placed in a 35-mm culture petri dish and covered with paraffin oil to prevent evaporation. The prepared dish was equilibrated overnight at 37 °C in a 5 % CO_2 incubator.

On Day 25, washed 2-cell embryos were transferred to equilibrated M16 medium droplets under a stereomicroscope and cultured from Day 1.5 to Day 5.0 d.p.c. at 37 °C and 5% CO_2. This *in vitro* system supported the development of the

preimplantation stages. The growth and quality of the embryos were subsequently evaluated by daily morphological assessment and developmental scoring.

3.13.1.3 Developmental Progress and Morphological Quality Assessment

Embryo development was monitored daily using an inverted microscope connected to PixeLINK software. Morphology was scored according to the Association for the Study of Reproductive Biology (ASEBIR) scoring system (Prados et al., 2012; Saiz et al., 2018), which categorises embryos as normal or abnormal based on stage-specific criteria. This scoring system was applied from the 2-cell stage to the blastocyst stage. The detailed criteria can be found in Table 3.4 and Appendix I. These evaluations ensured a systematic assessment of developmental progress and morphological quality under *in vitro* conditions.

Table 3.4
Assessment Time and Criteria for Embryonic Scoring

Embryonic stage	**Assessment time**	**Criteria**
2-cell 8-cell	1.5 d.p.c 2.5 d.p.c	▪ Stage-specific characteristics: ▪ Number of blastomeres ▪ Size of blastomeres ▪ Degree of fragmentation
Morula	3.0 d.p.c	▪ Cavitation and compaction activity ▪ Degree of fragmentation
Blastocyst	4.0 d.p.c	▪ Degree of expansion of the blastocoel ▪ Characteristics of trophectoderm (TE) cells and Inner Cell Mass

This table summarises the outline of the morphological assessment criteria and the corresponding developmental time points for preimplantation mouse embryos, based on Saiz et al., (2018) and Prados et al., (2012). Embryos were categorised as normal or abnormal based on stage-specific characteristics (number and symmetry of blastomeres), compaction and cavitation activity, expansion of the blastocoel, morphology of trophectoderm (TE) and inner cell mass (ICM), and the degree of cytoplasmic fragmentation. Fragmentation of more than 25% was considered abnormal. d.p.c: day post coitum.

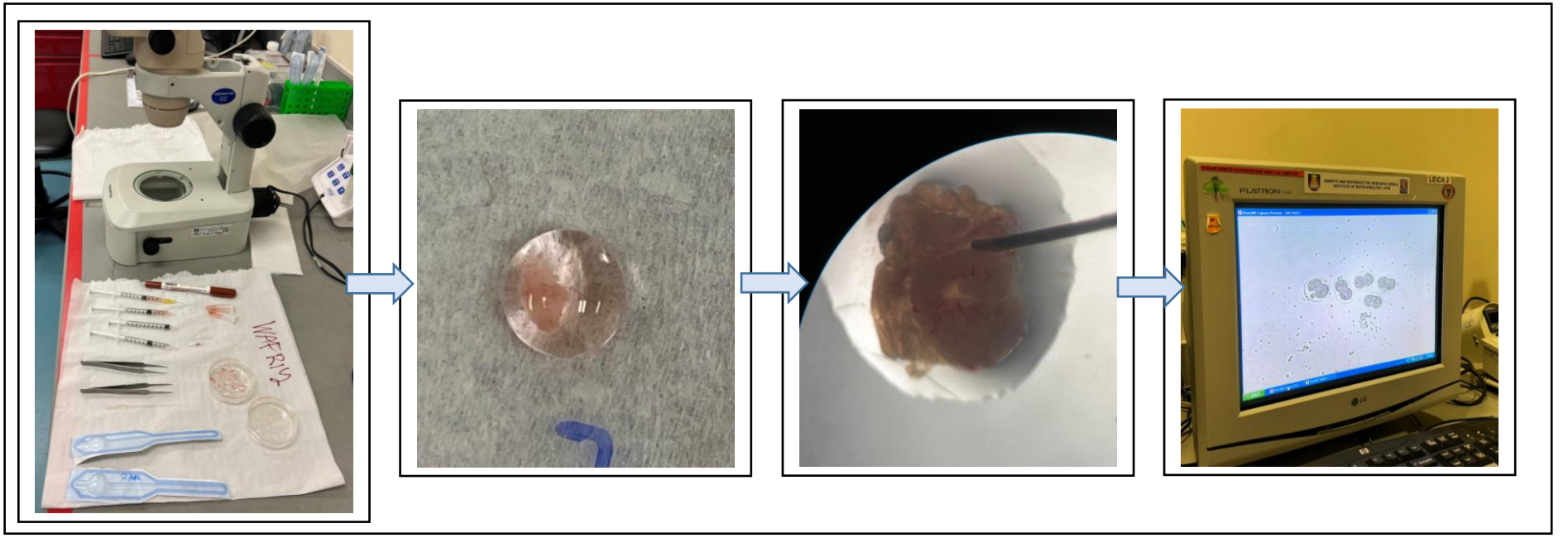


Figure 3.11 Overview of Oviductal Flushing Technique until Microscopic Analysis
This figure shows the apparatus and equipment needed for oviductal flushing and overview of oviductal flushing technique starting with isolation of oviducts followed by flushing at infundibulum. Next, daily microscopic analysis using an inverted microscope was conducted from 1.5 to 5.0 days post coitum.

3.13.2 Transmission Electron Microscopy (TEM)

TEM was used to analyse the ultrastructural features of 2-cell embryos derived from OVA-induced allergic asthma model. The aim of this analysis was to characterise ultrastructural changes associated with maternal inflammation and oxidative stress. In parallel, the potential protective effects of maternal TRF supplementation on the ultrastructure of the 2-cell embryo were also investigated.

3.13.2.1 Preparation of Chemicals

To prepare for TEM, several reagents were prepared or diluted to the appropriate working concentrations in preparation for transmission electron microscopy. The reagents for primary and post-fixation were 0.2 M sodium cacodylate buffer, 2.5 % glutaraldehyde solution and 1 % osmium tetroxide solution. For dehydration, acetone solutions of 35 %, 50 %, 75 %, 95 % and 100 % were prepared by diluting 100 % acetone with Millipore water.

The resin mixture for infiltration and embedding was prepared using the Agar 100 Resin Kit by mixing 10 mL of Agar 100 resin, 6 mL of Dodecenyl Succinic Anhydride (DDSA), 5.5 mL of Methyl Nadic Anhydride (MNA) and 0.5 mL of Benzyl Dimethylamine (BDMA). Staining reagents included ready-to-use uranyl acetate and freshly prepared lead citrate. A detailed description of reagent preparation can be found in Appendix J.

3.13.2.2 Primary and Post-Fixation

A volume of 2.5% glutaraldehyde was added to a well of a 12-well plate. Five embryos were placed in the fixative and the plate was wrapped in aluminium foil to protect from light. The embryos were incubated at 4 °C for 12–24 hours. During this fixation, glutaraldehyde cross-linked the proteins by binding to nucleophilic sites particularly to abundant amino groups, thereby preserving the embryonic structure (Tizro et al., 2019).

After primary fixation, the embryos were washed in 0.1 M cacodylate buffer at room temperature for 10 minutes. This step was repeated three times to remove excess glutaraldehyde. The embryos were then transferred to 1% osmium tetroxide in

cacodylate buffer and incubated at 4 °C for 1–2 hours. Osmium tetroxide interacts with lipid components in cell membranes, enhances membrane contrast and stabilises macromolecular structures. Fixation was followed by a further series of three 10-minute washes in cacodylate buffer at room temperature.

3.13.2.3 Dehydration

After fixation, the embryos were dehydrated by a graded acetone series: 35%, 50%, 75%, 95% and 100% (Table 3.5). The first steps of dehydration were performed in a 12-well plate. The embryos were immersed in 35% and 50% acetone for 10 minutes each, then transferred to 75% acetone and left overnight.

The next day, dehydration was continued in the radiation capsules. Before use, the capsules were coated with 10 µL of poly-L-lysine, air dried in a fume hood, and left overnight at room temperature. The capsules were then rinsed with distilled water and filled with 75% acetone. Five embryos were pipetted into each capsule and processed as follows:

- 75% acetone was removed by tilting the capsule at a 45° angle
- 95% acetone was added and left for 5 minutes
- 100% acetone was added for 10 minutes and repeated three

The loss of the embryo was monitored at each step to ensure the integrity of the sample. Complete dehydration was essential to prevent collapse during electron microscopy due to the vacuum.

Table 3.5
Acetone Dehydration Steps

Acetone concentration	**Duration**	**Container**
35%	10 min	12-well plate
50%	10 min	12-well plate
75%	Overnight	12-well plate
95%	5 min	Beam capsule
100%	10 min ×3	Beam capsule

3.13.2.4 Infiltration, Embedding and Polymerization

After dehydration, the embryos were subjected to resin infiltration to replace the remaining liquid with an embedding medium. A 1:1 mixture of 100% acetone and epoxy resin was added to each capsule and allowed to stand for 1 hour. This was followed by a 1:3 mixture (acetone: resin) for 2 hours. Finally, the embryos were immersed in 100% resin for a further 2 hours. After infiltration, the capsules were placed in an oven at 60 °C for 42–48 hours to allow the resin to polymerise. The fully polymerised resin blocks were then ready for sectioning.

3.13.2.5 Block Trimming and Ultrathin Sectioning

The polymerised blocks containing the embryos were cut into a trapezoidal shape using an ultramicrotome equipped with a diamond knife to expose the embedded samples (Figure 3.12A). Ultra-thin sections (60–80 nm) were made to allow penetration of the electron beam. The sections floated on the water in the knife trough and were carefully collected with a loop and placed on a 200-mesh copper grid in preparation for staining.

3.13.2.6 Copper Grid Staining

The sections mounted on copper grids were stained with uranyl acetate and lead citrate to increase contrast for TEM imaging. One drop of uranyl acetate was applied to each grid and incubated for 10 minutes at room temperature followed by five rinses in double distilled water. A drop of lead citrate was then applied and left for 10 minutes. To prevent precipitation of lead citrate, sodium hydroxide (NaOH) pellets were added to the staining dish to absorb atmospheric CO_2. The grids were rinsed again in double distilled water, dried with filter paper and allowed to air dry before being stored in a grid case.

3.13.2.7 Embryo Visualisation

Stained grids were examined with a transmission electron microscope at 80–120 kV (Figure 3.12 B). Microscopic images were taken at different magnifications which are low (4,200×), moderate (8,200×) and high (20,500–23,000×). These detailed imaging steps enabled a comprehensive ultrastructural analysis of the embryonic cells which is summarised in Table 3.6.

Table 3.6
Transmission Electron Microscopy Magnification Levels and Structural Features Assessed

Magnification	**Structural Features Assessed**
Low (4,200×)	To assess overall organelle distribution.
Moderate (8,200×)	To observe mitochondrial clusters, microvilli, perivitelline space, and features of cellular degradation
High (20,500×–23,000×)	To visualize fine mitochondrial structures including membranes, cristae, and matrix.

This table explains structural features assessed at each magnification in the ultrastructural study of murine 2-cell embryos. Each magnification level was selected to analyse specific structural details ranging from general organelle distribution to fine mitochondrial morphology.

3.13.2.8 Ultrastructural Assessment

The ultrastructural assessment of the embryo sections was carried out using the ImageJ software (available at http://rsbweb.nih.gov/ij/). Both quantitative and qualitative analyses were performed to assess the structure and distribution of the organelles.

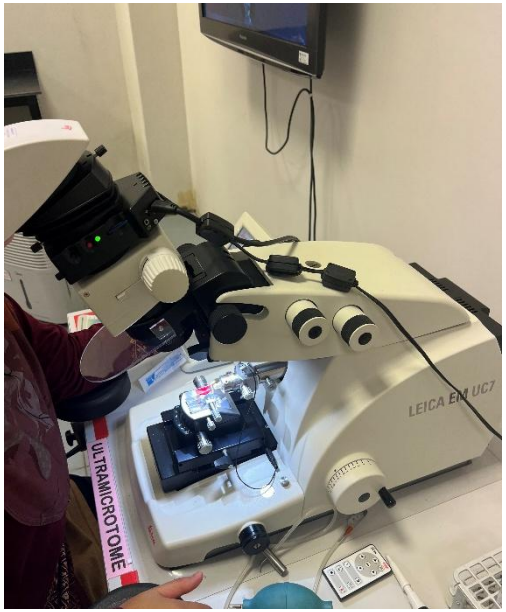
For the quantitative analysis, a stereological approach known as counting technique was used to estimate the relative volume (Vv) of the target organelles. The Vv refers to the proportion of the cytoplasmic volume occupied by a specific organelle which was expressed as percentage. It provides a quantitative estimate of how much space a targeted organelle occupies relative to the total cytoplasmic area. Using ImageJ, a stereological grid was superimposed on the transmission electron micrographs. The number of intersection points overlying the organelles of interest was counted and

compared to the total number of points over the cytoplasm. This method allowed estimation of the proportional volume occupied by specific organelles in the cytoplasm. The Vv was calculated using the following formula:

Vv (*targeted organelle*) = [number of dots (*organelle*)/number of dots (*cytoplasm*)] ×100 (%)

In addition to the quantitative analysis, a qualitative assessment of the morphology of the organelles was also carried out. Structural features such as the shape, integrity and internal organisation of the organelles especially the mitochondria were visually assessed using ImageJ. This allowed the identification of ultrastructural abnormalities or degeneration associated with pathological conditions.

A)



B)

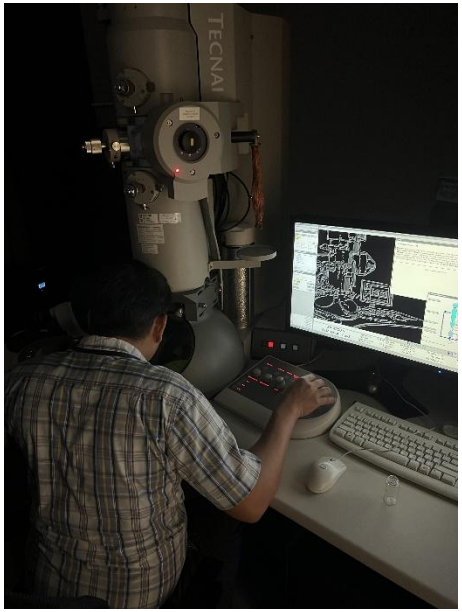


Figure 3.12 Ultramicrotome and Transmission Electron Microscope

This figure shows the equipment used in transmission electron microscopy. A) Ultramicrotome (Leica EMUC7) used for block trimming and ultrathin sectioning. B) Transmission electron microscope (TECNAI G2) used for embryo visualisation.

3.13.3 Fluidigm qPCR

To assess gene expression in 2-cell embryos, qPCR was performed using the Fluidigm microfluidic platform known as Fluidigm Biomark HD System (Figure 3.13). This high-throughput system enables simultaneous analysis of multiple genes from low-throughput samples with high sensitivity and precision. In this study, genes involved in oxidative stress response (*Nox1, Sod1, Gpx4*), mitochondrial fusion (*Opa1, Mfn1, Mfn2*), mitochondrial bioenergetics (*Atp5e, Cox4i1*) and apoptosis regulation (*Bax, Bcl2*) were analysed.

3.13.3.1 Embryo Processing

Two-cell embryos were pooled to 15 embryos per biological replicate (n = 1). A total of 45 embryos per experimental group (225 embryos in total) were processed. Under a stereomicroscope, embryos were rinsed three times in PBS to remove M2 medium and then transferred to a 10 µL droplet of Acid Tyrode's solution for 1–2 min until the zona pellucida dissolved. Denuded embryos were additionally washed three times in PBS and immediately prepared for the next steps.

3.13.3.2 Embryo Storage

The embryos were placed in 200 µL nuclease-free PCR tubes with 1 µL resuspension buffer. Using a 135 µm EZ-squeeze plastic pipette, 15 embryos per tube were transferred, frozen in liquid nitrogen (1–2 min) and stored at –80 °C until processing.

3.13.3.3 Primer Design

Forward and reverse primers for 2 reference genes (*Actb, Gapdh*) and 10 target genes (*Nox1, Sod1, Gpx4, Mfn1, Mfn2, Opa1, Atp5e, Cox4i1, Bax, Bcl2*) were designed with D3 Assay Design and synthesised by Fluidigm, USA. The primer sequences are listed in Table 3.7. Stock oligonucleotides were supplied at the manufacturer's standard concentration and diluted to the working concentration immediately prior to use.

Table 3.7
List of Primer Sequence for Reference and Targeted Genes

Gene symbol	NCBI ID	Sequence (5' → 3')
Actb	NM_007393	Forward: CCCTAAGGCCAACCGTGAAA Reverse: AGCCTGGATGGCTACGTACA
Gapdh	NM_008084	Forward: CAAGGTCATCCCAGAGCTGAA Reverse: CAGATCCACGACGGACACA
Nox1	NM_172203	Forward: GTGCCGACAACAAGCTCAAA Reverse: GCAAAGGCACCTGTCTCTCTA
Sod1	NM_011434	Forward: CTCACTCTCAGGAGAGCATTCC Reverse: TTCCACCTTTGCCCAAGTCA
Gpx4	NM_001367995	Forward: TTACGAATCCTGGCCTTCCC Reverse: TAGCCGGCTGCAAACTCC
Opa1	NM_133752	Forward: GACTGACAAGCAGCTTCCTAA Reverse: GTTCTGTCATGAAGCGGGAA
Mfn1	NM_024200	Forward: GTGAGCTTCACCAGTGCAAA Reverse: GTTGGCACAGTCGAGCAAA
Mfn2	NM_001285922	Forward: AAGTCGAGGCTCTGGATTCA Reverse: TGTTGAGTTCGCTGTCCAAC
Atp5e	NM_025983	Forward: CTTCGGGCAGCAGCATAAAA Reverse: ACTCAGCACTTCAGGCTTCA
Cox4il	NM_001293559	Forward: CAGCCTTTCCAGGGATGAGAA Reverse: TTCATTGGTGCCCCTGTTCA
Bax	NM_007527.3	Forward: GCGTGGTTGCCCTCTTCTA Reverse: CTGATCAGCTCGGGCACTTTA
Bcl2	NM_009741	Forward: ATGTGTGTGGAGAGCGTCAA Reverse: GATGCCGGTTCAGGTACTCA

This table contains the nucleotide sequences of the forward and reverse primers (5' to 3') that were used for quantitative PCR (q-PCR) to amplify selected genes. The reference genes included *Actb* and *Gapdh*, which were used for normalisation. Target genes were selected based on their involvement in oxidative stress response (*Nox1*, *Sod1*, *Gpx4*), mitochondrial fusion (*Opa1*, *Mfn1*, *Mfn2*), mitochondrial bioenergetics (*Atp5e*, *Cox4il*) and apoptosis regulation (*Bax*, *Bcl2*).

3.13.3.4 Cell Lysis

Frozen embryos in tubes were thawed at room temperature. To lyse the embryos, 0.5 µL lysis enhancer was added and the samples were incubated at 70 °C for 20 min in a thermocycler. Complete lysis of the embryos was confirmed microscopically.

3.13.3.5 DNase I Treatment

To eliminate genomic DNA, 0.5 µL DNase I (1 U μL^{-1}) and 0.22 µL DNase buffer were added followed by incubation at 25 °C for 15 min. The enzyme activity was terminated with 0.55 µL 25 mM Ethylenediaminetetraacetic Acid (EDTA) and heating at 70 °C for 10 min.

3.13.3.6 cDNA Synthesis and Specific Target Preamplification (STA)

For each sample, 7.5 µL of STA mix was prepared by combining 5 µL of CellsDirect 2× Reaction Mix, 0.5 µL of SuperScript III RT/Platinum Taq Mix, 1 µL of pooled primers (500 nM each) and 1 µL of DNA suspension buffer. A total volume of 7.5 µL of STA mix was added to each lysed embryo. After addition of the STA mix, cDNA synthesis and pre-amplification were performed in the PCR machine. The Fluidigm PCR machine was set to thermal cycling conditions: 50 °C 20 min → 95 °C 2 min → 18 cycles of 95 °C 15 s → 60 °C 4 min.

3.13.3.7 Exonuclease I Treatment

Residual primers were removed by adding 0.8 µL exonuclease I (20 U μL^{-1}), 0.4 µL exonuclease buffer and 2.8 µL nuclease-free water. The samples were incubated for 30 min at 37 °C and inactivated for 15 min and at 80 °C.

3.13.3.8 Primer Validation and Serial Dilution Optimisation

Primer efficiency and dynamic range were assessed using a Flex Six™ Gene Expression Integrated Fluidic Circuit (IFC) according to the manufacturer's guidelines. The pre-amplified cDNA was serially diluted 1:1, 1:5, 1:10, 1:20 and 1:40 (triplicate). The 1:10 dilution was chosen as the primers achieved an amplification efficiency of 90 – 110 % and an $R^2 \geq 0.98$.

3.13.3.9 Integrated Fluidic Circuit (IFC) Loading and qPCR Cycling

Assay and sample mixes (5 µL each) were loaded into a 192.24 IFC as shown in Figure 3.14 which assays and samples were separated. The IFC enables parallel analysis of 192 samples against 24 primer assays allowing for high-throughput gene expression profiling. The IFC was only used for designated assay and samples. The assay mix contained 3 µL assay loading reagent, 00.6 µL combined primers (50 µM) and 2.4 µL DNA suspension buffer. The sample mix contained 3 µL of 2× SsoFast EvaGreen Supermix (low ROX), 0.3 µL of 20× DNA binding dye, 1.35 µL of processed sample and 1.35 µL of DNA suspension buffer.

The Fluidigm machine was set to thermal cycling conditions: 30 cycles of 95 °C 15 s → 60 °C 60 s followed by a melting curve of 60 °C → 95 °C. Finally, the cycle threshold (Ct) and calibration curves were generated using Fluidigm RT-PCR Analysis Software.

3.13.3.10 Calculation of Results

The cycle threshold (Ct) values were exported for each gene and sample. Fold-change expression was calculated using the 2^(−ΔΔCt) method using *Actb* and *Gapdh* as reference genes and the PBS group as calibrator. Genes of interest (GOIs) with fold-change values > 1 compared to PBS were categorised as upregulated while values < 1 were considered downregulated.

Figure 3.13 Fluidigm Biomark HD System
This figure shows the Fluidigm Biomark HD System used for high-throughput qPCR analysis. This microfluidics-based platform enables the precise and simultaneous quantification of multiple gene targets in small volume samples and is therefore suitable for low throughput materials such as preimplantation embryos.

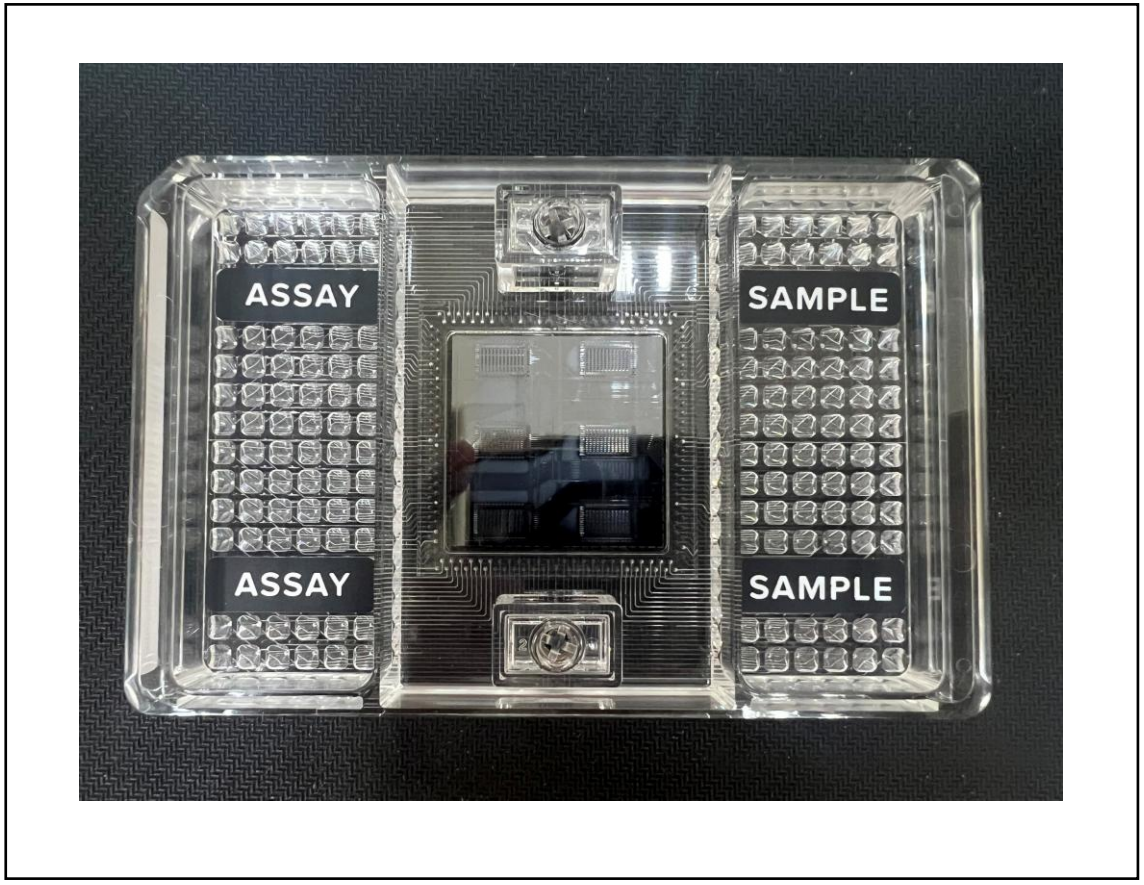


Figure 3.14 The 192.24 Integrated Fluidic Circuit
This figure shows the layout of the 192.24 IFC used in the Fluidigm qPCR workflow. The chip enables parallel analysis of 192 samples against 24 primer assays, allowing for high-throughput gene expression profiling

3.14 Statistical Analysis

Quantitative data were expressed as mean ± SD or mean ± standard error of the mean (SEM) depending on the context. Developmental progress, morphological grading and relative embryo volume were expressed as percentages. The normality of the data was tested using the Shapiro-Wilk test. For normally distributed data, a one-way ANOVA followed by Tukey's Multiple Comparison Test was used to determine group differences. Non-normally distributed data were analysed using the Kruskal-Wallis test. Categorical or qualitative data were analysed using the chi-square test. A p-value of < 0.05 was considered statistically significant. All statistical analyses and graphical representations were performed using GraphPad Prism version 8 software.

CHAPTER 4
RESULTS

4.1 Overview of Results Chapter

This experiment consists of Experiment I: Effects of Different Doses of Ovalbumin on Maternal Serum Biomarkers and Embryonic Outcomes and Experiment II: The Protective Effect of Maternal Tocotrienol-rich Fraction Supplementation on Embryo Viability in Ovalbumin-Induced Allergic Asthma Mice.

In Experiment I, systemic inflammatory and oxidative stress biomarkers in maternal serum were analysed to assess the physiological effects of allergic asthma. Subsequently, the effects of maternal asthma on the developmental potential and morphological quality of 2-cell embryos were analysed. Three different ovalbumin (OVA) doses were tested: a low dose (50 µg/200 µL; OVA1), a medium dose (100 µg/200 µL; OVA2) and a high dose (150 µg/200 µL; OVA3). Phosphate Buffered Saline (PBS, vehicle control) groups served as controls. The SHAM group acted as untreated. In the first phase of the experiment, the systemic condition of the mothers was analysed by measuring serum levels of interleukin (IL)-4 and immunoglobulin (Ig)-E as indicators of allergic inflammation. In parallel, serum levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and total antioxidant capacity (TAOC) were determined to assess the status of oxidative. Then, the experiment analysed embryonic outcomes.

In Experiment II, the protective effects of maternal tocotrienol-rich fraction (TRF) supplementation on embryo viability in OVA-induced allergic asthma mice were investigated. In this experiment, a dose of 150 µg/200 µl OVA (OVA3) was used which was considered the optimal dose from Experiment I. Mice in the TOVA group received TRF at a dose of 60 mg/kg body weight while mice in the POVA group received palm olein (vehicle for TRF which is stripped of tocotrienol). Initially, the primary focus was on the protective effects of maternal TRF supplementation on maternal serum biomarkers (systemic inflammation, oxidative stress and pregnancy hormones). Subsequently, the protective effects of maternal TRF supplementation on the developmental potential, morphological quality, ultrastructural features and gene expression of 2-cell embryos were investigated.

4.2 The Effects of Different Doses of Ovalbumin on Maternal Serum Biomarkers

The maternal parameters were used to determine which dose of OVA elicited the most severe allergic and oxidative responses. Thus, identifying the most appropriate dose for eliciting adverse maternal effects in subsequent embryo-related studies.

4.2.1 Maternal Inflammation (IL-4 and IgE)

Serum levels of IL-4 and IgE were quantified by using ELISA. As shown in Figure 4.1A, serum IL-4 levels were significantly increased in all OVA-treated groups compared to PBS group ($p < 0.0001$). IL-4 levels increased progressively in all groups from 139 pg/mL in OVA1 to 153.8 pg/mL in OVA2 and reached the highest level in OVA3 at 184.3 pg/mL indicating a clear dose-dependent effect. Of note, IL-4 levels were significantly higher in the PBS group than in the SHAM group ($p < 0.001$) indicating a mild immunological effect of the induction technique. The differences between all OVA groups were statistically significant confirming that increasing OVA doses led to stronger T helper (Th)2-type immune activation.

Serum IgE levels also increased with increasing OVA dose (Figure 4.1B). While no significant difference was observed between the SHAM and PBS groups (ns), all OVA groups showed a significant increase in IgE levels compared to the PBS group ($p < 0.0001$). IgE levels increased in a dose-dependent manner from 2083 ng/ml in OVA1 to 3587 ng/ml in OVA2 and peaked at 5533 ng/mL in OVA3 reflecting a strong allergic response.

Overall, these results indicate that OVA elicited a significant and dose-dependent increase in serum IL-4 and IgE with the 150-µg dose (OVA3) producing the most pronounced pro-allergic effects. Thus, OVA3 can be considered the optimal dose for establishing a robust murine model of allergic asthma for subsequent mechanistic or therapeutic studies.

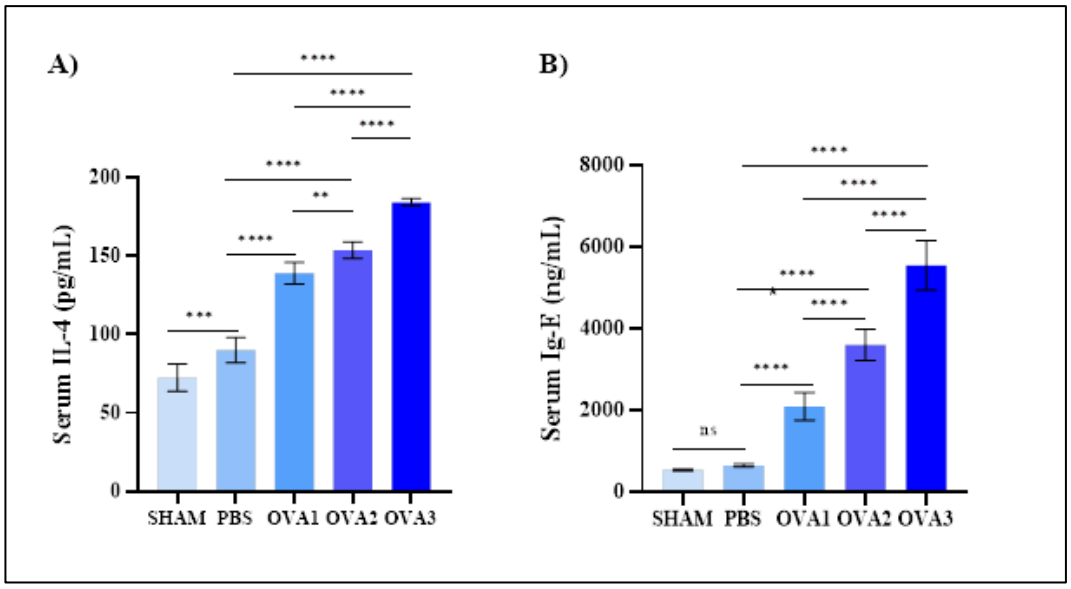


Figure 4.1 Effects of Different Ovalbumin Doses on Maternal Inflammation (A) Serum interleukin (IL)-4 level (pg/ml) and (B) Serum immunoglobulin (Ig)-E level (ng/ml) were measured in SHAM (untreated), PBS (vehicle control) and OVA-treated groups receiving increasing doses of OVA (OVA1: 50 µg/200 µl; OVA2: 100 µg/200 µl; OVA3: 150 µg/200 µl). Both IL-4 and Ig-E showed a significant dose-dependent increase, with the highest concentrations observed in OVA3. Data are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ns: not significant, **p < 0.01, ***p < 0.001, **p < 0.0001.

4.2.2 Maternal Oxidative Stress and Antioxidant Capacity (8-OHdG and TAOC)

To further evaluate the systemic effects of OVA sensitisation, serum levels of 8- hydroxy-2′-deoxyguanosine (OHdG) and total antioxidant capacity (TAOC) were measured by using ELISA. As shown in Figure 4.2A, the SHAM and PBS groups had low and comparable 8-OHdG levels with no significant difference between them. In contrast, all OVA-sensitised groups showed increased 8-OHdG levels indicating increased oxidative DNA damage. A clear dose-dependent trend was observed with levels increasing from 9.34 ng/mL in OVA1 to 47.38 ng/mL in OVA2 and peaking at 86.46 ng/mL in OVA3. These increases were statistically significant compared to PBS and also between OVA groups ($p < 0.01$ to $p < 0.0001$) suggesting that higher doses of OVA exacerbate oxidative damage.

As shown in Figure 4.2B, the SHAM and PBS groups maintained a high antioxidant capacity (5.49 and 5.73 U/mL, respectively) with no significant difference. In contrast, TAOC was significantly reduced in all OVA groups in a dose-dependent manner. The lowest value was found in OVA3 (0.63 U/mL) which was significantly lower than in PBS group ($p < 0.0001$). OVA2 and OVA1 also showed a mean reduction ($p < 0.0001$). These results show that increasing OVA exposure impairs systemic antioxidant defences.

Overall, OVA sensitisation led to a dose-dependent increase in oxidative stress (8-OHdG) with concomitant suppression of antioxidant capacity (TAOC) with the 150-µg dose (OVA3) having the most pronounced adverse effects. This argues in favour of using OVA3 as the optimal sensitising dose.

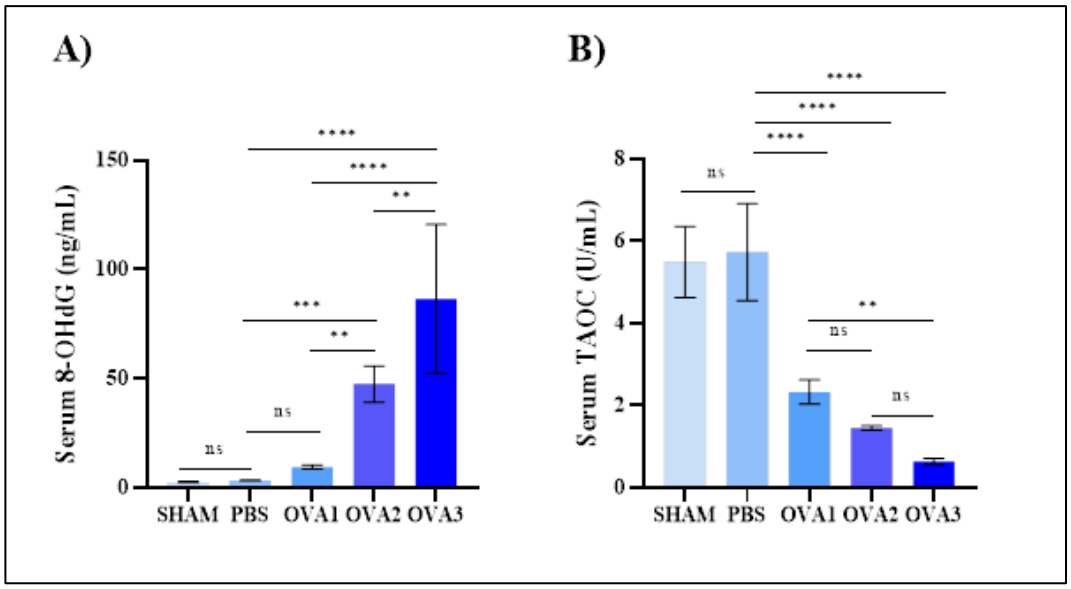


Figure 4.2 Effects of Different Ovalbumin Doses on Maternal Oxidative Stress and Antioxidant Capacity

(A) Serum 8-hydroxy-2'-deoxyguanosine (8-OHdG) level (ng/mL) and (B) total antioxidant capacity (TAOC) (U/mL) were measured in SHAM, PBS and OVA-treated mice (OVA1: 50 µg/200 µL; OVA2: 100 µg/200 µL; OVA3: 150 µg/200 µL). OVA sensitisation led to a dose-dependent increase in 8-OHdG levels and a corresponding decrease in TAOC. Data are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. ns: not significant, **p < 0.01, ***p < 0.001, ****p < 0.0001.

4.3 The Effects of Different Doses of Ovalbumin on Developmental Potential and Morphological Quality

The developmental potential and morphological quality of 2-cell embryos were evaluated based on Association for the Study of Reproductive Biology (ASEBIR) grading system (Saiz et al., 2018).

As shown in Table 4.1, a total of 168, 150, 77, 49 and 16 two-cell embryos were collected from the SHAM, PBS, OVA1, OVA2, and OVA3 groups, respectively. The SHAM and PBS groups exhibited the highest proportions of morphologically normal embryos at 81.0% (136/168) and 90.7% (136/150) with no significant difference between them.

In contrast, OVA-treated groups showed a marked dose-dependent reduction in normal morphology. In OVA1, only 55.8% (43/77) of embryos were normal ($p < 0.0001$ vs. PBS). This declined further in OVA2 with only 22.4% (11/49) morphologically normal embryos ($p < 0.0001$ vs. PBS; ns vs. OVA1). Strikingly, in OVA3, no embryos were morphologically normal (0%) representing a significant reduction compared to PBS ($p < 0.0001$), OVA1 ($p < 0.0001$) and OVA2 ($p < 0.05$).

Normal 2-cell embryos were characterised by evenly sized blastomeres with <10% fragmentation whereas abnormal embryos showed unevenly sized blastomeres and >10% fragmentation (Table 4.2).

The detrimental effects were further reflected in developmental progress. In the SHAM and PBS groups, 76.79% and 71.33% of embryos developed to the hatched blastocyst stage respectively, with no significant difference. However, developmental rates were significantly reduced in OVA1 (46.75%; $p < 0.0001$ vs. PBS) and remained comparably low in OVA2 (48.98%; $p < 0.0001$ vs. PBS; ns vs. OVA1). In OVA3, only 1 of 16 embryos progressed to the hatched blastocyst stage (6.25%; $p < 0.0001$ vs. PBS, $p < 0.001$ vs. OVA1, $p < 0.05$ vs. OVA2).

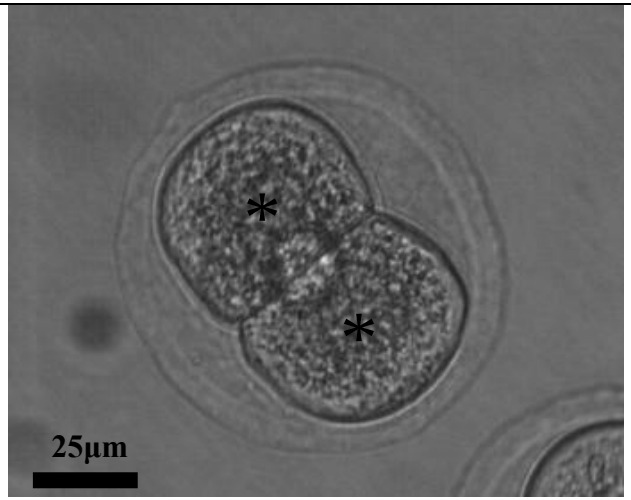
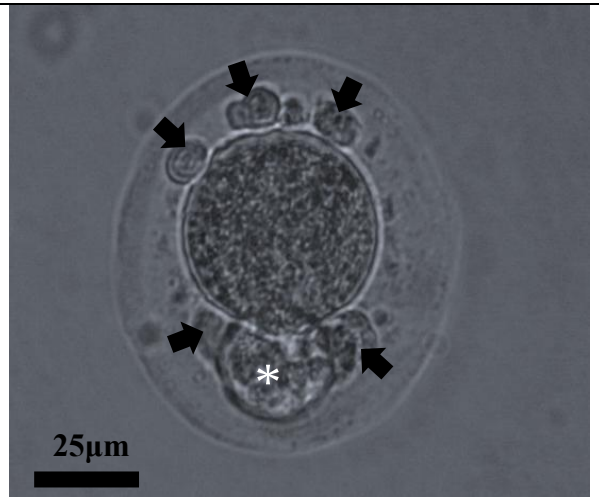
Together, these findings demonstrate that increasing maternal OVA doses are strongly associated with higher rates of embryonic morphological abnormalities and severely compromised developmental progress with the most profound impairment observed in OVA3.

Table 4.1
Effects of Different Concentrations of Ovalbumin on Morphology and Developmental Rate of 2-cell Embryos

	2-cell embryo			**Hatched blastocyst**	
	Total Number, N	Morphology		Total Number, N	Developmental rate (%)
		Normal % (N)	Abnormal % (N)		
SHAM	168 ns-*	81.0 (136)	19.0 (32)	129 ns-*	76.79
PBS	150	90.7 (136)	9.3 (14)	107	71.33
OVA1	77 ****	55.8 (43)	44.2 (34)	36 ****	46.75
OVA2	49 ****, ns-&	22.4 (11)	77.6 (38)	24 ****, ns-&	48.98
OVA3	16 ****, &&&&, $	0.0 (0)	100.0 (16)	1 ****, &&&, $	6.25

This table presents the total number of 2-cell embryos collected, their morphological classification and the course of development up to the hatched blastocyst stage. OVA1, OVA2 and OVA3 represent increasing doses of ovalbumin (OVA): 50 µg/200 µL, 100 µg/200 µL and 150 µg/200 µL respectively. SHAM represents untreated control. PBS represents control with phosphate-buffered saline. The values are given as a percentage (number) or absolute number. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. ns-*: not significant compared to the PBS group. *: significant compared to the PBS group. ns-&: not significant compared to the OVA1 group. &: significant compared to the OVA1 group. $: significant compared to the OVA2 group.

Table 4.2
Representative Morphology of Normal and Abnormal 2-cell Embryos

	Normal	**Abnormal**
2-cell embryo		

This table shows the representative images illustrating the morphological differences between normal and abnormal 2-cell embryo. A normal 2-cell embryo is characterised by even-sized blastomeres (indicated by black asterisk). In contrast, abnormal embryos display uneven blastomere size (white asterisk) with cytoplasmic fragmentation (black arrow). Images viewed under an inverted microscope at 40× magnification. Scale bar: 25 µm.

4.4 The Effects of Maternal Tocotrienol-rich Fraction Supplementation on Maternal Serum Biomarkers

This study demonstrates the protective role of TRF in mitigating systemic inflammation and oxidative stress in OVA-induced allergic asthma murine model. TRF supplementation appeared to exert anti-inflammatory, antioxidant and hormonal regulatory effects. Thereby, improving maternal physiological balance during early pregnancy.

4.4.1 Maternal Inflammation (IL-4 and IgE)

As shown in Figure 4.3A, maternal serum IL-4 levels were markedly elevated in the OVA3 group (184.3 ± 2.307) compared with the PBS control (90.04 ± 8.093, $p < 0.0001$) confirming the successful induction of Th2-mediated allergic response. Similarly, the POVA group (palm olein + OVA) exhibited significantly higher IL-4 levels (200.0 ± 3.536) than both the PBS and OVA3 groups ($p < 0.0001$, $p < 0.001$), indicating that palm olein alone did not alleviate inflammation and may have even exacerbated the immune response.

In contrast, TRF supplementation (TOVA group) resulted in a significant reduction in IL-4 (141.6 ± 2.250) concentration compared to both OVA3 and POVA groups ($p < 0.0001$) although values remained higher than those in PBS group ($p < 0.001$). This pattern indicates a partial but meaningful attenuation of systemic inflammation suggesting that TRF modulates Th2 cytokine production rather than completely suppressing it.

Similarly, Figure 4.3B shows that serum IgE levels followed the same trend as IL-4. The OVA3 group showed a marked elevation in IgE (5533 ± 609.4) compared with PBS (529.3 ± 23.80, $p < 0.0001$) and POVA did not differ significantly from OVA3 (5794 ± 66.31, ns) reinforcing the absence of a protective effect from palm olein. Conversely, the TOVA group exhibited a significant reduction in IgE (2033 ± 76.27) compared with both OVA3 and POVA ($p < 0.0001$) confirming that TRF supplementation effectively suppressed allergen-induced IgE overproduction. Nonetheless, IgE levels in TOVA remained slightly higher than in PBS group ($p < 0.0001$) indicating incomplete but substantial immune regulation.

These findings highlight the immunomodulatory potential of TRF in allergic asthma during pregnancy. By lowering IL-4 and IgE, TRF likely downregulates the Th2-dominant inflammatory cascade responsible for airway hypersensitivity and systemic allergic responses. This dual anti-inflammatory and anti-allergic action is particularly relevant for maternal health as chronic inflammation during early gestation can disrupt hormonal balance, impair placental development and compromise embryonic outcomes. Therefore, maternal TRF supplementation may provide a protective advantage by reducing immune overactivation and promoting a more favourable intrauterine environment for embryo development.

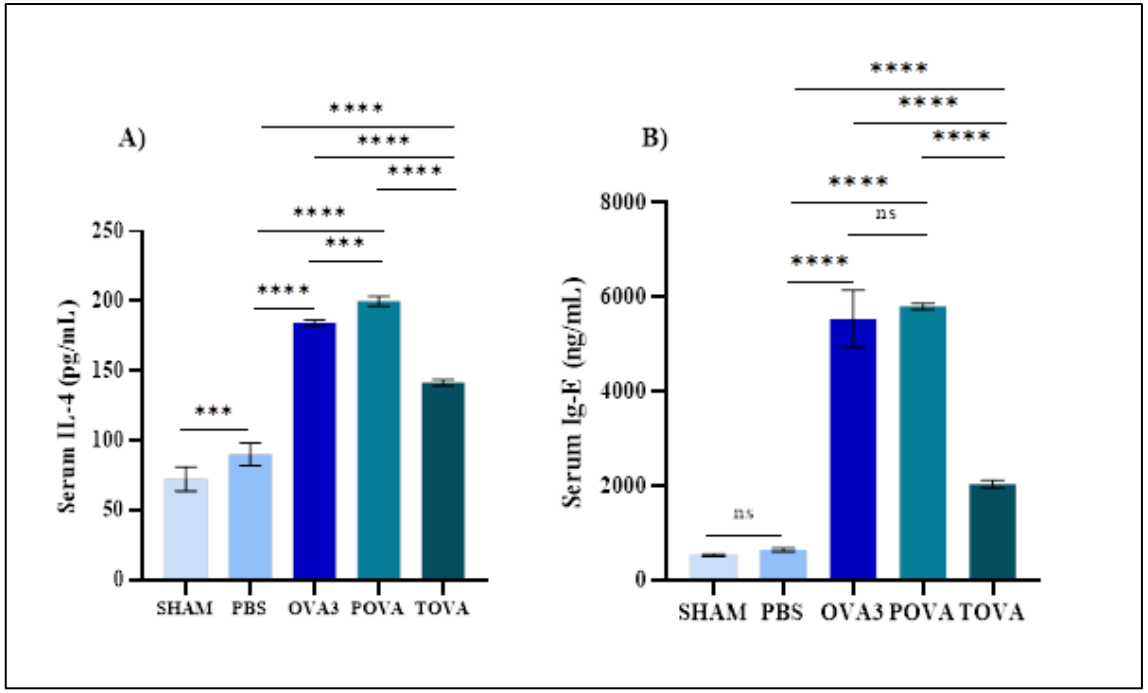


Figure 4.3 Effects of Maternal Tocotrienol-rich Fraction Supplementation on Systemic Inflammation

(A) Serum interleukin (IL)-4 level (pg/ml) and (B) Serum immunoglobulin (Ig)-E level (ng/ml) were measured in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). The OVA3 and POVA groups exhibited significantly elevated IL-4 and IgE levels compared with the SHAM and PBS groups. Maternal TRF supplementation (TOVA) markedly reduced both IL-4 and IgE levels relative to the OVA3 and POVA groups. Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ns = not significant, ***$p < 0.001$, ****$p < 0.0001$.

4.4.2 Maternal Oxidative Stress and Antioxidant Capacity (8-OHdG and TAOC)

As shown in Figure 4.4A, serum 8-OHdG levels were significantly elevated in the OVA3 group (86.46 ± 334.090) compared to the PBS group (3.193 ± 0.177, $p < 0.0001$), indicating increased oxidative DNA damage following asthma induction. Similarly, the POVA group exhibited significantly higher 8-OHdG levels (89.5 ± 3.832) than the PBS group ($p < 0.0001$), with no significant difference from the OVA3 group, suggesting that palm olein supplementation did not alleviate oxidative stress. In contrast, TRF supplementation (TOVA group) markedly reduced 8-OHdG concentrations (11.8 ± 0.570) compared to both the OVA3 and POVA groups ($p < 0.0001$), demonstrating a potent antioxidant effect of TRF in mitigating oxidative DNA damage. Although the TOVA group showed slightly higher 8-OHdG levels than the SHAM and PBS controls, the differences were not statistically significant (ns).

As illustrated in Figure 4.4B, TAOC was significantly decreased in the OVA3 group (0.630 ± 0.082) compared to the PBS group (5.730 ± 1.177, $p < 0.0001$) reflecting diminished systemic antioxidant defence in asthmatic mice. The POVA group displayed similarly reduced TAOC values (1.251 ± 0002) with no significant difference from the OVA3 group, further confirming the absence of antioxidant benefit from palm olein. TRF supplementation (TOVA group) did not significantly elevate TAOC values (1.278 ± 0.013) relative to the OVA3 and POVA groups suggesting that while TRF effectively mitigated oxidative DNA damage (as shown by reduced 8-OHdG), it did not substantially enhance overall serum antioxidant capacity in this model.

In summary, maternal TRF supplementation effectively attenuated oxidative DNA damage in OVA-induced allergic asthma as evidenced by decreased 8-OHdG levels. However, this protective effect was not paralleled by a significant recovery of systemic antioxidant capacity implying that TRF's antioxidant action may be exerted more locally or through specific molecular pathways rather than through a broad elevation of circulating antioxidant levels.

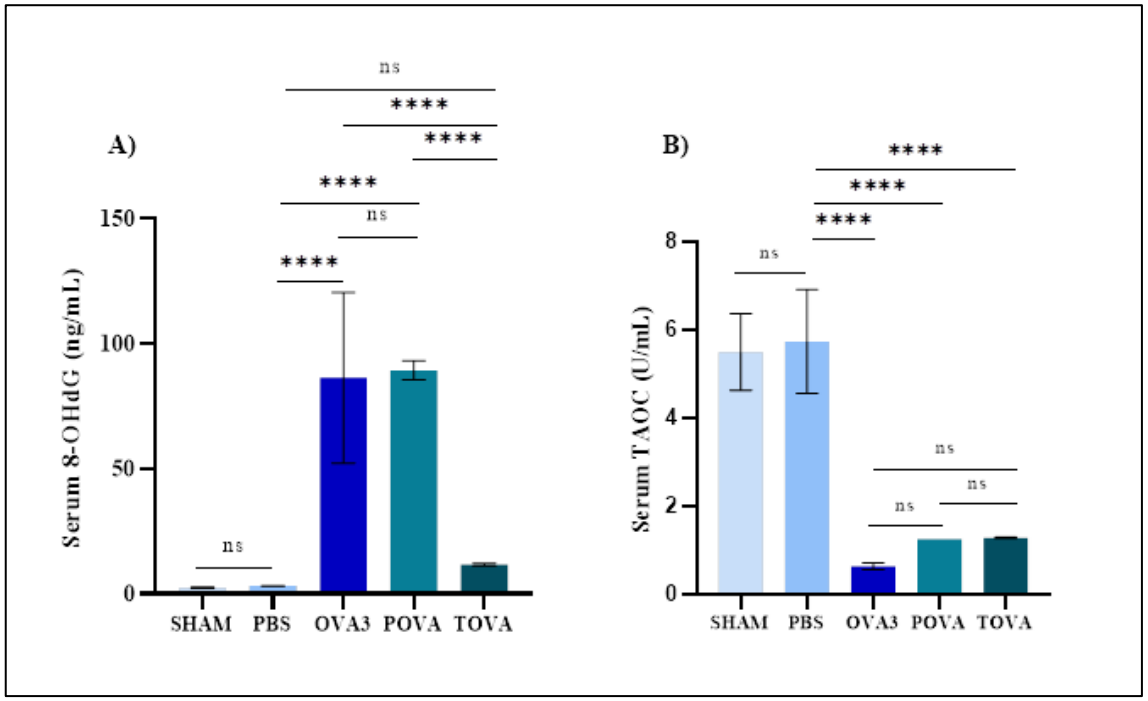


Figure 4.4 Effects of Maternal Tocotrienol-rich Fraction Supplementation on Systemic Oxidative Stress and Antioxidant Capacity
(A) Serum 8 hydroxy-2′-deoxyguanosine (8-OHdG) level (ng/mL) and (B) Serum total antioxidant capacity (TAOC) (U/mL) were measured in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). The OVA3 and POVA groups exhibited significantly elevated 8-OHdG level and decreased TAOC compared with the PBS group. Maternal TRF supplementation (TOVA) markedly reduced 8-OHdG level relative to the OVA3 and POVA groups but did not significantly improve TAOC. Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ns = not significant, ****$p < 0.0001$.

4.4.3 Maternal Pregnancy Hormones (P_4 and E_2)

To assess the potential modulatory effects of TRF supplementation on systemic pregnancy hormones, serum concentrations of progesterone (P_4) and estradiol (E_2) were measured.

As shown in Figure 4.5A, serum P_4 levels were slightly elevated in the OVA3 group (4.064 ± 0.816) compared to the SHAM (3.563 ± 1.272) and PBS (3.326 ± 1.540) groups. The POVA group exhibited a modest reduction in P_4 levels (2.932 ± 0.034) whereas the TOVA group demonstrated a slight increase relative (3.973 ± 0.201) to the other groups. However, these variations were not statistically significant (ns).

In Figure 4.5B, serum E_2 levels followed a similar pattern except for the OVA3 group (129.2 ± 74.40) showed a significant reduction in E_2 compared to the SHAM (226.6 ± 172.1) and PBS (190.8 ± 149.7) groups, consistent with the findings in the POVA group (124.3 ± 1.169). Although TRF supplementation (TOVA group) modestly increased E_2 concentrations (186.3 ± 7.909) compared to OVA3 and POVA, the differences did not reach statistical significance (ns).

In summary, maternal TRF supplementation appeared to slightly normalise serum P_4 and E_2 levels in asthmatic mice although these effects were not statistically significant. The findings suggest that TRF may exert a mild restorative influence on systemic pregnancy hormones under allergic asthma conditions. However, further investigations across different gestational stages or with extended supplementation are warranted to validate its endocrine-modulating potential.

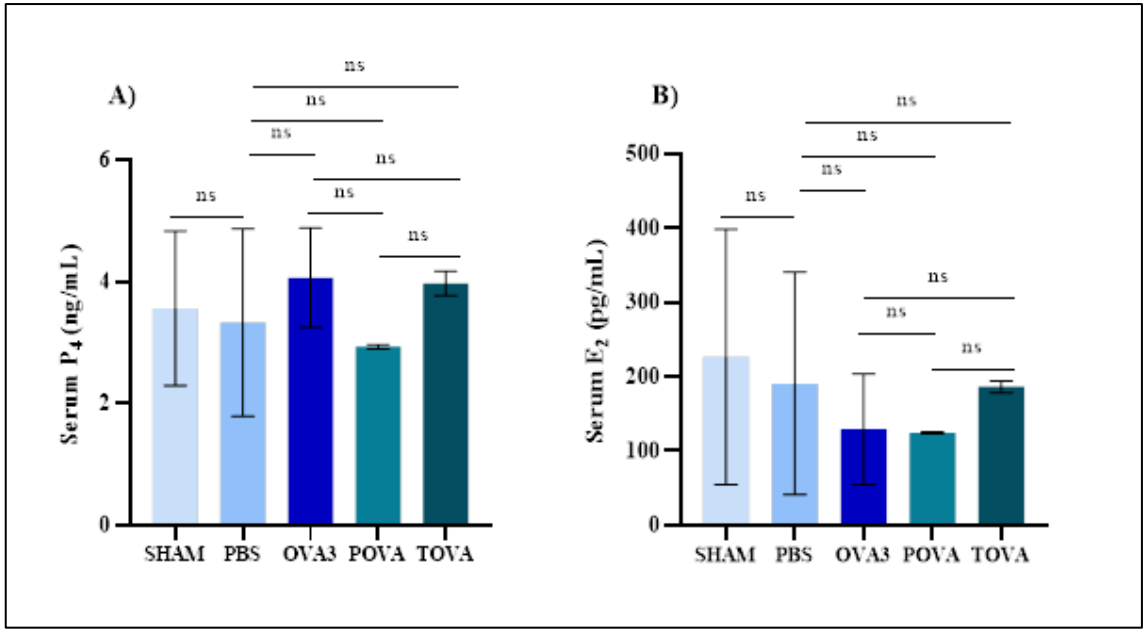


Figure 4.5 Effects of Maternal Tocotrienol-rich Fraction Supplementation on Pregnancy Hormones

(A) Serum progesterone (P_4) level (ng/mL) and (B) Serum oestrogen (E_2) (pg/mL) were measured in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). There were no statistically significant differences among the experimental groups. Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ns = not significant.

4.5 The Effects of Maternal Tocotrienol-rich Fraction Supplementation on Developmental Potential and Morphological Quality

Following the assessment of maternal physiological parameters, this section investigates the effects of maternal TRF supplementation on developmental potential and morphological quality of 2-cell embryos in OVA-induced allergic asthma mice. Developmental potential was evaluated from 1.5 to 4.5 days post coitum (d.p.c.) encompassing the key preimplantation stages (2-cell, 8-cell, morula, blastocyst and hatched blastocyst). Embryo morphology was assessed using a modified ASEBIR grading system (Saiz et al., 2018) to ensure a standardised and objective evaluation of developmental quality. Based on this system, embryos were classified as either normal or abnormal.

The following subsections describe the protective effects of TRF supplementation on developmental potential and morphological quality in 2-cell embryos.

4.5.1 Developmental Potential of 2-cell Embryos

A total of fifty 2-cell embryos per group (SHAM, PBS, OVA3, POVA, and TOVA) were cultured *in vitro* to evaluate developmental potential across the preimplantation stages (Table 4.3).

At the 8-cell stage, a significant reduction in the developmental progress was observed in the OVA3 group (42%) compared to the PBS group (92%, $p < 0.0001$). Similarly, the POVA group exhibited a markedly lower percentage of 8-cell embryos (38%, $p < 0.0001$ vs. PBS) indicating that palm olein supplementation did not alleviate the developmental impairment induced by allergic asthma. In contrast, TRF supplementation (TOVA group) significantly increased the proportion of embryos reaching the 8-cell stage (76%) compared to OVA3 ($p < 0.001$) and POVA ($p < 0.0001$) suggesting that TRF improved early cleavage development.

At the morula stage, both PBS and SHAM groups maintained high developmental rates (88% and 90%, respectively). In contrast, the OVA3 and POVA groups demonstrated markedly reduced morula formation (22% and 24%, respectively; $p < 0.0001$ vs. PBS) indicating impaired development and lack of benefit from palm olein. Importantly, the TOVA group showed a significantly higher morula formation

rate (58%) compared to OVA3 ($p < 0.001$) and POVA ($p < 0.001$) reflecting the protective effect of TRF supplementation.

At the blastocyst stage, both SHAM and PBS groups exhibited high developmental rates (80% and 78%, respectively). Conversely, the OVA3 and POVA groups displayed substantially lower blastocyst formation rates (16% and 18%, $p < 0.0001$ vs. PBS). TRF supplementation significantly enhanced blastocyst development in the TOVA group (54%) compared to OVA3 ($p < 0.01$) and POVA ($p < 0.01$) demonstrating a restorative effect on embryo development.

Finally, at the hatched blastocyst stage, a similar pattern was observed. The SHAM and PBS groups recorded high hatching rates (76% and 74%, respectively) whereas the OVA3 and POVA groups showed significantly lower percentages (14% and 12%, $p < 0.001$ vs. PBS). Notably, the TOVA group exhibited a markedly higher proportion of hatched blastocysts (52%) compared to OVA3 ($p < 0.05$) and POVA ($p < 0.05$) further supporting the beneficial role of TRF in promoting late preimplantation development.

Overall, these findings indicate that maternal TRF supplementation effectively mitigated the adverse effects of allergic asthma on developmental potential of 2-cell embryos enhancing progression from early cleavage to the hatched blastocyst stage.

4.5.2 Morphological Quality of 2-cell Embryos

Table 4.4 presents the morphological assessment of preimplantation embryos from 2-cell to blastocyst stages. Embryos were classified as either normal or abnormal based on a modified ASEBIR grading system (Saiz et al., 2018).

At the 2-cell stage, all embryos in the SHAM group (100%) and the majority in the PBS group (96%) were morphologically normal with no significant difference between the two control groups. In contrast, both the OVA3 and POVA groups recorded 0% normal embryos, with all 2-cell embryos displaying abnormal morphology. These differences were significant compared to the PBS group ($p < 0.05$) confirming that OVA-induced asthma adversely affected early embryonic morphology and that palm olein supplementation failed to confer protection. Notably, TRF supplementation (TOVA group) improved the proportion of normal 2-cell embryos to 66%, although this increase did not reach statistical significance (ns vs. PBS). Normal 2-cell embryos

exhibited even-sized blastomeres and <10% fragmentation whereas abnormal embryos displayed uneven blastomeres and >10% fragmentation (Table 4.5).

At the 8-cell stage, all embryos in the SHAM and PBS groups remained morphologically normal (100%). Conversely, none of the 8-cell embryos in the OVA3 and POVA groups were classified as normal, reaffirming the detrimental impact of allergic asthma and the ineffectiveness of palm olein in maintaining morphology. TRF supplementation markedly improved morphology with 78.9% of embryos in the TOVA group classified as normal, though the difference was not statistically significant (ns vs. PBS). Morphological assessment showed that normal 8-cell embryos possessed uniformly sized blastomeres while abnormal embryos exhibited uneven blastomeres and >10% fragmentation (Table 4.5).

At the morula stage, high percentages of normal embryos were maintained in both the SHAM (84.4%) and PBS (84.1%) groups. However, the OVA3 (72.7%, $p < 0.001$) and POVA (50%, $p < 0.0001$) groups displayed a significant reduction in normal morphology compared to PBS indicating impaired compaction and developmental delay. Remarkably, TRF supplementation restored normal morphology in the TOVA group to 89.7%, significantly higher than both OVA3 ($p < 0.05$) and POVA ($p < 0.01$). Morphological assessment revealed that normal morulae displayed complete blastomere compaction while abnormal ones exhibited incomplete compaction (Table 4.5).

At the blastocyst stage, normal morphology remained high in the SHAM (95%) and PBS (94.9%) groups. The OVA3 group showed a significant reduction in normal blastocysts (87.5%, $p < 0.001$) while the POVA group exhibited an even greater decline (66.7%, $p < 0.0001$ vs. PBS). Supplementation of TRF in the TOVA group restored the normal morphology rate to 96.3%, significantly higher than both OVA3 ($p < 0.05$) and POVA ($p < 0.01$). Normal blastocysts exhibited fully expanded blastocoel cavities and a clearly identifiable inner cell mass (ICM) including homogeneous trophectoderm (TE) cells whereas abnormal blastocysts displayed partially expanded blastocoel cavities and a poorly defined or absent ICM including non-homogenous TE cells (Table 4.5).

In summary, OVA-induced allergic asthma in maternal mice adversely affected both the development and morphology of preimplantation embryos particularly beyond the 2-cell stage. TRF supplementation (TOVA group) markedly improved developmental progression across all stages and significantly restored normal morphology at the morula and blastocyst stages. These findings highlight the protective

effects of TRF in enhancing embryonic development and morphological quality under allergic asthma conditions.

Table 4.3
Effects of Maternal Tocotrienol-rich Fraction Supplementation on Developmental Progress of 2-cell Embryos

	2-cell	**8-cell**	**Morula**	**Blastocyst**	**Hatched blastocyst**
	N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**
SHAM	50 (100.0)	47 (94.0) ns-*	45 (90.0) ns-*	40 (80.0) ns-*	38 (76.0) ns-*
PBS	50 (100.0)	46 (92.0)	44 (88.0)	39 (78.0)	37 (74.0)
OVA3	50 (100.0)	21 (42.0) ****	11 (22.0) ****	8 (16.0) ****	7 (14.0) ***
POVA	50 (100.0)	19 (38.0) ****, ns-&	12 (24.0) ****, ns-&	9 (18.0) ****, ns-&	6 (12.0) ***, ns-&
TOVA	50 (100.0)	38 (76.0) ns-*, &&&, $$$$	29 (58.0) ***, &&&, $$$	27 (54.0) ns-*, &&, $$	26 (52.0) ns-*, &, $

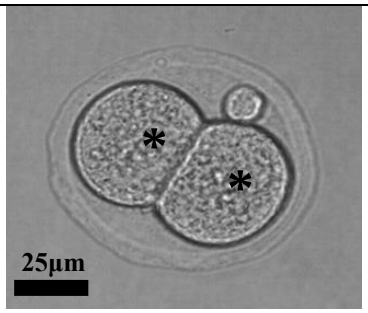
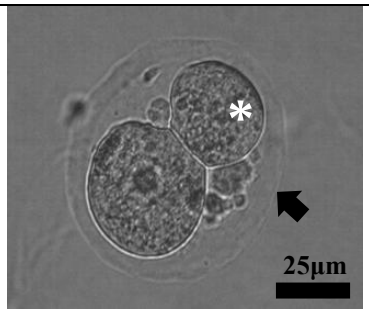
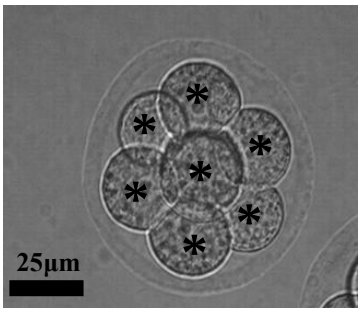
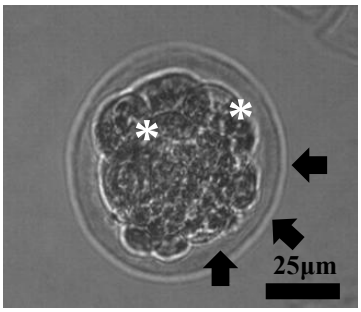
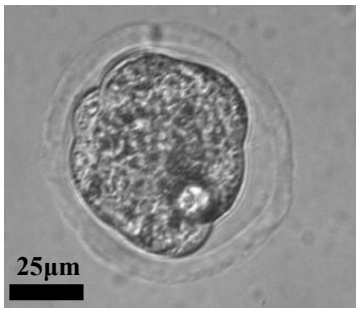
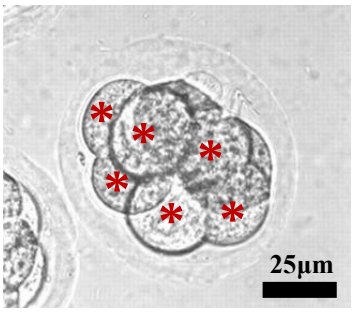
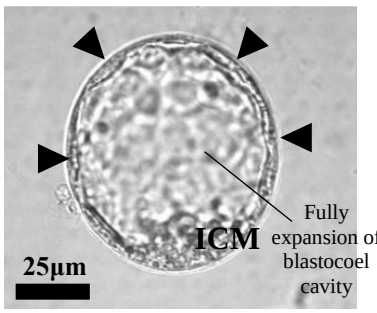
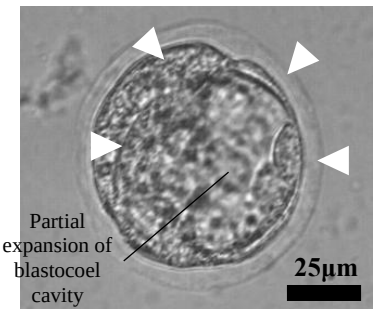
This table presents the developmental progression of 2-cell embryos across preimplantation embryonic stages (2-cell – hatched blastocyst stage). SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). The values are given as total number (N) and percentage (%). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. ns-*: not significant compared to the PBS group. *: significant compared to the PBS group. ns-&: not significant compared to the OVA3 group. &: significant compared to the OVA3 group. $: significant compared to POVA group.

Table 4.4
Effects of Maternal Tocotrienol-rich Fraction Supplementation on Morphological Assessment of Preimplantation Embryos

	2-cell		**8-cell**		**Morula**		**Blastocyst**	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
SHAM	50 (100.0) ns-*	0 (0.0)	47 (100.0) ns-*	0 (0.0)	38 (84.4) ns-*	7 (15.6)	38 (95.0) ns-*	2 (5.0)
PBS	48 (96.0)	2 (4.0)	46 (100.0)	0 (0.0)	37 (84.1)	7 (15.9)	37 (94.9)	2 (5.1)
OVA3	0 (0.0) *	50 (100.0)	0 (0.0) *	21 (100.0)	8 (72.7) ***	3 (27.3)	7 (87.5) ***	1 (12.5)
POVA	0 (0.0) *, ns-&	50 (100.0)	0 (0.0) *, ns-&	19 (100.0)	6 (50.0) ****, ns-&	6 (50.0)	6 (66.7) ****, ns-&	3 (33.3)
TOVA	33 (66.0) ns-*, ns-&, ns-$	17 (34.0)	30 (78.9) ns-*, ns-&, ns-$	8 (21.2)	26 (89.7) ns-*, &, $$	3 (10.3)	26 (96.3) ns-*, &, $$	1 (3.7)

This table presents the percentage of normal and abnormal morphology of preimplantation embryos across preimplantation embryonic stages (2-cell – blastocyst stage). SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). The values are given as total number (N) and percentage (%). Statistical analysis was performed using the Kruskal–Wallis test for the 2-cell and 8-cell stages, and one-way ANOVA for the morula and blastocyst stages. ns-*: not significant compared to the PBS group. *: significant compared to the PBS group. ns-&: not significant compared to the OVA3 group. &: significant compared to the OVA3 group. ns-$: not significant compared to POVA group. $: significant compared to POVA group.

Table 4.5
Representative Morphology of Normal and Abnormal Preimplantation Embryos (2-cell – Blastocyst)

	Normal	Abnormal
2-cell		
8-cell		
Morula		
Blastocyst		

This table shows the representative images illustrating the morphological differences between normal and abnormal 2-cell, 8-cell, morula and blastocyst. Normal embryos exhibit even-sized blastomeres (black asterisks), complete compaction and minimal fragmentation. In contrast, abnormal embryos show uneven or smaller blastomeres (white asterisks), incomplete or absent compaction (red asterisks) and cytoplasmic fragmentation (black arrows). At the blastocyst stage, black arrowheads indicate homogeneous trophectoderm (TE) cells characteristic of normal development, whereas white arrowheads denote non-homogeneous TE cells typically observed in abnormal blastocysts. The presence of the inner cell mass and the degree of blastocoel expansion are also indicated, with abnormal blastocysts showing partial expansion and poorly defined ICM structure. Images viewed under an inverted microscope at 40×magnification. Scale bar:25 µm.

4.6 The Effects of Maternal Tocotrienol-rich Fraction Supplementation on Ultrastructural Features

Following the assessment of preimplantation development and morphology, this study further examined the ultrastructural features of 2-cell embryos in OVA-induced allergic asthma mice. Specifically, 2-cell stage embryos were subjected to both qualitative and quantitative ultrastructural analyses using transmission electron microscopy (TEM).

A total of fifteen 2-cell embryos were collected from each experimental group for analysis of organelle distribution, morphology and volume density. The qualitative assessment focused on the spatial organisation and structural integrity of intracellular components whereas the quantitative evaluation determined the relative volume (Vv) of selected organelles following stereological principles (T. Santos et al., 2024). The summary of detailed numerical values was provided in Appendix K (Table K.1)

Particular emphasis was placed on mitochondrial parameters such as mitochondrial distribution patterns and clustering as mitochondria together with lipid droplets are central to adenosine triphosphate (ATP) generation during early embryogenesis. Other ultrastructural indices assessed included microvillar projection, perivitelline space condition and cellular degradation machinery which reflect cytoplasmic organisation and developmental competence that also elucidated embryo viability.

4.6.1 Distribution of Total Organelles

The distribution of total organelles within 2-cell embryos was heterogeneous across all groups with organelles predominantly localised in the peripheral cytoplasmic region adjacent to the zona pellucida. Accordingly, analyses focused on this region. At 4,200× magnification, organelles such as mitochondria and lipid droplets were consistently identified, along with the zona pellucida structure across all groups. Nonetheless, notable variations were evident in both the distribution and abundance of cytoplasmic organelles between experimental groups (Figure 4.6 and Table K.1, Appendix K).

In the SHAM and PBS groups, total organelles were abundant and evenly distributed throughout the peripheral region of cytoplasm (Figures 4.7A and 4.7B)

corresponding to high relative volumes of total organelles (36.25 ± 0.210% and 36.52 ± 5.851%, respectively). No significant difference was observed between these two control groups indicating normal organelle organisation under non-asthmatic conditions.

Conversely, embryos from the OVA3 group exhibited a marked reduction in cytoplasmic total organelle content (Figure 4.7C) reflected by a significantly lower relative volume of total organelles (11.12 ± 2.047%; $p < 0.001$) compared with the PBS group. This suggests that maternal allergic asthma compromises cytoplasmic organelle abundance at the 2-cell stage.

Palm olein supplementation (POVA) produced a modest improvement (Figure 4.7D) with a relative organelle volume of 16.40 ± 1.571%, still significantly lower than PBS ($p < 0.01$). This indicates that palm olein alone conferred limited restorative benefit.

Notably, TRF supplementation (TOVA) led to a substantial recovery in organelle abundance (24.57 ± 0.404%), significantly higher than the OVA3 group ($p < 0.05$) and trending above the POVA group although not statistically significant (Figure 4.6). Consistently, TEM micrographs revealed denser cytoplasmic organelle populations in TOVA embryos (Figure 4.7E) supporting the protective and restorative potential of TRF in mitigating asthma-induced cytoplasmic alterations.

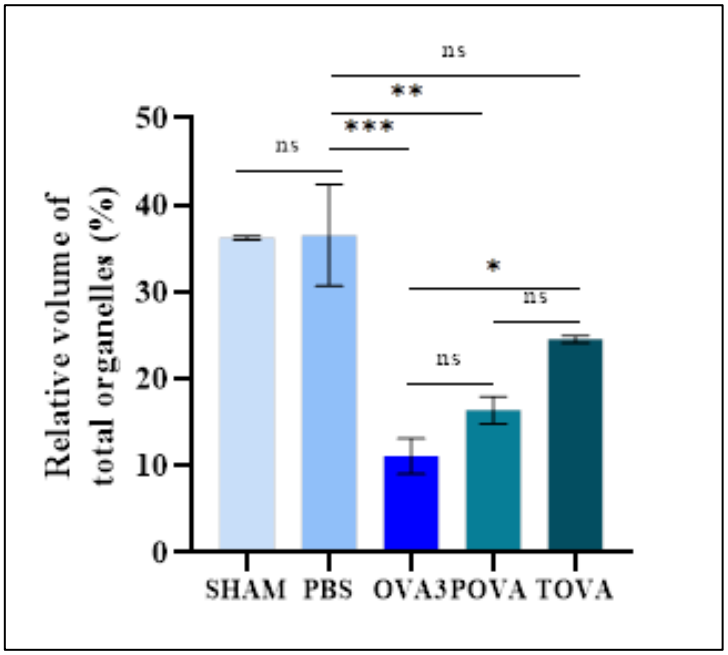


Figure 4.6 Effects of Maternal Tocotrienol-rich Fraction Supplementation on the Relative Volume of Total Organelles (%) in 2-Cell Embryos
The figure illustrates the mean relative volume of total organelles SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). The OVA3 group showed a marked reduction in the relative volume of total organelles compared to SHAM and PBS. Supplementation with TRF (TOVA) partially restored the relative volumes compared to OVA3. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ns: not significant. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.
Detailed numerical values are provided in Appendix K (Table K.1).

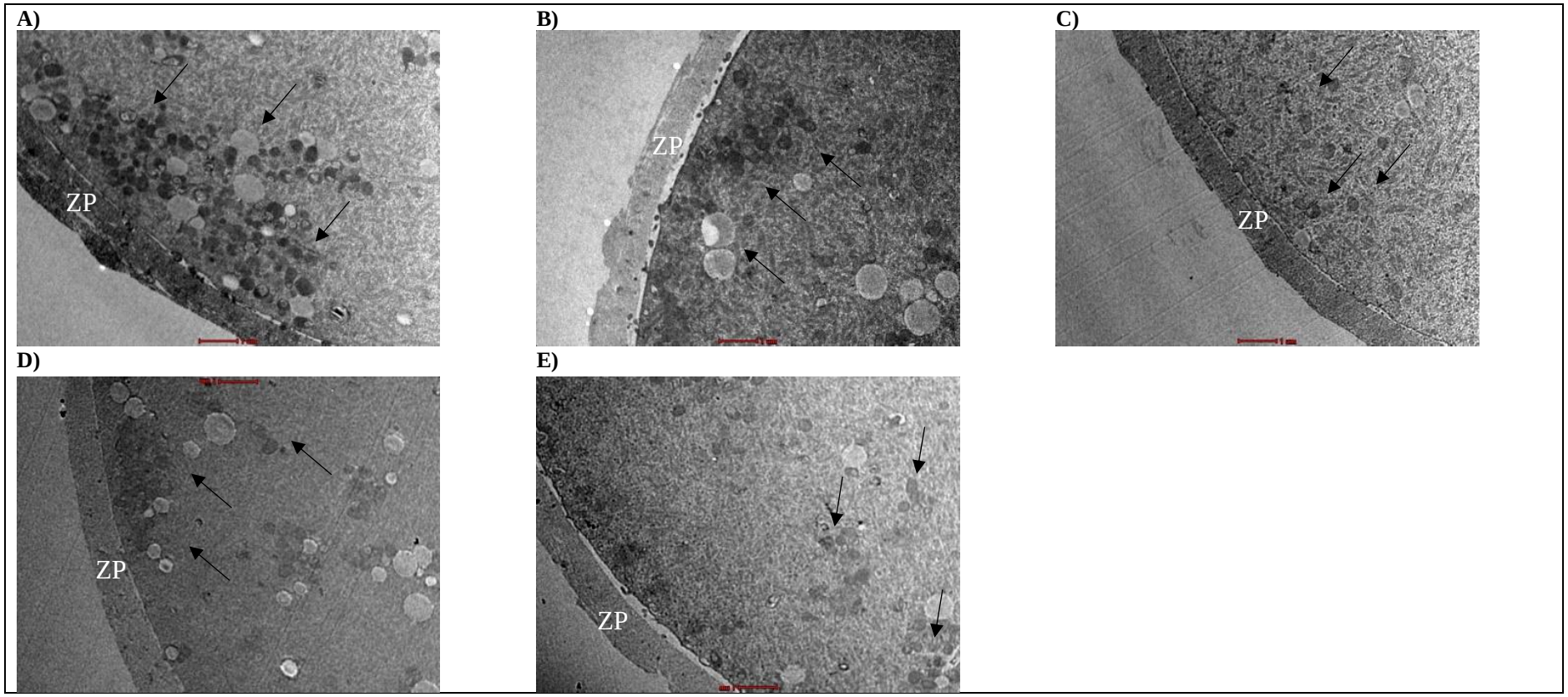


Figure 4.7 Organelles Distribution in 2-cell Embryos

This figure presents the transmission electron micrographs of organelles distribution embryos from SHAM (A), PBS (B), OVA3 (C), POVA (D), and TOVA (E) groups. Mitochondria and lipid droplets were mainly found at the edge of the cytoplasm near the zona pellucida (ZP). SHAM and PBS groups showed many organelles while OVA3 had noticeably fewer. POVA showed some recovery, and TOVA showed a clearer improvement. The ZP remained intact in all groups. Black arrow: peripheral region. Images taken at 4200X magnification. Scale bar: 1 µm.

4.6.2 Mitochondrial Distribution

Given the critical role of mitochondria in cellular energy production and early embryonic development, both the qualitative features and quantitative volume of mitochondria were evaluated in 2-cell stage embryos.

Quantitative analysis (Figure 4.8; Table K.1, Appendix K) revealed that 2-cell embryos from the SHAM and PBS groups exhibited high relative volumes (Vv) of total mitochondria (21.54 ± 1.560% and 20.65 ± 1.053%, respectively) with no significant difference between them. At 4,200× magnification, mitochondria were consistently localised along the peripheral cytoplasmic region adjacent to the zona pellucida (Figure 4.9A and 4.9B).

In contrast, 2-cell embryos from the OVA3 group demonstrated a marked reduction in mitochondrial content with a significantly lower relative volume (7.33 ± 3.266%; $p < 0.01$ vs. PBS) and visibly fewer mitochondria distributed within the cytoplasm (Figure 4.9C). A similar reduction was observed in the POVA group (7.52 ± 0.565%; $p < 0.01$ vs. PBS) accompanied by sparse mitochondrial presence (Figure 4.9D). These findings indicate that OVA-induced allergic asthma whether untreated or supplemented with palm olein adversely affects mitochondrial abundance during early embryogenesis.

However, maternal TRF supplementation (TOVA group) produced a partial recovery in mitochondrial volume. The relative mitochondrial volume increased to 15.59 ± 1.811%, which was higher than in the OVA3 and POVA groups, although not statistically significant. Mitochondria in TOVA embryos were again predominantly distributed at the cytoplasmic periphery (Figure 4.9E) reflecting a more normalized ultrastructural pattern.

As mitochondria were indistinguishable within TEM sections at lower magnifications, quantitative morphological analysis was not feasible. Nonetheless, qualitative assessment at higher magnifications (20,500×–23,000×) revealed that spherical and round-shaped mitochondria were the predominant morphological forms across all groups.

Although not statistically compared, embryos from the SHAM, PBS and TOVA groups exhibited a higher frequency of spherical and round mitochondria relative to the OVA3 and POVA groups. The round-shaped mitochondria were characterised by a dense matrix and the absence of vacuoles and distinct cristae (Figure 4.10A). In

addition, round-shaped mitochondria featuring a central vacuole encircled by cristae (Figure 4.10B).

Evidence of mitochondrial degeneration was present in embryos from all groups. Damaged mitochondria appeared with ruptured or thinned outer membranes leading to leakage of mitochondrial matrix into cytoplasm (Figure 4.10C) that is known as morphological hallmark of mitochondrial stress or early degeneration.

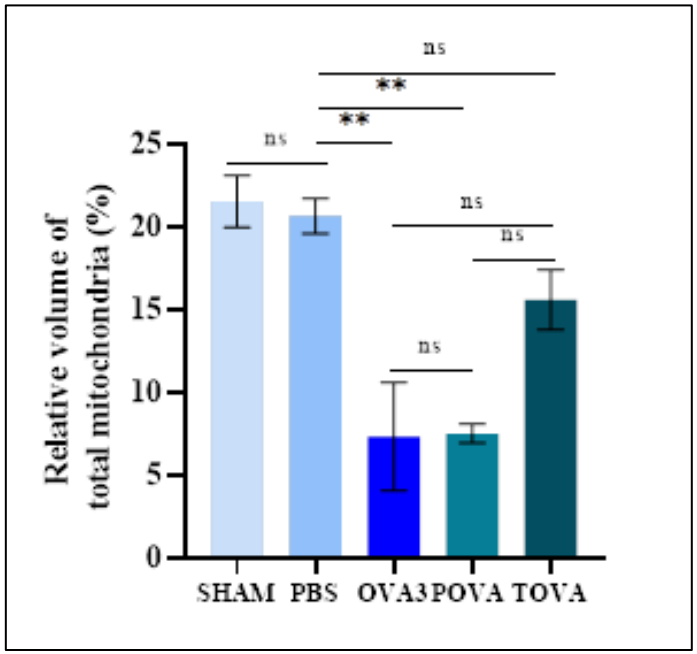


Figure 4.8 Effects of Maternal Tocotrienol-rich Fraction Supplementation on the Relative Volume of Total Mitochondria (%) in 2-Cell Embryos
The figure illustrates the mean relative volume of total mitochondria in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). A significant reduction in mitochondrial volume was observed in the OVA3 and POVA groups compared with PBS ($p \leq 0.01$). TRF supplementation (TOVA) partially restored mitochondrial content, showing a higher mean volume compared with OVA3 and POVA, though not statistically significant. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. ns = not significant. *$p < 0.05$. **$p < 0.01$. Detailed numerical values are provided in Appendix K (Table K.1).

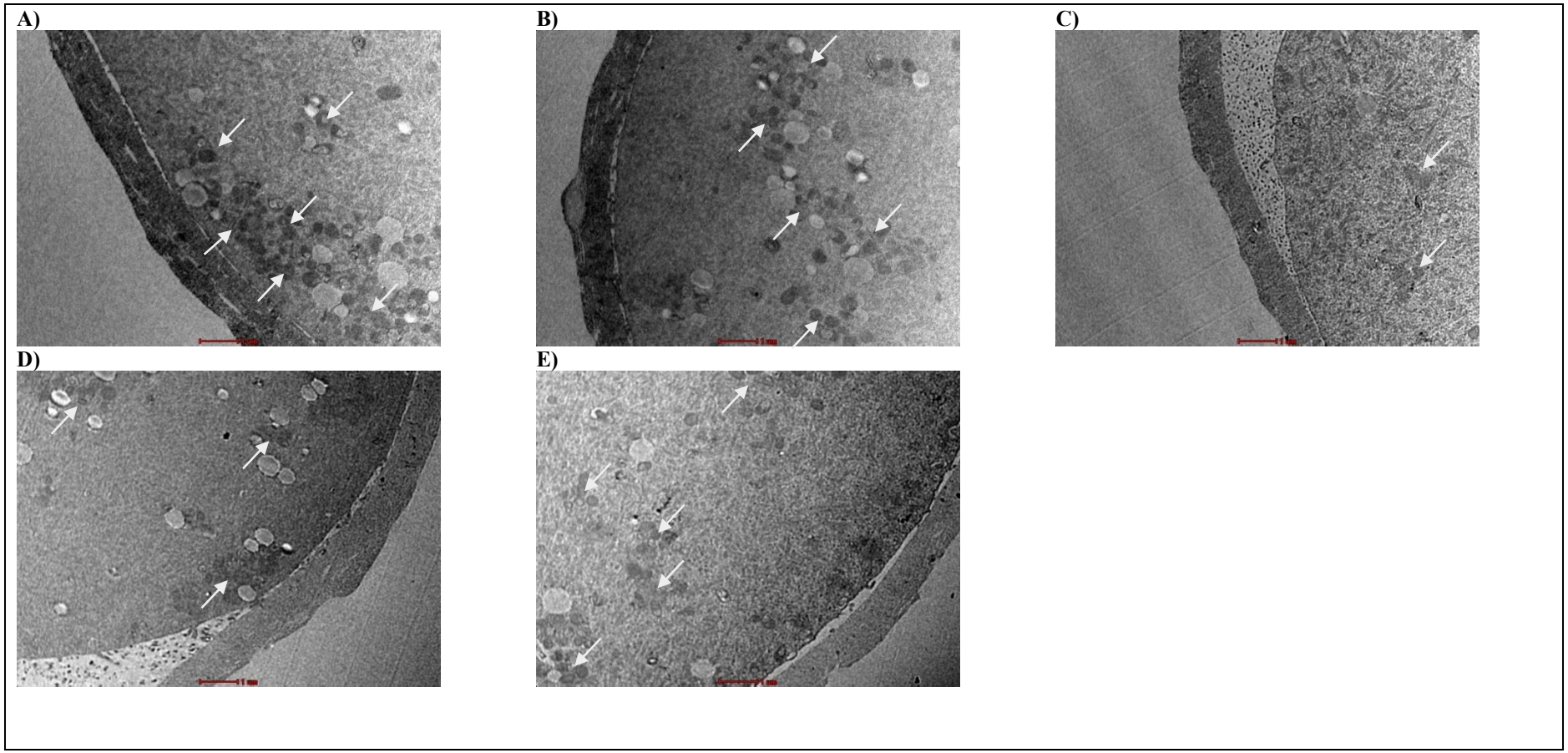


Figure 4.9 Mitochondrial Distribution in 2-cell Embryos

This figure presents the transmission electron micrographs of mitochondria distribution from SHAM (A), PBS (B), OVA3 (C), POVA (D), and TOVA (E) groups. The SHAM and PBS groups exhibited abundant mitochondria concentrated at the cytoplasmic periphery adjacent to the zona pellucida. In contrast, the OVA3 and POVA groups showed noticeably fewer mitochondria, whereas the TOVA group demonstrated an increased mitochondrial presence. White arrows indicate mitochondria. Images were captured at 4,200× magnification; scale bar = 1 µm.

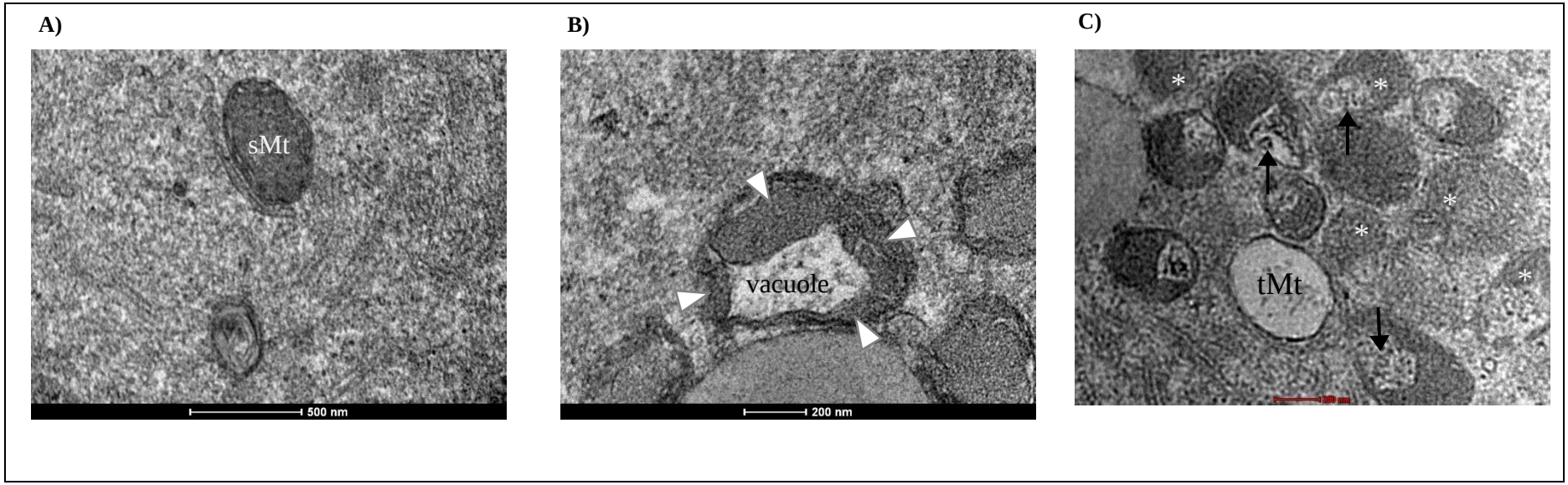


Figure 4.10 Representative Image of Spherical, Round and Damaged Mitochondria Observed in 2-cell Embryos
This figure presents the transmission electron micrographs of mitochondria morphologies in the cytoplasm of 2-cell embryos. (A) Spherical mitochondrion; (B) round mitochondrion; (C) damaged mitochondria exhibiting structural degeneration. sMt: spherical mitochondrion. White arrowhead: peripheral cristae. Black arrow: ruptured mitochondrial membrane. White asterisk: mitochondria fused with the embryonic cytoplasm. Images were captured at 20,500× or 23,000× magnification. Scale bar = 200 nm or 500 nm.

4.6.3 Mitochondrial Clusters

Figure 4.11 illustrates the relative volume (Vv) of mitochondrial clusters (%) in 2-cell embryos. Detailed numerical values are provided in Appendix K (Table K.1). The SHAM and PBS groups exhibited the highest mitochondrial cluster volumes, measured at 19.25 ± 1.617% and 16.70 ± 0.877%, respectively with no significant difference between them. These findings indicate normal mitochondrial clustering under non-asthmatic conditions.

In contrast, embryos derived from the OVA3 group showed a marked reduction in mitochondrial cluster volume (0.646 ± 0.333%, $p < 0.0001$ vs. PBS) suggesting severe disruption of mitochondrial dynamics as a consequence of maternal allergic asthma.

Supplementation with palm olein in the POVA group resulted in a modest increase in mitochondrial clustering (5.81 ± 0.521%), though values remained significantly lower than those of the PBS group ($p < 0.01$). Despite being higher than the OVA3 group, the difference was not statistically significant reflecting the limited restorative potential of palm olein.

In contrast, maternal supplementation with TRF in the TOVA group produced a notable improvement in mitochondrial clustering. The relative volume rose to 13.12 ± 2.161%, significantly higher than both the OVA3 ($p < 0.001$) and POVA ($p < 0.05$) groups. This suggests that TRF effectively restores mitochondrial clustering in embryos exposed to maternal allergic asthma.

At higher magnification (8200×), ultrastructural analysis provided further insight into the morphology and dynamics of mitochondrial clusters. As shown in Figures 4.12A to 4.12E, mitochondria within clusters appeared to fuse into enlarged or irregularly shaped masses where individual mitochondria were no longer clearly distinguishable. Such fused, cluster-like morphologies were frequent observed in SHAM, PBS and TOVA groups but sparse in the OVA3 and POVA groups corroborating the quantitative data of reduced relative volume of mitochondrial clustering under asthmatic conditions.

Additionally, evidence of active mitochondrial fusion was captured in Figures 4.12F and 4.12G, where two mitochondria were seen undergoing matrix merging and partial unification of outer membranes indicative of ongoing fusion events that is critical for maintaining mitochondrial dynamics and energy homeostasis in the early embryo.

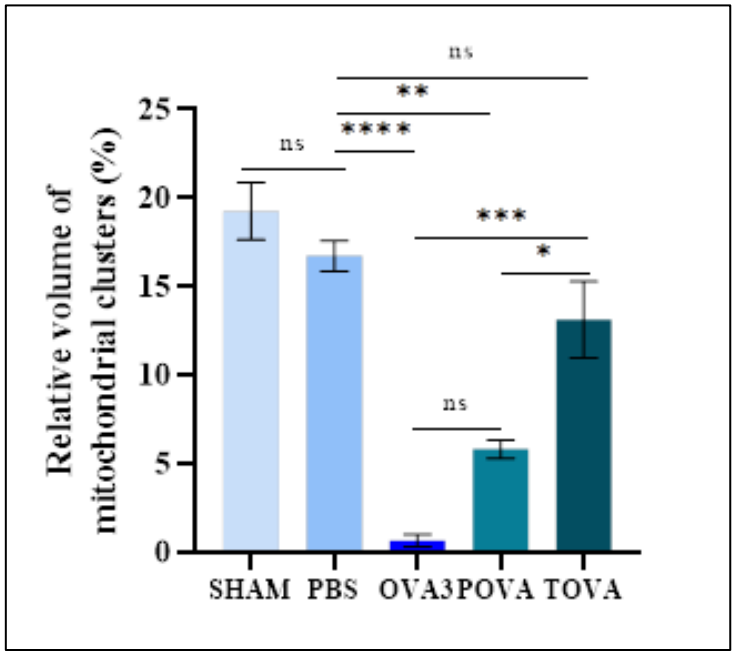


Figure 4.11 Effects of Maternal Tocotrienol-rich Fraction Supplementation on the Relative Volume of Mitochondrial Clusters (%) in 2-Cell Embryos

The figure illustrates the mean relative volume of mitochondrial clusters in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). A significant reduction in mitochondrial clusters was observed in the OVA3 and POVA groups compared with PBS ($p < 0.001$, $p < 0.01$). TRF supplementation (TOVA) partially restored mitochondrial clusters, showing a higher mean volume compared with OVA3 and POVA ($p < 0.01$, $p < 0.05$). Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. ns: not significant. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. ****$p < 0.0001$.

Detailed numerical values are provided in Appendix K (Table K.1).

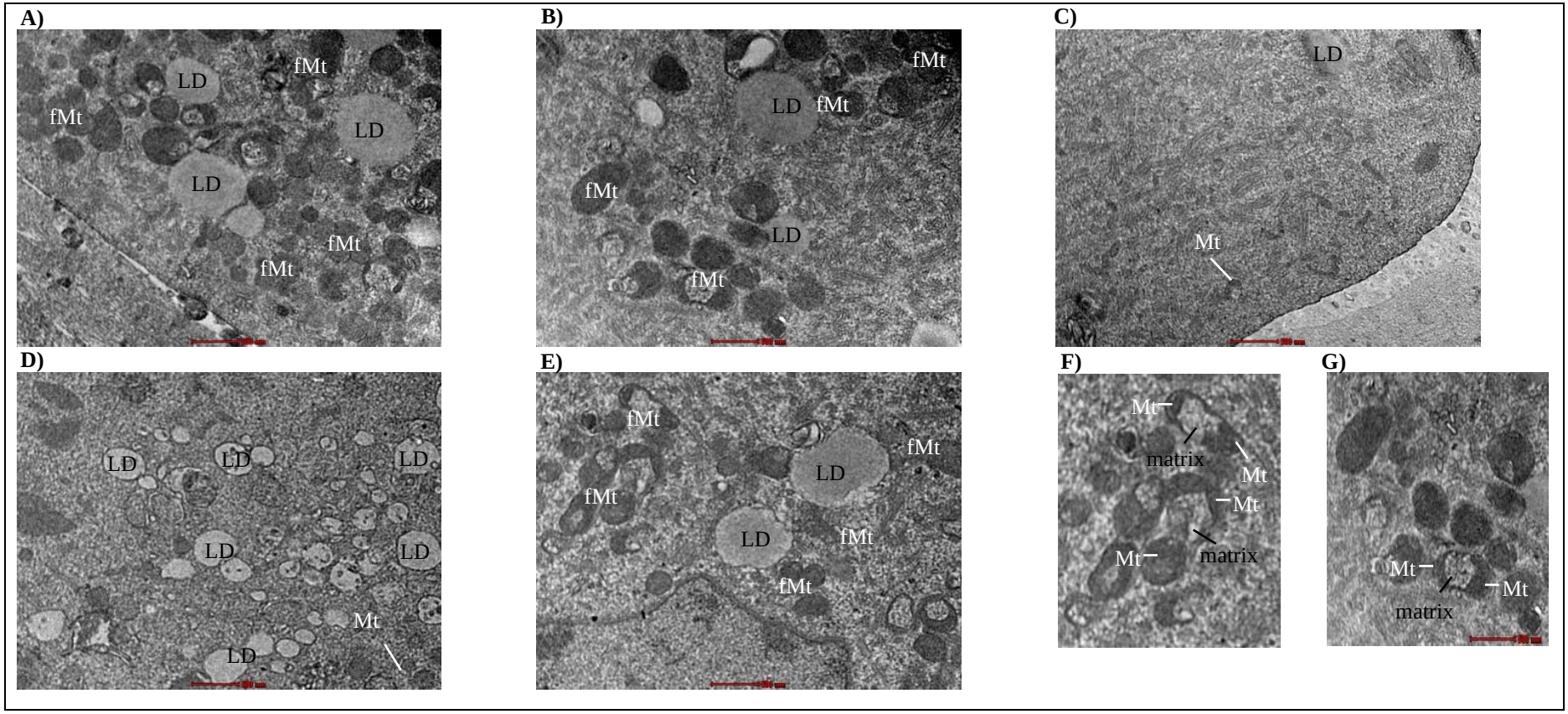


Figure 4.12 Mitochondrial Clusters in 2-cell Embryos

This figure presents the transmission electron micrographs of mitochondria clusters from SHAM (A), PBS (B), OVA3 (C), POVA (D), and TOVA (E) groups. Mitochondrial clusters are prominent in SHAM, PBS, and TOVA groups, while reduced clustering is observed in OVA3 and POVA groups. Panels (F) and (G) illustrate ongoing mitochondrial fusion events, showing partial merging of mitochondrial membranes and matrix continuity. Images were captured at 4200× magnification. Scale bar: 500 nm. fMt: fused mitochondria; Mt: mitochondrion; LD: lipid droplet.

4.6.4 Lipid Droplets and Peridroplet Mitochondria

Figure 4.13 presents the relative volume (Vv) of lipid droplets and peridroplet mitochondria in 2-cell embryos. Detailed numerical values are provided in Appendix K (Table K.1).

4.6.4.1 Lipid Droplet Volume

The SHAM group exhibited the highest lipid droplet volume (6.22 ± 0.567%) followed closely by the PBS group (5.95 ± 1.114%) with no significant difference between them. These findings indicate normal lipid storage and utilisation under non-asthmatic conditions. In contrast, a reduction was observed in the OVA3 group (0.05 ± 0.658%), though the difference did not reach statistical significance.

Supplementation with palm olein in the POVA group increased lipid droplet volume (6.54 ± 1.885%) compared to OVA3 ($p < 0.05$) suggesting partial recovery. The TOVA group (2.64 ± 0.191%) also demonstrated some restoration relative to OVA3 but the value remained significantly lower than in SHAM and POVA groups (ns) indicating that TRF supplementation produced a partial but incomplete recovery of lipid storage capacity.

4.6.4.2 Peridroplet Mitochondria Volume

The SHAM group again recorded the highest peridroplet mitochondrial volume (10.12 ± 1.379%) followed by the PBS group (7.48 ± 0.477%). In contrast, the OVA3 group displayed a marked reduction (0.48 ± 0.480%, $p < 0.01$ vs. PBS) reflecting a severe loss of peridroplet mitochondria and consequently impaired lipid–mitochondrial interactions under maternal allergic asthma conditions.

The POVA group (3.53 ± 1.156%) showed modest, though statistically non-significant improvement compared to OVA3. Notably, TRF supplementation in the TOVA group significantly enhanced peridroplet mitochondrial volume (4.64 ± 0.353%, $p < 0.05$ vs. OVA3) indicating that TRF restored mitochondrial–lipid droplet associations disrupted by maternal allergic asthma.

4.6.4.3 Ultrastructural Observations

Representative ultrastructural images in Figure 4.14 show peridroplet mitochondria at 4200× magnification. Lipid droplets were consistently located in similar spatial regions across all experimental groups and frequently positioned in close proximity to mitochondrial clusters or individual mitochondria (Figure 4.12).

A distinctive feature was the physical attachment of mitochondria to lipid droplets forming peridroplet mitochondria. In these configurations, mitochondria appeared anchored to the lipid droplet surface with the outer mitochondrial membrane merging seamlessly with the lipid droplet membrane. This direct membrane continuity suggests a functional coupling between mitochondria and lipid droplets likely facilitating efficient lipid and energy metabolism during early embryonic development.

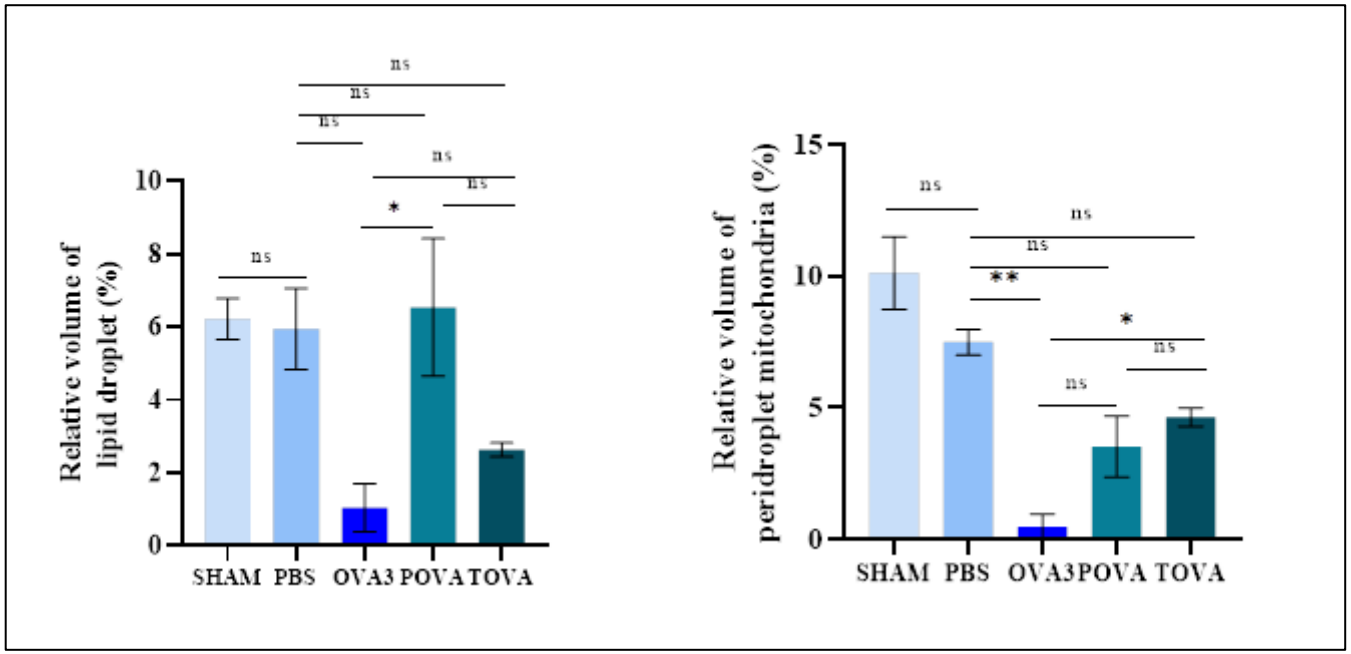


Figure 4.13 Effects of Maternal Tocotrienol-rich Fraction Supplementation on the Relative Volume of Lipid Droplets (%) in 2-Cell Embryos
The figure illustrates the mean relative volume of lipid droplets and peridroplet mitochondria in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). The OVA3 group exhibited a significant reduction in the relative volume of both lipid droplets ($p < 0.05$) and peridroplet mitochondria ($p < 0.01$) compared to the PBS group. The POVA and TOVA groups showed partial recovery, with TOVA-treated embryos displaying higher peridroplet mitochondrial volume ($p < 0.05$ vs. OVA3). Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. **$p < 0.01$; *$p < 0.05$; ns: not significant.
Detailed numerical values are provided in Appendix K (Table K.1).

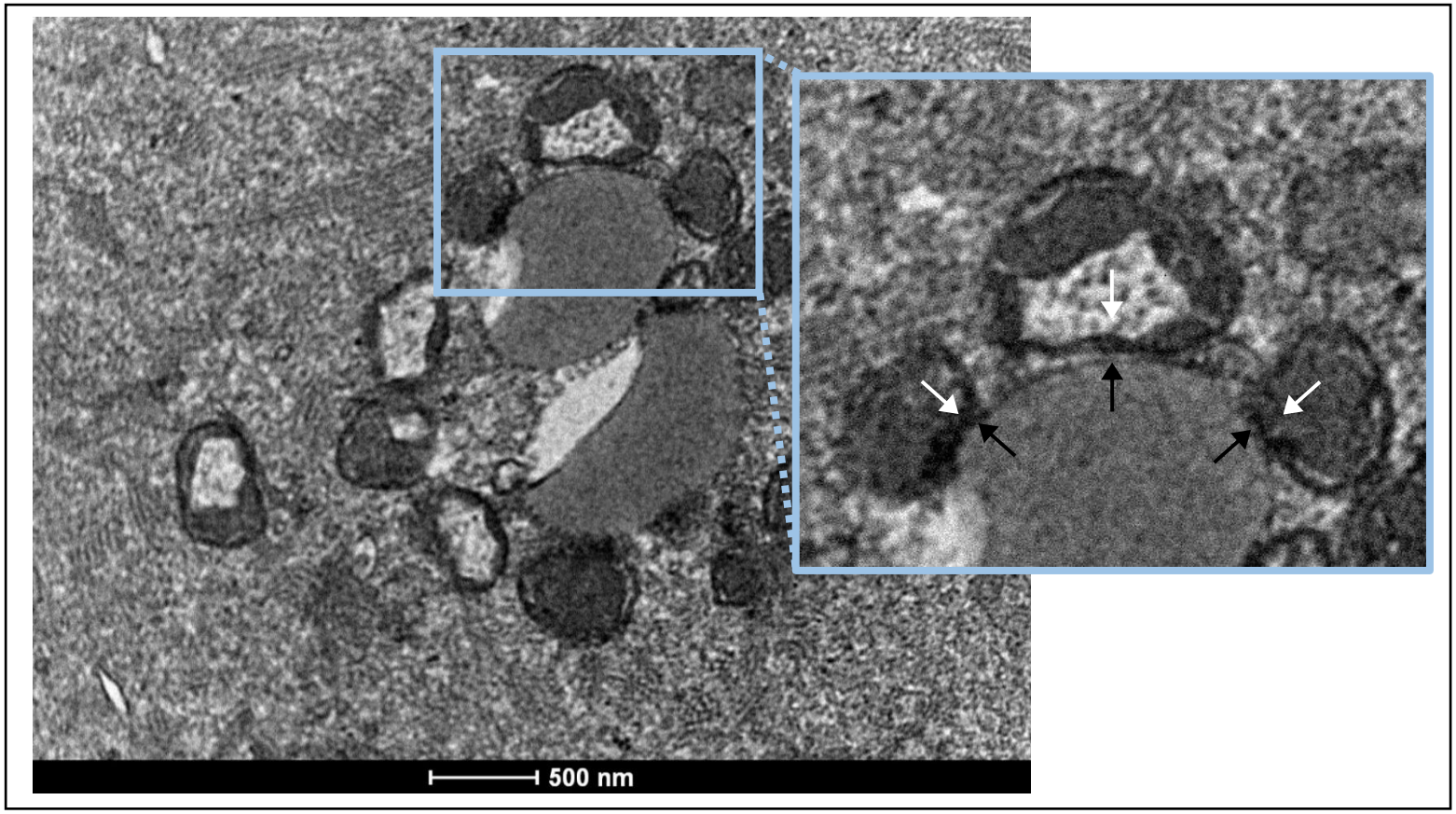


Figure 4.14 Peridroplet Mitochondria in 2-cell Embryos

This figure presents the transmission electron micrographs of peridroplet mitochondria. The inset shows an enlarged view of the attachment site where the outer mitochondrial membrane is closely opposed to, and partially continuous with, the lipid droplet membrane. White arrows indicate mitochondrial membranes, while black arrows indicate lipid droplet membranes. Images were captured at 8200× magnification. Scale bar: 500 nm.

4.6.5 Microvilli, Perivitelline Space and Cellular Degradation Machinery

To evaluate membrane integrity and overall cellular quality, the embryonic membrane of 2-cell embryos was examined for the presence of microvilli. Prominent membrane projections characteristic of microvilli was clearly visible in the SHAM, PBS and TOVA groups (Figures 4.15A, 4.15B and 4.15E) indicating intact membrane specialization and normal morphological development. In contrast, these projections were absent or markedly reduced in the OVA3 and POVA groups (Figures 4.15C and 4.15D) suggesting loss of membrane surface structures and compromised cellular polarity under asthmatic conditions.

Examination of the perivitelline space (PVS) revealed group-dependent differences. Embryos from the SHAM and PBS groups displayed clear PVS with minimal cellular debris (Figures 4.15A and 4.15B) reflecting a healthy extracellular environment. Conversely, the OVA3, POVA and even TOVA groups showed PVS partially occupied with cellular fragments and debris (Figures 4.15C to 4.15E) consistent with cellular stress, degeneration or impaired cytoplasmic clearance mechanisms.

Components of the cellular degradation machinery, notably autophagosomes and lysosomes were identified within the cytoplasm of embryos in all experimental groups (Figure 4.16). Although not quantified, their presence indicates activation of autophagy-related pathways likely in response to oxidative and metabolic stress induced by maternal allergic asthma.

Further ultrastructural analysis (Figure 4.17A and 4.17B) demonstrated the formation of autolysosomes where lysosomes were observed approaching and fusing with autophagosomes confirming active autophagic processing within the embryonic cytoplasm which might lead to apoptosis. Taken together, these qualitative observations suggest that maternal asthma disrupts microvillar integrity and enhances autophagic activity while TRF supplementation (TOVA) partially restores membrane specialization despite the persistence of cytoplasmic stress markers.

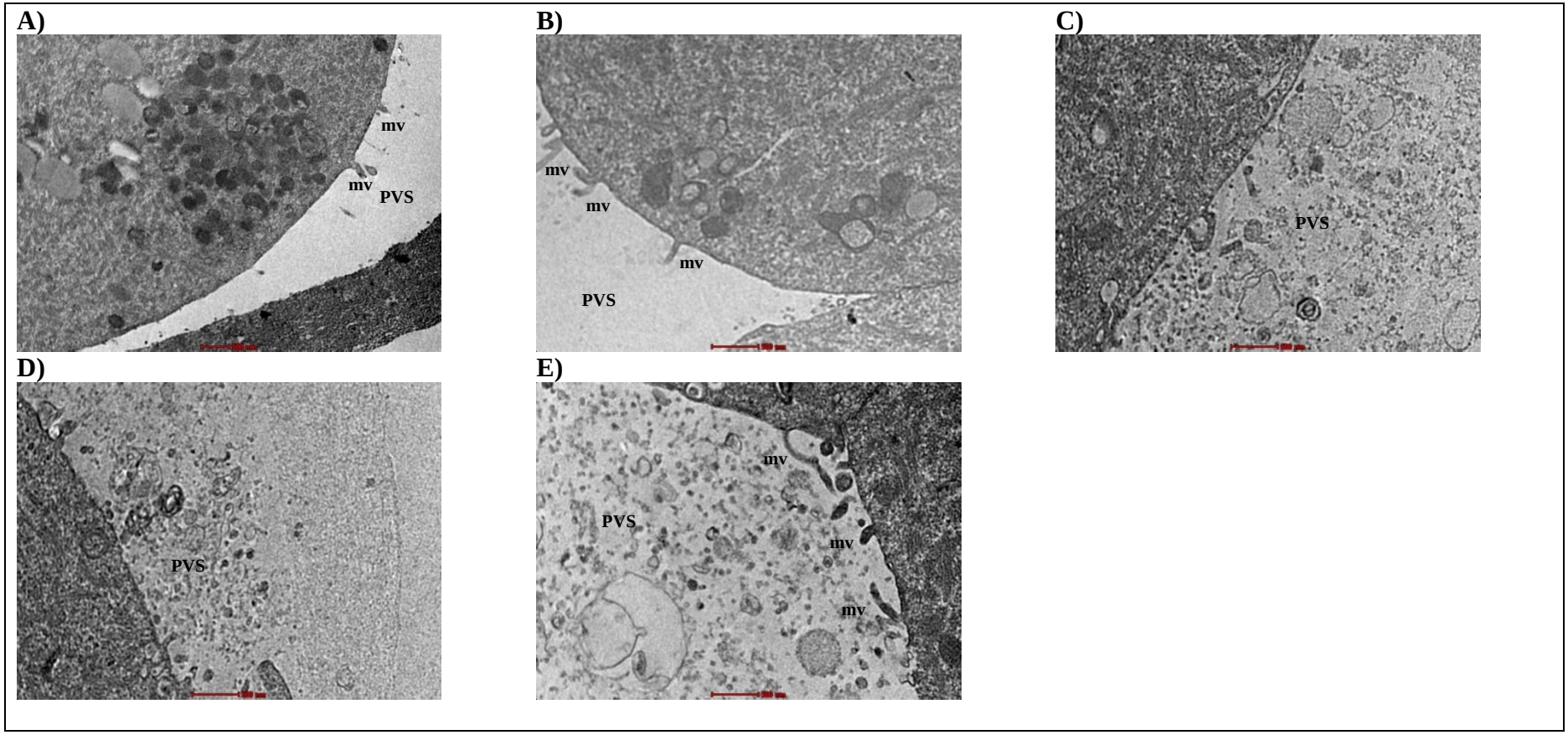


Figure 4.15 Microvilli and Perivitelline Space in 2-cell Embryos

This figure presents the transmission electron micrographs of the microvilli (mv) and perivitelline space (PVS) in 2-cell embryos from the SHAM (A), PBS (B), OVA3 (C), POVA (D), and TOVA (E) groups. Prominent microvilli and clear PVS are visible in the SHAM and PBS groups, while reduced microvillar projections and increased debris within the PVS are observed in OVA3, POVA, and TOVA groups. Images were captured at 8200× magnification. Scale bar: 500 nm.

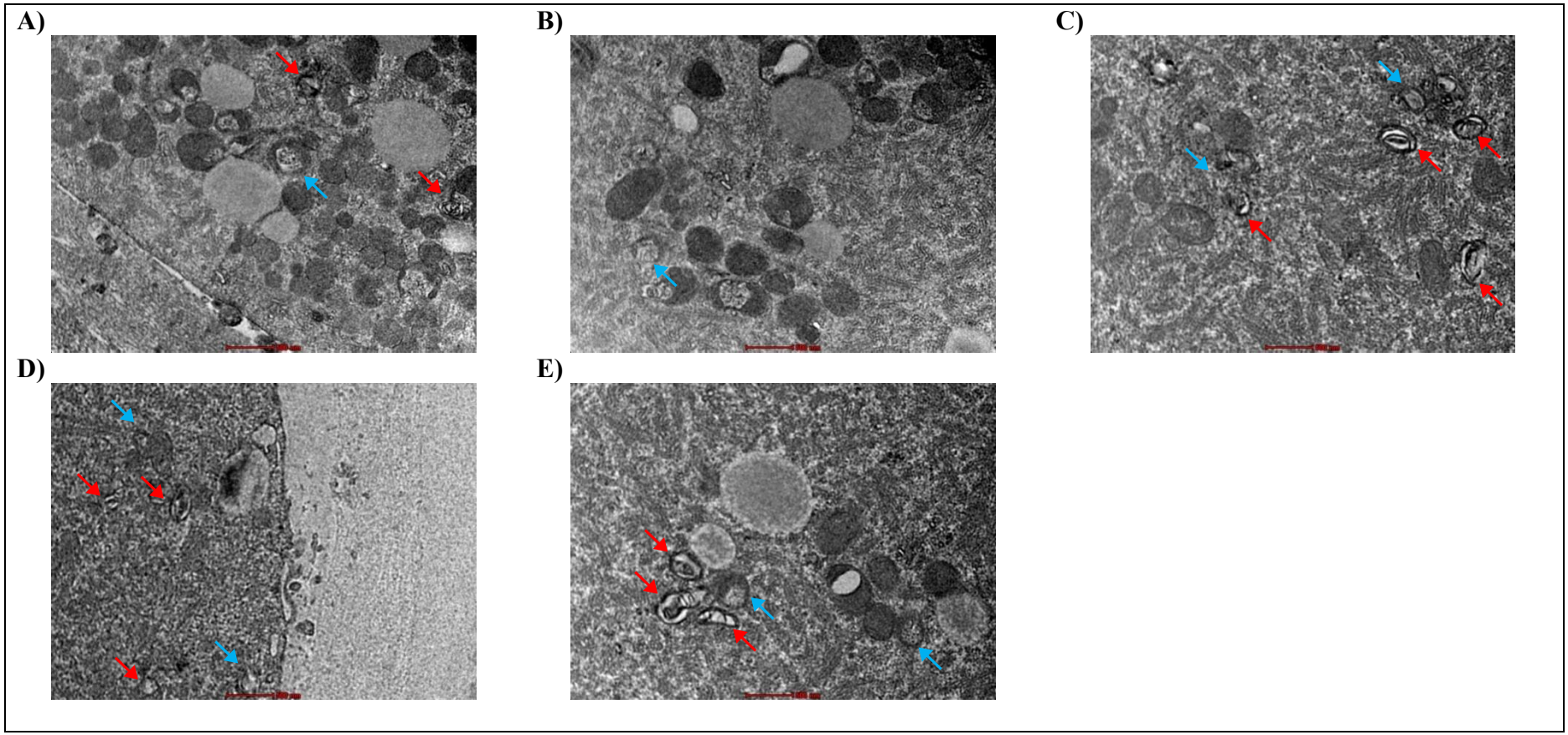


Figure 4.16 Cellular Degradation Machinery in 2-cell Embryos

This figure presents the transmission electron micrographs of cellular degradation machinery from SHAM (A), PBS (B), OVA3 (C), POVA (D) and TOVA (E) groups. Red arrows indicate lysosomes and blue arrows indicate autophagosomes. These organelles were observed within the embryonic cytoplasm, suggesting activation of cellular degradation and autophagy pathways. Images were captured at 8200× magnification. Scale bar: 500 nm.

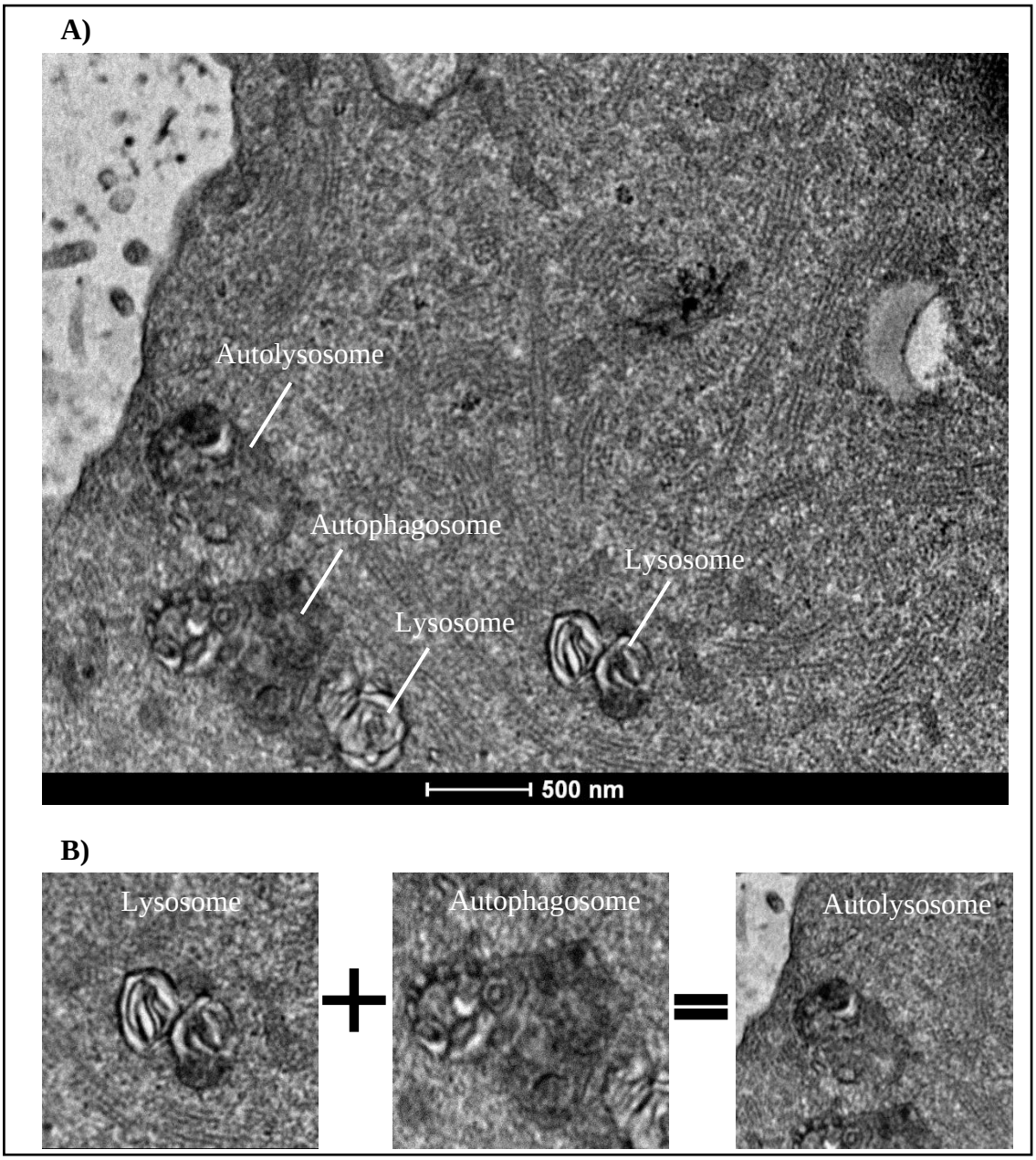


Figure 4.17 Formation of Autolysosome Under Transmission Electron Microscope
This figure presents the transmission electron micrographs of the formation of autolysosomes. (A) Representative image illustrating components of the cellular degradation machinery, including autophagosomes and lysosomes. (B) Sequential stages in the formation of an autolysosome, where lysosomes are seen approaching and fusing with autophagosomes. Images were captured at 8200× magnification. Scale bar: 500 nm.

4.7 The Effects of Maternal Tocotrienol-rich Fraction Supplementation on Gene Expression

Building upon the findings on embryonic development, morphology and ultrastructure, this section further elucidates the molecular mechanisms underlying the observed phenotypic alterations by analysing gene expression profiles of preimplantation embryos. In this study, 2-cell stage embryos were subjected to quantitative polymerase chain reaction (qPCR) analysis.

A total of 225 embryos were collected from all experimental groups (n = 3, with each replicate comprising 15 embryos per group). Four functional gene categories were examined:

i. Oxidative stress genes: *Nox1*, *Sod1* and *Gpx4*
ii. Mitochondrial fusion genes: *Mfn1, Mfn2* and *Opa1*
iii. Mitochondrial bioenergetic genes: *Atp5e* and *Cox4i1*
iv. Apoptosis genes: *Bax* and *Bcl2*

These genes were selected to elucidate the molecular mechanisms contributing to the detrimental effects on preimplantation embryos observed in Sections 4.3–4.5 (development, morphology and ultrastructure) and to assess the protective potential of maternal TRF supplementation in mitigating asthma-induced embryonic stress.

The expression profiles of all 10 targeted genes are summarised in Table 4.5. Gene expression was analysed using relative quantification (RQ) where differences in the expression levels of each gene of interest (GOI) among the experimental groups were evaluated. The results were presented as fold-change values relative to the PBS group which served as the baseline control.

The PBS group was assigned a relative expression value of 1.0-fold for all GOIs. Expression levels above 1.0-fold were interpreted as upregulated whereas levels below 1.0-fold were considered downregulated. Two reference genes, *Actb* and *Gapdh*, were used for normalization to ensure accuracy and consistency across samples.

This approach enabled the assessment of gene expression modulation in SHAM, OVA3, POVA, and TOVA groups relative to normal conditions (PBS group) providing a molecular basis for the developmental and ultrastructural changes described in earlier sections.

Table 4.6
Expression of Genes of Interest in 2-cell Embryos

	SHAM	PBS	OVA3	POVA	TOVA
Oxidative stress genes					
Nox1	↓	-	↑	↑	↓
Sod1	↑	-	↓	↓	↑
Gpx4	↑	-	↓	↓	↑
Mitochondrial fusion genes					
Mfn1	↑	-	↓	↓	↑
Mfn2	↑	-	↓	↓	↑
Opa1	↑	-	↓	↓	↑
Mitochondrial bioenergetics genes					
Atp5e	↑	-	↓	↓	↑
Cox4i1	↑	-	↓	↓	↑
Apoptotic genes					
Bax	↓	-	↑	↑	↓
Bcl2	↑	-	↓	↓	↑

This table summarises the expression patterns of genes of interest (GOIs) related to oxidative stress, mitochondrial fusion, mitochondrial bioenergetics and apoptosis in 2-cell embryos from SHAM, PBS, OVA3, POVA, and TOVA group. ↑: gene is upregulated (>1.0-fold). ↓: gene is downregulated (<1.0-fold). –: no significant change (≈1.0-fold).

4.7.1 Oxidative Stress Genes (*Nox1, Sod1, Gpx4*)

To evaluate oxidative stress status in 2-cell embryos, the expression levels of oxidative stress-related genes (*Nox1, Sod1,* and *Gpx4*) were quantified using qPCR and normalized to respective controls.

As shown in Figure 4.18A, expression of *Nox1*, a gene associated with reactive oxygen species (ROS) generation was significantly elevated in the OVA3 group (13.20 ± 0.018-fold) compared to the PBS group (1.0-fold, $p < 0.001$). A similar increase was observed in the POVA group (12.16 ± 2.799-fold, $p < 0.001$) with no significant difference between OVA3. In contrast, the TOVA group showed a marked suppression of *Nox1* expression (0.748 ± 0.022-fold) compared to both OVA3 and POVA ($p < 0.001$) returning to baseline levels comparable to SHAM and PBS groups. These results indicate that maternal allergic asthma induces oxidative stress via *Nox1* upregulation whereas TRF supplementation effectively mitigates this response.

In Figure 4.18B, *Sod1* expression was markedly downregulated in the OVA3 group (0.229 ± 0.064-fold) compared to PBS (1.0-fold, $p < 0.0001$) reflecting impaired antioxidant defence. The POVA group also showed reduced *Sod1* expression relative to PBS (0.532 ± 0.032-fold, $p < 0.01$) although slightly higher than OVA3 ($p < 0.05$). Notably, the TOVA group demonstrated a significant upregulation of *Sod1* (2.516 ± 0.082-fold) compared to both OVA3 and POVA ($p < 0.0001$) suggesting restoration of antioxidant capacity following TRF supplementation. Despite this recovery, *Sod1* levels in TOVA remained significantly higher than in PBS ($p < 0.0001$) possibly reflecting a compensatory response to prior oxidative insult.

As shown in Figure 4.18C, *Gpx4*, a critical enzyme in lipid peroxide detoxification, exhibited a similar trend with *Sod1*. Both OVA3 (0.206 ± 0.052-fold) and POVA (0.331 ± 0.042-fold) groups showed lower *Gpx4* expression compared to PBS (1.0-fold) although not statistically significant. However, the TOVA group displayed significantly higher *Gpx4* expression (4.659 ± 0.433-fold) than both OVA3 and POVA ($p < 0.0001$) even surpassing PBS levels ($p < 0.0001$). This upregulation reinforces TRF's role in restoring redox balance through enhanced antioxidant gene expression.

In combination, these findings indicate that maternal OVA exposure disrupts oxidative homeostasis in 2-cell embryos by upregulating pro-oxidant (*Nox1*) and downregulating antioxidant (*Sod1* and *Gpx4*) genes. TRF supplementation effectively

counteracts these molecular disruptions, restoring antioxidant defence mechanisms and supporting embryonic resilience under oxidative stress conditions.

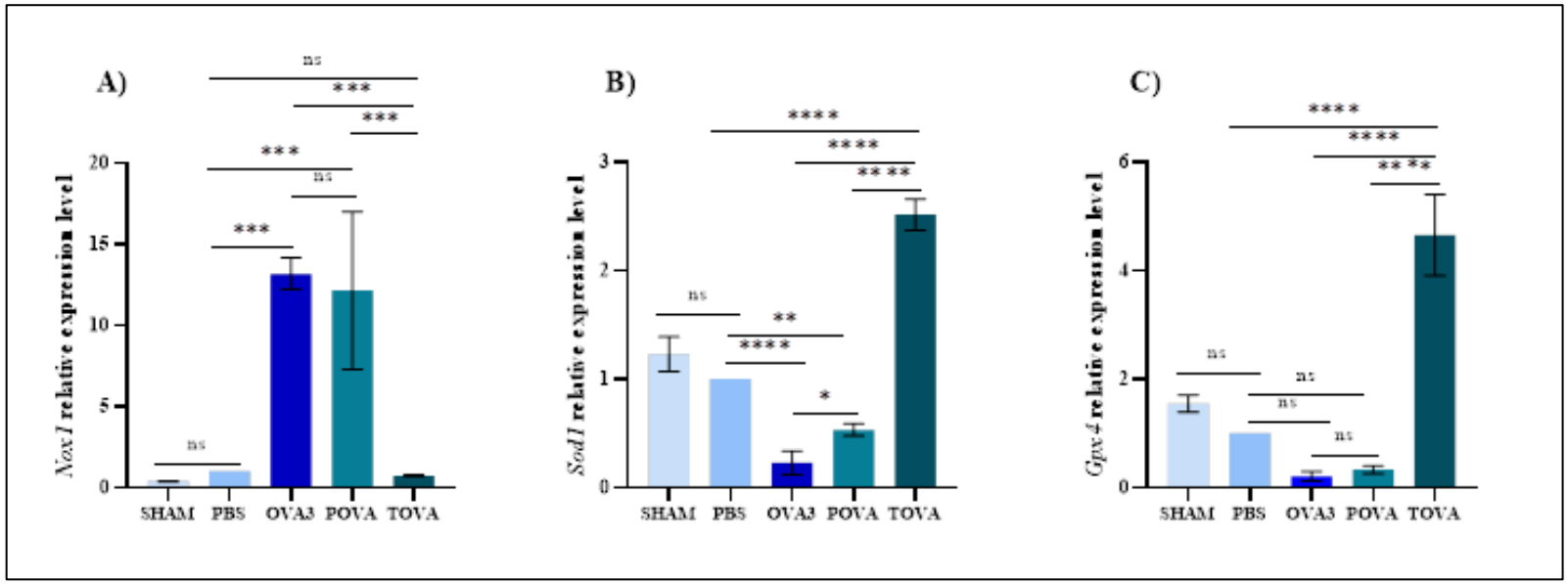


Figure 4.18 Effects of Maternal Tocotrienol-rich Fraction Supplementation on Oxidative Stress Genes Expression in 2-cell Embryos (A) Nox1 gene, (B) Sod1 gene and (C) Gpx4 gene were measured in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). Nox1 expression is significantly upregulated in OVA3 and POVA, while TOVA shows a marked reduction comparable to OVA3 and POVA. Sod1 expression is markedly downregulated in OVA3, partially restored in POVA, and strongly upregulated in TOVA. Gpx4 shows a similar pattern which reduced in OVA3 and POVA (ns), but significantly upregulated in TOVA. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

4.7.2 Mitochondrial Fusion Genes (*Mfn1, Mfn2, Opa1)*

To investigate the potential protective effects of maternal TRF supplementation on mitochondrial fusion activity during preimplantation development, the expression levels of mitochondrial fusion genes (*Mfn1, Mfn2, Opa1*) were quantified using qPCR and normalised to respective controls.

As shown in Figure 4.19A–B, the expression of Mfn1 and Mfn2, both encoding proteins involved in mitochondrial outer membrane (MOM) fusion was reduced in the OVA3 and POVA groups compared to SHAM and PBS although the differences were not statistically significant. In contrast, the TOVA group demonstrated a significant upregulation of both Mfn1 (2.273 ± 0.604-fold) and Mfn2 (2.072 ± 0.370-fold) compared to OVA3 and POVA ($p < 0.05$) suggesting that TRF supplementation promotes mitochondrial fusion which was otherwise suppressed under asthmatic conditions.

Expression of *Opa1*, a key mediator of mitochondrial inner membrane (MIM) fusion, is shown in Figure 4.19C. The SHAM and PBS groups exhibited moderate expression with no significant difference between them. Both OVA3 (0.108 ± 0.007-fold) and POVA (0.138 ± 0.012-fold) groups displayed reduced *Opa1* expression consistent with impaired inner membrane fusion. However, the TOVA group showed a significant restoration of *Opa1* expression (2.049 ± 0.560-fold) compared to both OVA3 and POVA ($p < 0.01$) indicating that TRF supplementation reversed MIM fusion impairment associated with maternal allergic asthma.

Jointly, these findings demonstrate that maternal TRF supplementation markedly improves mitochondrial health in 2-cell embryos from allergic asthma–induced mice. The OVA3 and POVA groups exhibited reduced expression of mitochondrial fusion (*Mfn1*, *Mfn2*, *Opa1*) genes signifying impaired mitochondrial fusion dynamics. Conversely, TRF supplementation (TOVA group) significantly restored their expression suggesting improved mitochondrial fusion dynamics. These results reinforce TRF's potential role in reversing asthma-induced mitochondrial dysfunction during early embryonic development.

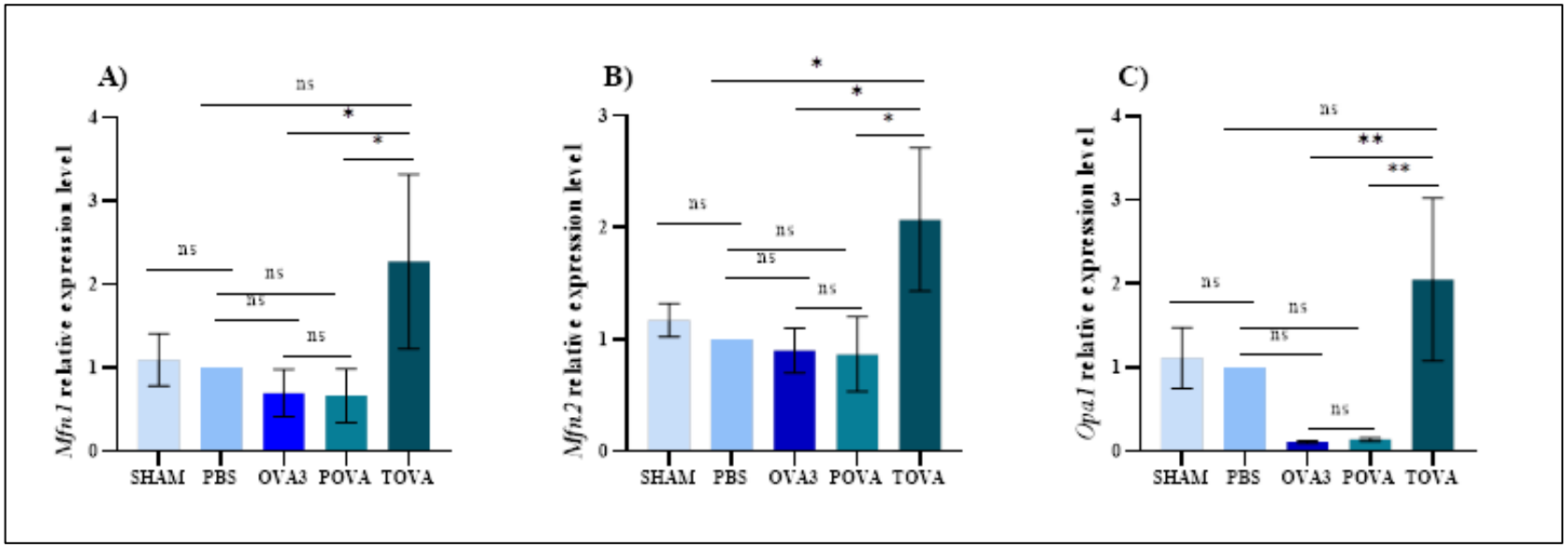


Figure 4.19 Effects of Maternal Tocotrienol-rich Fraction Supplementation on Mitochondrial Fusion Genes Expression in 2-cell Embryos (A) Mfn1 gene, (B) Mfn2 gene and (C) Opa1 gene were measured in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). Mfn1 expression shows no significant difference among SHAM, PBS, OVA3, and POVA groups; however, TOVA embryos exhibit significantly higher Mfn1 expression compared to both OVA3 and POVA. Mfn2 expression follows a similar trend, with significant upregulation in TOVA embryos relative to OVA3 and POVA including PBS. Opa1 was notably reduced in OVA3 and POVA compared to SHAM and PBS (ns) but significantly upregulated in TOVA. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ns: not significant. * $p < 0.05$. ** $p < 0.01$.

4.7.3 Mitochondrial Bioenergetics Genes (*Atp5e, Cox4il*)

To investigate the potential protective effects of maternal TRF supplementation on mitochondrial bioenergetics thru oxidative phosphorylation (OXPHOS) during preimplantation development, the expression levels of ATP synthesis-related and electron transfer-related genes (*Atp5e, Cox4i1*) were quantified using qPCR and normalised to respective controls.

As illustrated in Figure 4.20A, the expression of *Atp5e*, a mitochondrial gene encoding a subunit of ATP synthase was significantly downregulated in the OVA3 group (0.205 ± 0.012-fold) compared to PBS (1.0-fold, $p < 0.01$) reflecting compromised mitochondrial energy production. The POVA group (0.323 ± 0.069-fold) showed a similar trend indicating no substantial improvement. In contrast, the TOVA group exhibited a marked upregulation of *Atp5e* (1.304 ± 0.144-fold) compared to OVA3 and POVA ($p < 0.001$) suggesting enhanced mitochondrial bioenergetics capacity following TRF supplementation.

Similarly, Figure 4.20B shows that *Cox4i1*, a gene encoding a subunit of cytochrome c oxidase (complex IV) of the electron transport chain followed a comparable trend. Both OVA3 and POVA groups demonstrated reduced *Cox4i1* expression relative to SHAM and PBS, though not significant. Remarkably, the TOVA group showed a highly significant increase in *Cox4i1* expression (4.846 ± 0.111-fold) compared to all groups ($p < 0.0001$) indicating enhanced mitochondrial bioenergetics thru improved OXPHOS efficiency attributable to TRF.

These findings demonstrate that maternal TRF supplementation markedly improves mitochondrial health in 2-cell embryos from allergic asthma–induced mice. The OVA3 and POVA groups exhibited reduced expression of mitochondrial bioenergetics (*Atp5e, Cox4i1*) genes signifying impaired mitochondrial bioenergetics function. Conversely, TRF supplementation (TOVA group) significantly enhanced their expression suggesting improved mitochondrial ATP synthesis and OXPHOS capacity. These results reinforce TRF's potential role in reversing asthma-induced mitochondrial dysfunction during early embryonic development.

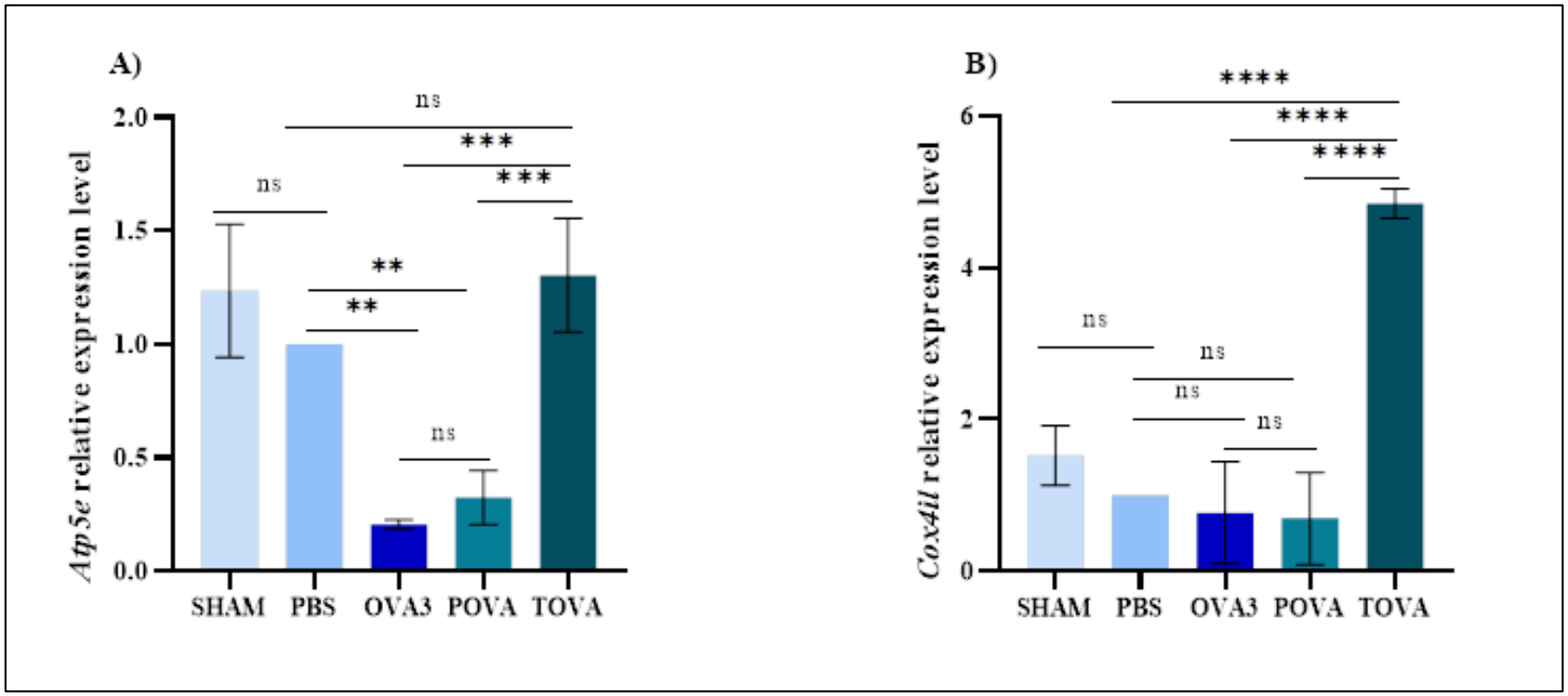


Figure 4.20 Effects of Maternal Tocotrienol-rich Fraction Supplementation on Mitochondrial Bioenergetics Genes Expression in 2-cell Embryos (A) Atp5e gene and (B) Cox4i1 gene were measured in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). Atp5e expression significantly reduced in OVA3 compared to PBS. TOVA embryos show a marked recovery, with expression levels significantly higher than both OVA3 and POVA, comparable PBS. Cox4i1 expression shows a dramatic upregulation in TOVA group, significantly higher than all other groups while no significant difference in other groups. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ns: not significant. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.

4.7.4 Expression of Apoptotic Genes (*Bax*, *Bcl2*)

To examine the influence of maternal TRF supplementation on apoptotic regulation in preimplantation embryos, the expression levels of the pro-apoptotic gene (*Bax*) and anti-apoptotic gene (*Bcl2*) were quantified using qPCR and normalised to the control group.

As shown in Figure 4.21A, expression of *Bax* was significantly elevated in the OVA3 (2.471 ± 0.287-fold) and POVA (2.429 ± 0.451-fold) groups compared to the PBS group (1.0-fold, $p < 0.05$) indicating enhanced pro-apoptotic activity in embryos exposed to asthma-induced oxidative stress. No significant difference was observed between OVA3 and POVA suggesting that palm olein supplementation did not alleviate apoptotic activation. In contrast, the TOVA group showed a marked reduction in *Bax* expression (0.330 ± 0.050-fold) relative to both OVA3 and POVA ($p < 0.001$) reflecting a protective, anti-apoptotic effect of TRF supplementation.

Conversely, Figure 4.21B illustrates the expression pattern of *Bcl2*, a gene that promotes cell survival by inhibiting apoptosis. Both OVA3 (0.480 ± 0.148-fold) and POVA (0.502 ± 0.026-fold) groups exhibited significant downregulation of *Bcl2* compared to the PBS group (1.0-fold, $p < 0.05$ and $p < 0.01$, respectively) confirming the apoptotic imbalance associated with maternal allergic asthma. Notably, the TOVA group demonstrated a significant upregulation of *Bcl2* (2.320 ± 0.078-fold) compared to other groups including PBS, OVA3, and POVA ($p < 0.0001$) suggesting robust anti-apoptotic and cytoprotective effects of TRF.

In summary, maternal allergic asthma (OVA3) increased *Bax* expression and suppressed *Bcl2* expression in 2-cell embryos reflecting heightened apoptotic activity and compromised embryonic viability. Palm olein (POVA) failed to reverse these changes. In contrast, maternal TRF supplementation (TOVA) significantly downregulated *Bax* while upregulating *Bcl2* indicating potent anti-apoptotic protection that preserves embryonic cell integrity and supports early developmental competence under asthmatic conditions.

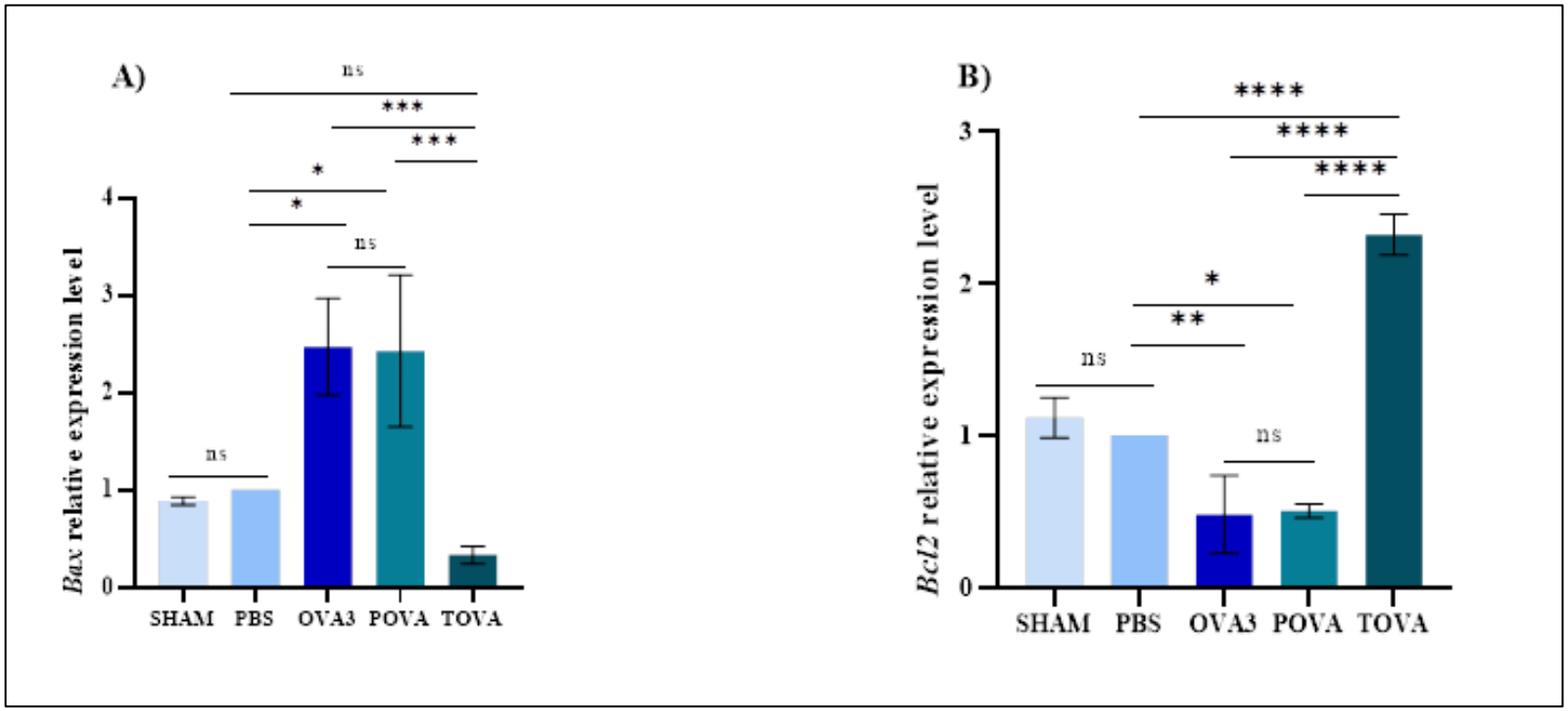


Figure 4.21 Effects of Maternal Tocotrienol-rich Fraction Supplementation on Apoptotic Genes Expression in 2-cell Embryos (A) Bax gene and (B) Bcl2 gene were measured in SHAM (untreated), PBS (vehicle control), OVA3 (OVA-treated group: 150 µg/200 µl), POVA (OVA-treated + palm olein) and TOVA (OVA-treated + TRF). Bax expression is significantly increased in OVA3 and POVA compared to PBS. In contrast, TOVA embryos exhibit a marked reduction in Bax expression relative to OVA3 and POVA. Bcl2 expression is significantly downregulated in OVA3 and POVA compared to PBS. The TOVA group shows a strong upregulation of Bcl2 expression compared to all other groups. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ns: not significant. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.

4.8 Summary of Findings

In Experiment I, maternal exposure to OVA produced dose-dependent detrimental effects on both systemic maternal physiology and preimplantation embryo quality. The severity of inflammation and oxidative stress increased progressively with OVA dose reaching the highest levels in the OVA3 group (150 µg/200 µL) followed by OVA2 and OVA1. Correspondingly, embryonic developmental outcomes were adversely affected in a similar dose-dependent manner. The number of retrieved 2-cell embryos decreased with increasing OVA concentration and the proportion of morphologically abnormal embryos was greatest in the OVA3 group. Additionally, developmental competence to the hatched blastocyst stage was significantly reduced at higher OVA doses.

Based on these collective findings, 150 µg/200 µL OVA (OVA3 group) was selected as the optimal dosage for inducing allergic asthma in Experiment II as it consistently produced marked and reproducible effects on both maternal and embryonic parameters.

In Experiment II, maternal supplementation with TRF was shown to exert significant protective effects in OVA-induced allergic asthma mice benefiting both maternal health and embryonic development. TRF supplementation markedly ameliorated maternal inflammation and oxidative stress confirming its anti-inflammatory and antioxidant properties under asthmatic conditions. These maternal improvements were accompanied by enhanced embryo viability.

At the embryonic level, TRF supplementation improved developmental progression and morphology as evidenced by higher percentages of 8-cell, morula, blastocyst and hatched blastocyst stages along with restored morphological integrity. Ultrastructural analyses revealed increased relative volumes of total organelles and mitochondria, enhanced mitochondrial clustering and more peridroplet mitochondria reflecting improved cellular bioenergetics and metabolic function.

Furthermore, qPCR gene expression analysis supported these observations by demonstrating that TRF supplementation downregulated oxidative stress genes (*Nox1*, *Sod1*, *Gpx4*), upregulated mitochondrial fusion genes (*Mfn1*, *Mfn2*, *Opa1*), and enhanced mitochondrial bioenergetics genes (*Atp5e, Cox4i1*). These molecular changes collectively contributed to the downregulation of pro-apoptotic (*Bax*) and upregulation

of anti-apoptotic (*Bcl2*) genes, thereby preventing apoptosis hence improving embryo viability.

Taken together, these findings provide compelling evidence that maternal TRF supplementation ameliorates asthma-induced systemic inflammation and oxidative stress, while restoring mitochondrial integrity, gene expression balance and developmental competence in preimplantation embryos. TRF thus holds strong potential as a maternal dietary antioxidant intervention to protect early embryonic development under inflammatory and oxidative stress conditions.

CHAPTER 5
DISCUSSION

5.1 Overview of Discussion Chapter

This chapter presents a comprehensive discussion of the study's findings in relation to its research objectives. Each section addresses one of the major experimental aims, integrating the corresponding results with relevant literature to provide a critical interpretation of the observed trends and underlying mechanisms. Section 5.2 discusses the impact of different ovalbumin (OVA) doses on maternal systemic health and developmental potential and morphological quality of 2-cell embryos, establishing the dose-dependent nature of allergic asthma–induced stress. Subsequent sections (5.3–5.6) explore the protective effects of maternal tocotrienol-rich fraction (TRF) supplementation on various aspects of embryonic viability such as developmental potential and morphological quality, ultrastructural features, and gene expression related to oxidative stress, mitochondrial fusion and bioenergetics, and apoptosis. Section 5.7 presents the mechanistic insights on 1) integrating key findings, 2) how maternal TRF supplementation protects mitochondrial fusion, and 3) how maternal TRF supplementation enhances interaction with lipid droplets. Then, Section 5.8 elucidates the implication of findings in clinical practice.

5.2 Impact of Different Ovalbumin Doses on Maternal Health and Preimplantation Embryos in a Murine Allergic Asthma Model

The findings from Experiment I provide crucial insight into the dose-dependent impact of OVA-induced allergic asthma on maternal systemic health and preimplantation embryo development in mice. Three OVA doses i.e., 50 µg/200 µL (OVA1), 100 µg/200 µL (OVA2), and 150 µg/200 µL (OVA3) were tested. The results demonstrated that the highest dose (OVA3) produced the most severe maternal inflammation and oxidative stress, accompanied by marked impairment in embryo morphology and developmental competence. These findings clearly establish a dose–response relationship between maternal allergic inflammation and embryonic outcomes.

5.2.1 High Dose of Ovalbumin (150µg/200µL) Induced the Most Pronounced Maternal Inflammation and Oxidative Stress Accompanied by Detrimental Effects on 2-cell Embryos

At the lowest dose (50 µg/200 µL, OVA1), mild inflammation and moderate oxidative stress caused a partial reduction in morphologically normal 2-cell embryos and a modest decline in development to the blastocyst stage. Some embryos displayed uneven blastomeres and fragmentation, although many still developed normally (Figures 4.0–4.1; Table 4.0).

At the intermediate dose (100 µg/200 µL, OVA2), stronger inflammation and oxidative stress further damaged embryo morphology and development, indicating increased oxidative injury and reduced antioxidant protection.

At the highest dose (150 µg/200 µL, OVA3), these adverse effects were most severe. Mice in this group showed marked elevations in serum interleukin (IL)-4, immunoglobulin (Ig)-E, and 8-hydroxy-2'-deoxyguanosine (OHdG) levels, alongside a significant decline in total antioxidant capacity (TAOC). This confirms that higher OVA exposure amplifies allergic inflammation and oxidative DNA damage, peaking at 150 µg/200 µL. Consequently, preimplantation embryos exhibited extensive fragmentation, uneven blastomeres, and developmental arrest, reflecting a profound loss of developmental potential (Figure 4.0–4.1 and Table 4.0).

The elevated IL-4 and IgE levels confirm the establishment of a T helper (Th)2-dominant allergic response, consistent with previous OVA-induced asthma models (Hajimohammadi et al., 2020; Y. Li et al., 2019; Sung et al., 2019). These systemic immune shifts can extend to the reproductive tract, altering maternal–embryo communication and endometrial receptivity (J. L. Robinson et al., 2024; H. Wang et al., 2020).

In parallel, increased 8-OHdG and reduced TAOC levels indicate maternal oxidative DNA damage and diminished antioxidant defence. Such an environment exposes embryos to excessive reactive oxygen species (ROS), which can cross the uterine barrier and compromise the embryonic microenvironment (Bazan-socha et al., 2022). Elevated ROS disrupt DNA integrity, mitochondrial function, and cell cycle progression, leading to abnormal cleavage patterns, cytoplasmic fragmentation, and developmental arrest (Deluao et al., 2022; Divvela et al., 2025).

Sustained oxidative stress during early development may also trigger epigenetic and transcriptional alterations, predisposing offspring to metabolic and allergic disorders later in life, aligning with the Developmental Origins of Health and Disease (DOHaD) framework (Deluao et al., 2022; Divvela et al., 2025). These findings highlight how maternal allergic asthma disrupts the delicate oxidative–antioxidative balance, impairing both immediate embryo viability and potential long-term health outcomes.

Considered all the findings, Experiment I established that 150 µg/200 µL OVA (OVA3) is the optimal dose for inducing a reproducible and severe murine allergic asthma model. This dose produced consistent and measurable systemic inflammatory and oxidative stress responses, alongside clear embryonic morphological abnormalities.

This robust and well-characterised model thus provided a reliable platform for Experiment II, which investigated the protective effects of maternal TRF supplementation. By using the OVA3 dose, the subsequent experiment could evaluate whether TRF's antioxidant and anti-inflammatory properties can counteract the detrimental maternal and embryonic effects observed in Experiment I, thereby elucidating potential mechanistic and therapeutic pathways for improving reproductive outcomes in allergic conditions.

5.3 Protective Effects of Maternal Tocotrienol-rich Fraction Supplementation in OVA-Induced Allergic Asthmatic Mice

Following the establishment of the optimal allergic asthma model in Experiment I, Experiment II aimed to determine whether maternal supplementation with TRF could mitigate the systemic inflammatory and oxidative stress responses induced by OVA and, in turn, protect preimplantation embryos.

The TRF, a potent form of vitamin E composed of α-, β-, γ-, and δ-tocotrienols, has been shown to exert antioxidant, anti-inflammatory, and immunomodulatory effects in various models of oxidative injury. Its ability to scavenge free radicals particularly ROS, stabilise cellular membranes, and downregulate pro-inflammatory cytokines makes it a promising candidate for restoring homeostasis in maternal tissues exposed to allergic inflammation.

5.3.1 Maternal Tocotrienol-rich Fraction Attenuates Maternal Systemic Inflammation

The findings of this study suggest that TRF supplementation modulates maternal immune balance during allergic asthma, primarily by attenuating the Th2-skewed immune response that characterises allergic inflammation. Maternal mice sensitised with OVA exhibited elevated systemic IL-4 and IgE levels, particularly in the OVA3 and POVA groups, both hallmark indicators of a Th2-dominant allergic profile (J. Wang et al., 2023). In contrast, TRF supplementation significantly reduced these elevations, demonstrating a clear anti-inflammatory role for TRF.

This anti-inflammatory effect was further supported by the finding that IL-4 production was suppressed in the TRF-supplemented (TOVA) group. By reducing IL-4, TRF helps to rebalance the Th2-predominant immune response, which is reflected by the corresponding reduction in IgE levels. Consequently, TRF mitigates the excessive Th2 inflammation that contributes to airway hyperreactivity and systemic allergic manifestations, thereby improving overall maternal health (Kleniewska & Pawliczak, 2025; Lebold et al., 2020). These findings are consistent with those of Zainal et al., (2019), who reported that TRF effectively modulated Th2-associated inflammatory markers (IL-4, IL-13, and IgE) in a female asthmatic rat model.

Although IL-4 and IgE levels in TRF-treated mice (TOVA) remained higher than in the PBS control group, the reduction was both robust and statistically significant, underscoring the immunomodulatory potential of TRF. This aligns with broader evidence indicating that controlling Th2 cytokines during pregnancy can improve maternal well-being and reduce adverse outcomes associated with allergic inflammation (Kleniewska & Pawliczak, 2025).

In summary, TRF acts as an anti-inflammatory and immunomodulatory agent by partially suppressing Th2-associated cytokines and immunoglobulins, thereby restoring immune equilibrium during allergic asthma. This immune regulation contributes to healthier maternal systemic conditions, which are essential for sustaining both maternal and embryonic well-being.

5.3.2 Maternal Tocotrienol-rich Fraction Supplementation Attenuates Systemic Oxidative Stress

The evaluation of serum 8-OHdG and TAOC levels in OVA-induced allergic asthma mice highlights the antioxidant protective role of TRF. Asthmatic mice in the OVA3 and POVA groups exhibited a marked increase in serum 8-OHdG, indicating elevated oxidative DNA damage, together with a reduction in TAOC, reflecting depleted systemic antioxidant defences. TRF supplementation substantially lowered serum 8-OHdG levels, demonstrating a strong ability to mitigate oxidative DNA injury. However, TRF did not significantly restore TAOC, suggesting that while it effectively reduces oxidative damage, it may not fully replenish overall systemic antioxidant capacity.

Despite the absence of significant TAOC restoration, TRF retains the ability to reduce oxidative DNA damage through targeted antioxidant action, possibly at the mitochondrial or cellular level, where ROS generation plays a pivotal role in asthma pathophysiology (Jaffer et al., 2015). This site-specific antioxidant activity is particularly important because oxidative stress is considered a key mediator of tissue injury and embryonic impairment in allergic asthma (Bazan-socha et al., 2022; Deluao et al., 2022; Divvela et al., 2025). By attenuating oxidative DNA damage, TRF may improve embryonic developmental outcomes, supporting its therapeutic potential in managing oxidative stress–related pregnancy complications such as preterm birth and low birth weight (Meakin et al., 2020; Murphy, 2015).

The findings indicate that TRF enhances specific antioxidant enzyme activities rather than broadly increasing circulating antioxidant levels. Several studies have demonstrated that TRF supplementation significantly elevates the activities of catalase (CAT) and superoxide dismutase (SOD) at the cellular level, both of which are crucial for neutralising ROS within mitochondria and other intracellular compartments (Khor et al., 2017; Sadikan et al., 2022). Therefore, the antioxidant action of TRF appears to be targeted toward improving the efficiency of endogenous enzymatic defences through direct ROS scavenging, rather than simply augmenting overall systemic antioxidant concentrations.

In summary, TRF supplementation reduces oxidative DNA damage without substantially increasing TAOC levels, by enhancing the function of endogenous antioxidant enzymes that mediate cellular and mitochondrial ROS detoxification.

Consequently, the attenuation of systemic oxidative stress contributes to improved maternal health, a condition essential for supporting normal preimplantation embryo growth under allergic asthma conditions.

5.3.3 Maternal Tocotrienol-rich Fraction Supplementation Exhibits No Significant Effect on Early Pregnancy Hormones

In this study, maternal TRF supplementation in OVA-induced allergic asthma mice did not produce statistically significant changes in systemic pregnancy hormones, specifically serum progesterone (P_4) and estrogen (E_2) levels. Although minor trends were observed, these fluctuations did not reach statistical significance. The findings therefore suggest that TRF may exert only a mild modulatory influence on endocrine regulation during early pregnancy under allergic conditions.

The quantification of P_4 and E_2 was performed at 1.5 days post coitum (d.p.c.), a stage that represents the immediate post-fertilisation period. In mice, 1.5 d.p.c. is considered too early for major endocrine changes to occur (S. Li & Winuthayanon, 2017). At this stage, ovarian and uterine signalling mechanisms are only beginning to respond to fertilisation, while the peak functional roles of P_4 and E_2, including the regulation of uterine blood flow, vascular remodelling, and endometrial preparation for implantation are typically observed at later stages (Soma-pillay et al., 2016).

Hence, it is plausible that the timing of sample collection influenced the outcome, as later stages such as 3.5–5.5 d.p.c., coinciding with implantation and early placental hormone activity, are more likely to reveal pronounced and biologically relevant hormonal differences. These time points are associated with critical endocrine transitions that support embryo implantation, trophoblast invasion, and early conceptus development (Soma-pillay et al., 2016).

In summary, the absence of significant TRF effects on P_4 and E_2 may be attributed to early sampling at 1.5 d.p.c., before the onset of major hormonal surges. Future studies should incorporate later time points or larger sample sizes to better evaluate the potential endocrine effects of TRF during pregnancy.

Nevertheless, this study provides compelling evidence that maternal TRF supplementation substantially reduces systemic inflammation and oxidative DNA damage in allergic asthma, with favourable trends in hormonal modulation. Collectively, these findings elucidate the therapeutic potential of TRF in ameliorating

allergic asthma–related reproductive risks and enhancing early embryonic development, forming a foundation for future mechanistic and translational research.

5.4 Protective Roles of Maternal Tocotrienol-rich Fraction Supplementation on Developmental Potential and Morphological Quality of 2-cell Embryos

The findings of this study demonstrate that maternal TRF supplementation significantly enhances both the developmental potential and morphological quality of 2-cell embryos in OVA-induced allergic asthma mice. TRF supplementation increased the proportion of 2-cell embryos progressing to the hatched blastocyst stage accompanied by improved morphological quality across key developmental milestones. These enhancements indicate that TRF effectively supports embryonic developmental potential and cellular integrity under conditions of inflammation and oxidative stress. Taken together, these results emphasie the protective role of TRF in maintaining embryo viability during maternal allergic asthma. The observed benefits are likely mediated through TRF's combined anti-inflammatory and antioxidant actions which collectively create a more favourable maternal–embryo environment conducive to early development.

5.4.1 Maternal Tocotrienol-rich Fraction Supplementation Improves Developmental Potential of 2-cell Embryos

Both the OVA3 and POVA groups exhibited a marked reduction in the number of embryos progressing from the 2-cell stage to the hatched blastocyst reflecting the profound impact of maternal systemic inflammation and oxidative stress during the early post-fertilisation period (1.5 d.p.c.). These maternal disturbances likely altered the intrauterine environment and compromised embryo survivability.

In contrast, maternal TRF supplementation in the TOVA group substantially enhanced embryonic development across all preimplantation stages with the most notable improvements observed at the 8-cell, morula, blastocyst and hatched blastocyst stages compared to both OVA3 and POVA groups. This clearly indicates that TRF effectively ameliorates the developmental impairments caused by allergic asthma.

The observed improvements align with the well-established antioxidant and anti-inflammatory roles of TRF which likely ameliorated the hostile uterine milieu

induced by asthma-related oxidative stress and inflammation (Listyoko et al., 2024). By reducing oxidative DNA damage and modulating inflammatory cytokines in maternal mice, TRF may have enhanced embryo survivability and supported the cellular energy metabolism essential for successful preimplantation development (Arribat et al., 2019; Maheshwari et al., 2022). These results are consistent with previous findings demonstrating TRF's capacity to inhibit ROS generation, suppress inflammatory signalling pathways, and activate endogenous antioxidant defences (Looi et al., 2024; Mutalip et al., 2018).

While these improvements are likely mediated through systemic maternal effects, there remains the possibility that TRF or its bioactive metabolites exert direct local actions within the reproductive tract. Owing to their lipophilic nature, tocotrienol compounds can cross biological membranes (Kabir et al., 2017; Kowalska, 2024) suggesting that TRF or its metabolites may have penetrated the uterine barrier, accumulated in endometrial tissues, or even reached the preimplantation embryos. Such actions could provide localised antioxidant protection and reduce oxidative stress at the cellular and mitochondrial levels within the embryos.

The presence of TRF or its metabolites in uterine fluid could further facilitate ROS scavenging within the embryonic microenvironment which directly protecting embryonic cells from oxidative injury. However, studies exploring the direct embryotropic effects of TRF remain limited. Future investigations should include *in vitro* embryo culture models supplemented with TRF to determine its direct impact on embryonic development, oxidative stress modulation, and cellular integrity which those are independent of maternal systemic influences. Such studies would clarify whether TRF acts solely via maternal improvement or also exerts direct protective effects on the embryo.

5.4.2 Maternal Tocotrienol-rich Fraction Supplementation Enhances Morphological Quality of 2-cell Embryos

Morphological assessment in this study demonstrated that maternal TRF supplementation significantly restored normal embryo morphology particularly at the morula and blastocyst stages. The TOVA group showed a notably higher proportion of embryos exhibiting even blastomere size, minimal fragmentation, complete compaction and full blastocoel expansion. The presence of a well-defined trophectoderm (TE) cells

and inner cell mass (ICM) in these blastocysts further indicates improved cellular differentiation and overall embryo quality. These morphological parameters are crucial as early embryo quality strongly predicts implantation potential and pregnancy success (Maheshwari et al., 2014; Sciorio et al., 2025; Yazdani et al., 2024).

The observation that 14 % and 16 % of embryos still progressed to the hatched blastocyst stage despite severe early morphological abnormalities in OVA and POVA group presents an apparent paradox. This finding suggests that preimplantation embryos possess intrinsic resilience mechanisms that permit partial developmental recovery at later stages. The recovery observed at the blastocyst stage may be attributed to embryonic self-correction processes (Munné et al., 2005). During early cleavage, oxidative stress can induce blastomere fragmentation, chromosomal instability, and cytoplasmic abnormalities (S. H. Lee & Rinaudo, 2024). As development proceeds, embryos may selectively eliminate damaged or aneuploid blastomeres through apoptosis and cellular fragmentation (Orvieto et al., 2020). The expulsion of abnormal cells enables the remaining viable blastomeres to continue proliferation and lineage specification, ultimately forming structurally competent blastocysts capable of hatching (X. Wang et al., 2023).

Thus, although maternal asthma-induced oxidative stress severely disrupts early morphology, a subset of embryos retains sufficient developmental plasticity to overcome initial damage. In contrast, TRF supplementation appears to reduce the extent of early oxidative insult, thereby minimising the need for extensive self-correction and preserving structural integrity from earlier developmental stages (Orvieto et al., 2020). Collectively, the protective effects of TRF on embryo morphology and competence are likely mediated by its capacity to attenuate maternal systemic inflammation and oxidative stress as demonstrated in Sections 5.2.1 and 5.2.2.

By reducing key allergic mediators such as IL-4 and IgE and lowering oxidative DNA damage, TRF creates a favourable uterine environment that promotes optimal embryo development (Arribat et al., 2019; Maheshwari et al., 2022). Moreover, tocotrienols have been reported to modulate mitochondrial function and reduce ROS production which may directly benefit embryonic cells by preserving energy metabolism and reducing oxidative injury (Nowak et al., 2012).

While these findings provide strong evidence for the protective effects of TRF on early embryo morphology, further research is warranted to elucidate the molecular and cellular mechanisms involved. Future studies should explore embryonic gene

expression, epigenetic modifications and mitochondrial dynamics to better understand how TRF influences developmental competence at the molecular level.

In summary, maternal TRF supplementation demonstrated clear protective effects on preimplantation embryo development and morphology in OVA-induced allergic asthma mice. By mitigating maternal inflammation and oxidative stress, TRF improved embryo viability reflecting a restoration of the early reproductive environment. The improvements in blastomere uniformity, compaction and blastocyst formation further suggest that TRF contributes to the stabilisation of cellular processes essential for embryogenesis.

These findings indicate that the protective effects of TRF extend beyond systemic maternal protection to include possible cellular and mitochondrial support within the embryo itself. Therefore, the subsequent section 5.4 explores the ultrastructural features particularly mitochondria integrity and physiology to elucidate underlying TRF's protective actions.

5.5 Protective Roles of Maternal Tocotrienol-rich Fraction Supplementation on Ultrastructural Features of 2-cell Embryos

The ultrastructural analysis of 2-cell embryos in this study revealed profound detrimental effects of maternal OVA-induced allergic asthma on embryonic organelle content, mitochondrial integrity and membrane specialization which those detrimental effects were partially reversed by maternal TRF supplementation. These findings provide novel insight into ultrastructural mechanisms underlying asthma-related embryotoxicity and the protective potential of TRF during early embryogenesis.

5.5.1 Maternal Tocotrienol-rich Fraction Supplementation Restores Organelle Distribution in 2-cell Embryos

Embryos derived from the OVA3 group exhibited a marked reduction in cytoplasmic organelle content with the relative volume declining to approximately 11.12%. This significant decrease suggests that maternal allergic asthma impairs the accumulation or maintenance of essential organelles during early embryogenesis. The deficit likely reflects the combined effects of systemic inflammation and oxidative stress which disrupt mitochondrial dynamics, lipid metabolism and overall cytoplasmic

organization (S. Chen et al., 2024; I.-W. Lee et al., 2024; Shcherbik & Pestov, 2019; Sukhorukov & Orekhov, 2024). Reduced organelle content may compromise ATP production, redox balance and cellular resilience, thereby predisposing embryos to developmental arrest or abnormal cleavage (Gałęska et al., 2025; May-Panloup et al., 2021).

Tocotrienol-rich fraction supplementation substantially restored cytoplasmic organelle volume to nearly 24.57%, a significant improvement compared with the OVA3 group and a positive trend relative to POVA. The increased abundance and normalized distribution of mitochondria and lipid droplets in the TOVA group indicate that TRF effectively ameliorates the cellular deficits induced by maternal asthma.

This protective effect likely arises from TRF's potent antioxidant and anti-inflammatory properties which reduce maternal systemic oxidative stress and inflammation, creating a more favourable uterine environment that supports organelle maintenance. Enhanced organelle content consequently improves embryonic energy production, metabolic competence and stress resilience (Arribat et al., 2019; Maheshwari et al., 2022).

In summary, early embryos are highly vulnerable to maternal systemic disturbances in which modest improvements in maternal physiology via TRF supplementation can translate into marked enhancements in embryonic cellular architecture.

5.5.2 Maternal Tocotrienol-rich Fraction Supplementation Restores Mitochondrial Distribution and Morphological Quality in 2-cell Embryos

Quantitative and qualitative analyses revealed significant alterations in mitochondrial abundance and distribution in embryos from asthmatic mothers. OVA3 embryos showed a sharp decline in mitochondrial volume (approximately 7.33%) indicating a loss of mitochondrial content at the critical 2-cell stage. This reduction corresponded with fewer visible mitochondria in the cytoplasm.

Systemic inflammation and oxidative stress associated with allergic asthma likely underlie this mitochondrial insufficiency as excessive ROS and cytokines impair mitochondrial biogenesis and promote degradation (Zong et al., 2024). The resulting energy deficiency compromises cleavage, developmental competence and viability (May-Panloup et al., 2021; Yildirim & Seli, 2024).

Maternal TRF supplementation partially restored mitochondrial volume to 15.59%, significantly higher than in OVA3 and POVA groups though not yet matching control levels. Restoration of peripheral mitochondrial distribution suggests normalization of spatial patterns critical for energy metabolism. TRF's antioxidative and anti-inflammatory actions likely preserve mitochondrial integrity and biogenesis consistent with prior evidence of tocotrienol-mediated mitochondrial protection (May-Panloup et al., 2021).

Morphological observations revealed predominantly spherical and round mitochondria in SHAM, PBS and TOVA embryos. In contrast, ring-shaped mitochondria with central vacuoles were rarely observed. Evidence of ruptured or thinned mitochondrial membranes was most pronounced in asthmatic embryos reflecting leakage of mitochondrial matrix and potential activation of apoptotic pathways (Suen et al., 2008; Zhao et al., 2015).

In summary, maternal allergic asthma markedly reduces mitochondrial abundance and alters morphology compromising embryonic development. TRF supplementation mitigates these effects, partially restoring mitochondrial number, distribution and morphology. Thus, underscoring the role of maternal health in maintaining embryonic mitochondrial integrity.

5.5.3 Maternal Tocotrienol-rich Fraction Supplementation Improves Mitochondrial Clustering in 2-cell Embryos

Maternal allergic asthma significantly disrupted mitochondrial clustering in 2-cell embryos as shown by the drastic reduction in cluster volume in the OVA3 group compared with PBS. Such disruption impairs mitochondrial organization and energy production which both are essential for developmental competence (Komatsu et al., 2014; I.-W. Lee et al., 2024).

TRF supplementation markedly improved mitochondrial clustering which was significantly higher than in both OVA3 and POVA groups ($p \leq 0.001$ and $p \leq 0.05$, respectively). This restoration aligns with previous findings that TRF promotes healthy mitochondrial ultrastructure and normal embryonic development (Sarbandi et al., 2018, 2021).

High-magnification transmission electron microscopy revealed that SHAM, PBS and TOVA embryos exhibited fused and irregularly shaped mitochondrial clusters

which is a hallmark of healthy fusion dynamics. On the other hand, OVA3 and POVA embryos displayed fewer fused mitochondria consistent with impaired fusion or excessive fragmentation (Hao et al., 2024). Observed fusion events (Figure 4.12) confirm that active mitochondrial dynamics were preserved in control and TRF-treated embryos but disrupted in asthmatic groups.

The beneficial influence of TRF on mitochondrial dynamics likely stems from its antioxidative action which enhances antioxidant enzyme levels (SOD1, CAT, GPx), upregulates anti-apoptotic genes such as *Bcl*, reduces ROS-mediated mitochondrial damage and maintains homeostasis which crucial for embryonic growth (Kamsani & Rajikin, 2017).

In summary, maternal asthma severely impairs mitochondrial clustering and fusion, and further compromising embryonic development. TRF supplementation significantly restores these dynamics underscoring its potential to preserve mitochondrial integrity in early embryos exposed to maternal inflammatory stress.

5.5.4 Maternal Tocotrienol-rich Fraction Supplementation Regulates Lipid Droplets and Modulates Mitochondria-lipid Droplets Interactions in 2-cell Embryos

SHAM and PBS embryos exhibited the highest relative lipid droplet volumes consistent with normal lipid storage under physiological conditions. Lipid droplets provide essential energy reserves during cleavage supporting the high metabolic demands of early embryos (Zadoorian et al., 2023).

OVA3 embryos showed a downward trend in lipid droplet volume, though not statistically significant suggesting that allergic asthma impairs lipid storage or mobilization which potentially limiting available energy during embryogenesis. TRF supplementation partially restored lipid droplet volume relative to OVA3, though levels remained below PBS implying that TRF's major benefits target mitochondrial function rather than direct lipid accumulation.

Physiologically, lipid droplets store triacylglycerols that release fatty acids for mitochondrial β-oxidation and ATP generation (Lipinska et al., 2023; Mau et al., 2022). This process becomes especially vital during embryonic genome activation at the 2-cell stage (Lipinska et al., 2023). Hence, disruptions observed in OVA3 embryos may hinder developmental progression.

TEM analyses also identified direct physical interactions between mitochondria and lipid droplets called peridroplet mitochondria where membranes are in continuity (Benador et al., 2018). These structures facilitate lipid oxidation and ATP generation (L. Cui & Liu, 2020). SHAM and PBS embryos exhibited abundant peridroplet mitochondria signifying healthy metabolic coupling whereas OVA3 embryos displayed a pronounced reduction. Supplementation of TRF significantly improved peridroplet mitochondrial volume restoring vital mitochondria–lipid droplets interactions that support fatty acid metabolism and embryonic viability (Lipinska et al., 2023; Mau et al., 2022).

Overall, healthy mitochondria–lipid droplets interactions are critical for preimplantation development. TRF's ability to restore these interactions highlights its therapeutic promise in improving reproductive outcomes under maternal inflammatory stress. Future studies should explore molecular mechanisms by which TRF modulates these interactions and their long-term developmental implications.

5.5.5 Maternal Tocotrienol-rich Fraction Supplementation Maintains Microvilli Structure, Perivitelline Space Integrity and Controls Cellular Degradation Machinery in 2-cell Embryos

Ultrastructural observations provide compelling evidence that maternal allergic asthma compromises the membrane integrity and cellular quality of early embryos which partially mitigated by TRF supplementation.

Prominent microvilli on embryonic membranes of SHAM, PBS and TOVA embryos indicate preserved membrane specialization essential for nutrient exchange, signalling and intercellular communication (Ansel et al., 2024; Bruce & Mihajlovic, 2017). Their absence in OVA3 and POVA embryos reflects disrupted architecture likely impairing these vital processes. The present study provides compelling ultrastructural evidence that maternal allergic asthma adversely affects the structural integrity and cellular quality of early embryos particularly at the 2-cell stage with partial mitigation by TRF supplementation.

A clear perivitelline space (PVS) with minimal debris was observed in SHAM and PBS embryos had signified a healthy extracellular milieu. In contrast, debris accumulation in OVA3, POVA and some TOVA embryos suggests increased cellular stress or impaired clearance mechanisms. This debris originating from apoptotic or

necrotic cells may interfere with embryo–maternal signalling and mechanical protection (Yazdani et al., 2024). While TRF improved several cellular features, persistent debris implies incomplete restoration of extracellular homeostasis.

On the other hand, the presence of autophagosomes, lysosomes and autolysosomes across all groups indicates active autophagy, a critical mechanism for removing damaged organelles and maintaining homeostasis under stress (Singla et al., 2020). The observed autolysosomes confirm ongoing autophagic flux which possibly heightened in embryos exposed to maternal oxidative or metabolic stress.

TRF's antioxidant and gene-modulatory properties likely temper excessive autophagic or apoptotic activity by reducing oxidative damage signals that trigger these degradation pathways (Amin et al., 2022; Sarbandi et al., 2021). Tocotrienols have been reported to modulate stress-related signalling and autophagy-associated genes (Lim et al., 2019) helping balance degradation and survival responses in stressed embryos.

In summary, TRF supplementation partially preserved microvilli and cellular integrity reflecting its protective effect on membrane morphology and function. However, residual debris and autophagic activity suggest incomplete protection indicating that optimization of TRF dosage, timing or combination therapy may further enhance embryonic resilience.

5.6 Protective Roles of Maternal Tocotrienol-rich Fraction Supplementation on Oxidative Stress, Mitochondrial Fusion, Mitochondrial Bioenergetics and Apoptotic Genes Expression in 2-cell Embryos

This study extends the findings on developmental, morphological and ultrastructural alterations by evaluating the expression of oxidative-stress-related, mitochondrial fusion, mitochondrial bioenergetics and apoptotic genes in 2-cell embryos. The results elucidate the potential molecular mechanisms through which maternal TRF supplementation protects embryos from the deleterious effects of allergic asthma.

5.6.1 Maternal Tocotrienol-rich Fraction Supplementation Attenuates Oxidative Stress-related Genes in 2-cell Embryos

The significant elevation of *Nox1* expression in the OVA3 group compared with PBS controls indicates increased ROS generation in embryos exposed to maternal allergic asthma. *Nox1* encodes NADPH oxidase 1, a major enzymatic source of ROS that drives oxidative-stress formation (Meo et al., 2016). Upregulated *Nox1* suggests that maternal asthma establishes a pro-oxidant intra-embryonic environment which potentially leading to cellular injury, impaired development and reduced embryo quality (X. J. Fu et al., 2014; Sela et al., 2015).

Excessive ROS from *Nox1* overactivity can damage membranes, disrupt mitochondrial function and compromise genomic stability. These processes are critical for embryonic survival and growth (Vermot et al., 2021). *Nox1*-induced ROS accumulation has been reported to halt proliferation and trigger senescence or apoptosis via DNA-damage responses (Kodama et al., 2013). Similar pathways may be activated in embryonic cells impairing their developmental potential.

Importantly, TRF supplementation in the TOVA group effectively suppressed *Nox1* expression to near-baseline levels demonstrating TRF's capacity to curb ROS overproduction. TRF's antioxidant properties are attributed to its ability to upregulate endogenous antioxidant enzymes such as SOD and GPx which enzymatically detoxify ROS (Deluao et al., 2022; Truong et al., 2016).

Conversely, *Sod1* expression was downregulated in OVA3 embryos reflecting weakened antioxidant defence. The SOD1 encodes superoxide dismutase 1, which catalyses the dismutation of superoxide radicals into hydrogen peroxide and oxygen, thus preventing oxidative injury (Moussa et al., 2019). Reduced *Sod1* correlates with higher oxidative burden and increased ROS-mediated vulnerability.

Likewise, *Gpx4* expression followed a comparable trend showing robust induction in the TOVA group which signifying enhanced detoxification of lipid peroxides and preservation of membrane integrity (Moussa et al., 2019).

In summary, maternal allergic asthma disturbs oxidative balance in 2-cell embryos by elevating pro-oxidant (*Nox1*) and reducing antioxidant (*Sod1*, *Gpx4*) gene activity. TRF supplementation effectively re-establishes redox equilibrium by downregulating *Nox1* while upregulating *Sod1* and *Gpx4*. These molecular corrections align with the morphological and ultrastructural recovery observed elucidating that TRF

not only scavenges ROS directly but also modulates gene regulatory networks to reinforce endogenous antioxidant defence.

5.6.2 Maternal Tocotrienol-rich Fraction Supplementation Enhances Mitochondrial Fusion and Bioenergetics in 2-cell Embryos

Gene-expression analysis revealed downregulation of *Mfn1*, *Mfn2*, and *Opa1* in the OVA3 group suggesting impaired mitochondrial fusion in embryos from asthmatic mothers. Mitochondrial fusion is a vital dynamic process maintaining mitochondrial morphology, distribution and function which are coordinated by the outer-membrane proteins MFN1/MFN2 and the inner-membrane protein OPA1 (Yildirim & Seli, 2024). Therefore, disruption of these fusion mediators may compromise ATP production and increase ROS production which may impair early embryonic development.

In this study, in comparisons among SHAM, PBS, OVA3 and POVA groups, no statistically significant differences were observed in *Mfn1*, *Mfn2* and *Opa1* gene expression. This suggests that transcriptional regulation of mitochondrial fusion genes may not be the sole determinant of developmental competence. Despite similar mRNA expression profiles, embryonic developmental outcomes at blastocyst and hatched stage varied substantially. This discrepancy indicates the involvement of post-transcriptional, translational, or post-translational regulatory mechanisms that decouple gene expression levels from functional mitochondrial performance (Zanfardino et al., 2025).

In contrast, TRF supplementation in the TOVA group significantly upregulated *Mfn1* and *Mfn2* and robustly restored *Opa1* expression implying enhanced mitochondrial fusion activity. This might be due to maternal factors appear to override the influence of fusion gene transcription under asthmatic conditions (Hemachandra Reddy, 2011). In this study, the elevated systemic oxidative DNA damage and inflammatory cytokines might the mechanism impairing embryo morphogenesis through broader bioenergetic and apoptotic pathways. These effects may compromise processes such as compaction, blastocoel expansion, and lineage specification independently of fusion transcript abundance (S. H. Lee & Rinaudo, 2024).

Collectively, these findings suggest that mitochondrial dynamics in preimplantation embryos are regulated not only at the transcriptional level but also through maternal redox status and downstream functional integrity. On the other hand, improved fusion supports mitochondrial-network integrity, facilitates mitochondrial

DNA repair and sustains bioenergetic efficiency which are key factors for early embryogenesis (S. Chen et al., 2024; Yildirim & Seli, 2024; Zou et al., 2021).

Restoration of mitochondrial function is essential for ATP synthesis via oxidative phosphorylation (OXPHOS) (Čunátová et al., 2021; Huang et al., 2019). The *Atp5e* gene (encoding an ATP-synthase subunit) and *Cox4i1* (a cytochrome c oxidase subunit) were markedly downregulated in OVA3 and POVA embryos signifying OXPHOS dysfunction and energy deficiency. Such mitochondrial deficits increase oxidative stress, activate apoptosis and threaten embryonic survival (Gualtieri et al., 2021; Lim et al., 2019; Zong et al., 2024).

TRF supplementation significantly upregulated *Atp5e* and *Cox4i1* beyond PBS control levels indicating enhanced ATP synthesis and improved electron-transport efficiency. This recovery may result from TRF's antioxidant effect that minimizes ROS-induced mitochondrial injury. Furthermore, TRF's modulation of mitochondrial-fusion and OXPHOS-related genes suggests a direct role in sustaining mitochondrial network homeostasis and energy metabolism during critical preimplantation windows.

In summary, TRF supplementation markedly improves mitochondrial health and bioenergetic function in embryos affected by maternal asthma as evidenced by increased expression of fusion and OXPHOS-related genes. These molecular findings complement the morphological and ultrastructural evidence highlighting the indispensable role of mitochondrial fitness in early embryonic development.

5.6.3 Maternal Tocotrienol-rich Fraction Supplementation Downregulates Pro-apoptotic and Upregulates Anti-apoptotic Genes in 2-cell Embryos

Embryos from OVA3 and POVA groups exhibited significantly increased expression of the pro-apoptotic gene (*Bax*) and concomitant downregulation of the anti-apoptotic (*Bcl2*) signifying heightened apoptotic signalling and cellular stress under maternal allergic asthma.

TRF supplementation reversed this pattern where *Bax* expression was markedly reduced while *Bcl2* was significantly elevated surpassing even the PBS control which demonstrated a strong anti-apoptotic influence. The downregulation of *Bax* coupled with upregulation of *Bcl2* indicates TRF's potent ability to stabilize cellular survival pathways under oxidative and inflammatory stress.

Mechanistically, TRF's antioxidative capacity likely reduces ROS accumulation, thereby modulating apoptosis-related transcription. The *Bcl2* inhibits mitochondrial cytochrome c release which further preventing apoptosome formation and caspase activation (Qian et al., 2022). Thus, TRF-induced *Bcl2* enhancement may suppress mitochondrial outer-membrane permeabilization and block *Bax*-mediated apoptosis. This was consistent with the ultrastructural findings of preserved mitochondrial morphology in TRF-treated embryos.

In summary, TRF's antioxidant properties confer anti-apoptotic protection by mitigating oxidative stress, stabilizing mitochondrial integrity, and rebalancing pro- and anti-apoptotic gene expression. This restoration of apoptotic equilibrium offers a promising therapeutic strategy for improving embryo viability in pregnancies complicated by maternal allergic asthma. Taken together, these molecular outcomes reinforce TRF's potential as a reproductive-protective antioxidant intervention in conditions marked by elevated oxidative stress and apoptosis.

5.7 Mechanistic Insights

This section presents an integrated discussion of the overall findings obtained in this study which investigated the impact of maternal allergic asthma on 2-cell embryo viability and evaluated the protective effects of maternal TRF supplementation. By combining microscopic, ultrastructural and molecular analyses, the present work provides a comprehensive understanding of how maternal systemic inflammation and oxidative stress influence embryonic cellular architecture and gene expression. Furthermore, it elucidates the mechanistic pathways through which TRF mitigates these detrimental effects, thereby improving embryo viability.

5.7.1 Integration of Maternal and Embryonic Findings

The integration of key findings was demonstrated by illustrating on how maternal stress induced by allergic asthma indirectly compromises the 2-cell embryo viability. Findings of this study demonstrated that maternal allergic asthma profoundly alters the uterine and systemic environments which are critical for early embryonic survival. Asthmatic mothers exhibited increased inflammatory and oxidative markers which translated into reduced 2-cell embryo viability due to propagation of

inflammatory and oxidative markers thru systemic circulation into reproductive microenvironment. The 2-cell stage embryos, in particular, showed marked vulnerability to maternal systemic disturbances characterised by impaired morphological quality, reduced cytoplasmic organelle volume, altered mitochondrial fusion, minimized mitochondria-lipid droplet interaction, lowered ATP synthesis and activated apoptotic signalling (Figure 5.1).

In particular, ultrastructural analyses revealed that embryos from OVA-induced allergic asthma mice exhibited significant reductions in cytoplasmic organelle volume, mitochondrial clustering and microvillar structures accompanied by increased cellular debris and disorganisation within the perivitelline space. These structural abnormalities were strongly associated with mitochondrial fragmentation and loss of peridroplet mitochondria reflecting disrupted energy metabolism and impaired developmental potential observed in this study.

Gene expression profiling further substantiated these morphological and ultrastructural observations. Two-cell embryos from asthmatic mothers exhibited upregulation of the oxidative stress-related gene (*Nox1*) and downregulation of antioxidant (*Sod1*) and mitochondrial fusion (*Mfn1*, *Mfn2*, *Opa1*) genes alongside increased expression of pro-apoptotic (*Bax*) and reduced expression of anti-apoptotic (*Bcl2*) genes. Cumulatively, these findings indicate that maternal allergic asthma triggers a cascade of oxidative, mitochondrial, and apoptotic disruptions that compromise embryonic viability at the earliest stage of development.

However, maternal supplementation with TRF significantly reversed many of these adverse effects. TRF-treated mothers produced embryos with improved morphology and higher developmental competence. Ultrastructurally, TRF supplementation restored cytoplasmic organelle content, improved mitochondrial abundance, and re-established mitochondrial clustering and peridroplet interactions. At the molecular level, TRF downregulated *Nox1* while upregulating *Sod1*, *Gpx4*, *Mfn1*, *Mfn2*, *Opa1*, *Atp5e*, Cox4i1, and *Bcl2*, thereby normalising oxidative stress and apoptotic balance and enhancing mitochondrial functionality.

This section presents the integration of key findings by combining and interpreting the results obtained from the maternal mice with the corresponding findings obtained in the 2-cell embryos. Through this integrated approach, this study helps to elucidate the relationship between maternal physiological alterations and embryonic developmental outcomes providing a comprehensive overview of how maternal allergic

asthma and TRF supplementation collectively influence early embryonic development. The subsequent section will further elaborate on the mechanistic insights underlying these integrated findings highlighting the potential cellular and molecular pathways involved in mediating the observed effects.

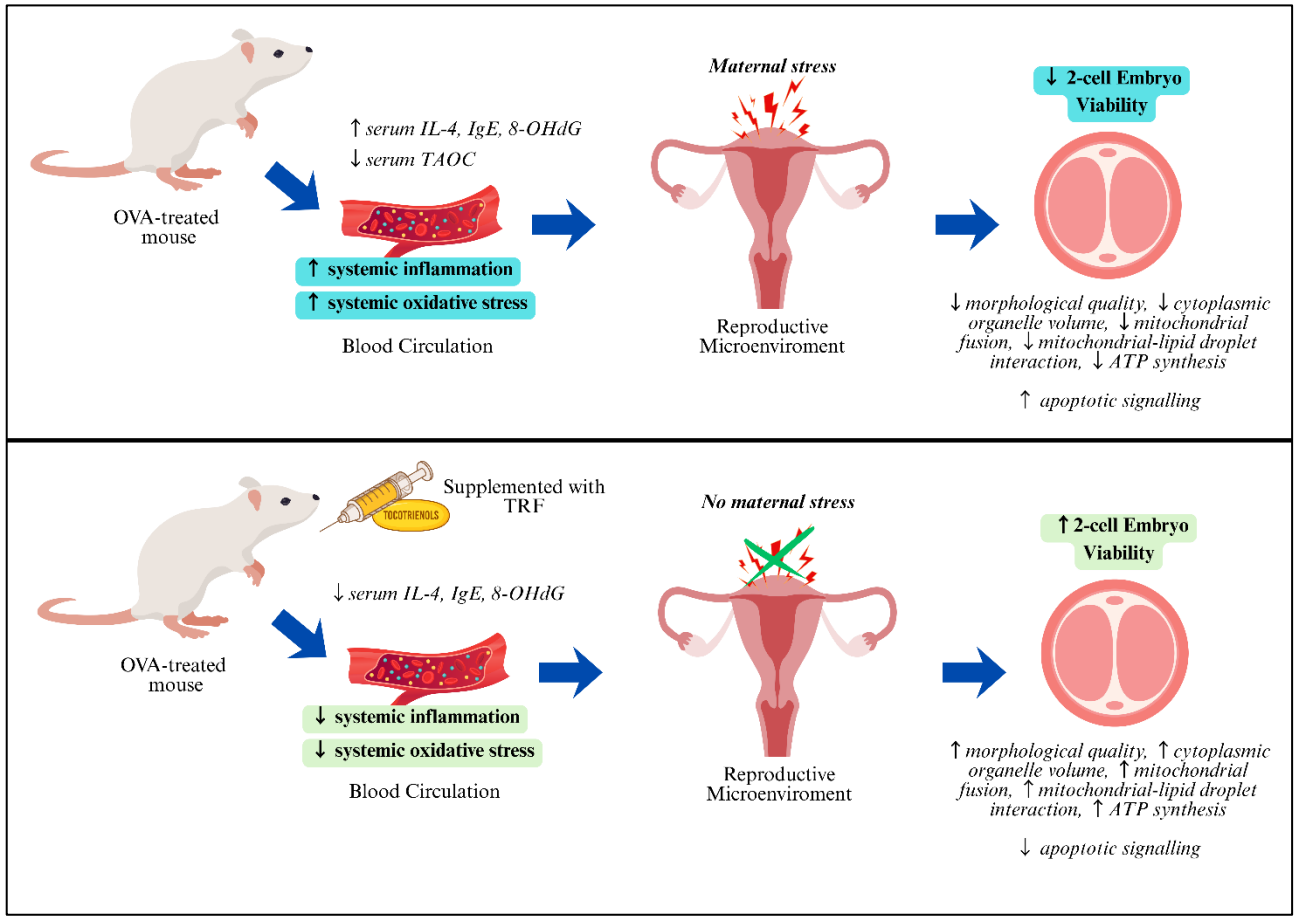


Figure 5.1 Integration of Maternal and Embryonic Findings

This figure demonstrates the integration of maternal and embryonic findings. In OVA-treated mice, increased systemic inflammation and oxidative stress disrupt the reproductive microenvironment leading to maternal stress and reduced 2-cell embryo viability. TRF supplementation mitigates these effects by lowering inflammatory and oxidative markers, alleviating maternal stress and improving embryonic viability.

5.7.2 Tocotrienol-rich Fraction Supplementation Enhances Mitochondrial Fusion Which Improves Embryo Viability

The findings provide mechanistic insight into on how maternal TRF supplementation enhances mitochondrial fusion which improves embryo viability in the OVA-induced allergic asthma model. As depicted in Figure 5.2, TRF supplementation attenuates oxidative stress, enhances mitochondrial fusion, improves ATP synthesis and suppresses apoptosis which collectively improving embryo viability and progression to the blastocyst stage.

OVA-induced allergic asthma is associated with systemic oxidative imbalance that can extend to the reproductive system compromising oocyte and early embryo quality. In the present study, maternal TRF supplementation upregulated the antioxidant defence system as indicated by increased *Sod1* and *Gpx4* expression and concurrent downregulation of Nox1, a major producer of ROS. This suggests that TRF effectively neutralizes oxidative radicals and preserves redox homeostasis within the embryo. The decrease in oxidative stress is essential for maintaining mitochondrial integrity as excess ROS is known to induce mitochondrial fragmentation and dysfunction during early embryogenesis (Gualtieri et al., 2021). By restoring the balance between ROS generation and detoxification, TRF likely prevents the oxidative damage that would otherwise impair mitochondrial dynamics.

Mitochondrial fusion is a critical adaptive response that enables the exchange of mitochondrial content, repair of damaged organelles, and maintenance of bioenergetic efficiency (Ojaimi et al., 2022; Yao et al., 2019; Yildirim & Seli, 2024). TRF supplementation increased the expression of fusion-related genes *Mfn1*, *Mfn2*, and *Opa1* suggesting an enhancement of mitochondrial connectivity within the 2-cell embryos. In contrast, embryos from untreated OVA mice likely experienced impaired mitochondrial morphology consistent with the fragmented mitochondrial network typically observed under oxidative stress. By promoting fusion, TRF helps preserve the structural and functional integrity of mitochondria enabling efficient energy metabolism and preventing the activation of stress-induced apoptotic pathways.

Mitochondrial fusion is closely linked to enhanced OXPHOS efficiency and ATP synthesis (Yao et al., 2019). The upregulation of *Atp5e* and *Cox4i1* components of the ETC complexes V and IV, respectively, suggests improved mitochondrial respiration and ATP production in TRF-supplemented embryos. This indicates that TRF

not only protects mitochondria structurally but also optimizes their bioenergetics capacity. Enhanced ATP availability is crucial during preimplantation stages supporting biosynthetic activities, ion transport, and developmental transitions, including compaction and blastocyst formation.

The mitochondrial improvements conferred by TRF also translated into reduced apoptotic signalling as reflected by decreased *Bax* and increased *Bcl2* expression. This suggests a shift toward anti-apoptotic conditions within the developing embryos. The maintenance of mitochondrial integrity and sufficient ATP levels likely suppresses cytochrome c release and caspase activation, thus preventing premature cell death (McIlwain et al., 2013; J. D. Yu & Miyamoto, 2021). Prevention of apoptosis is particularly important during cleavage-stage development because blastomere loss can critically impair subsequent morphogenesis and implantation potential.

These cumulative effects directly contribute to improved embryo viability. The synchronized regulation of antioxidant defences, mitochondrial fusion and bioenergetics enhancement, and apoptosis suppression ensure the preservation of cellular homeostasis during critical stages of preimplantation development. Embryos possessing stable mitochondrial function and adequate ATP reserves are more likely to maintain proper cleavage rates, sustain genomic activation and successfully reach the blastocyst stage (Chu et al., 2025; Phan et al., 2025). Therefore, the TRF-mediated restoration of mitochondrial performance translates into tangible improvements in embryonic survival and developmental competence highlighting energy metabolism as a central determinant of viability in the asthma-compromised maternal environment.

These molecular and cellular events converge to support successful embryonic development. The embryos derived from TRF-supplemented OVA-treated mice exhibited normal mitochondrial morphology, efficient mitochondrial fusion, and sufficient energy supply to progress through preimplantation stages that ultimately reaching the hatched blastocyst stage. This mechanistic cascade underscores the pivotal role of maternal antioxidant and mitochondrial fusion in overcoming the detrimental effects of systemic inflammation and oxidative stress on early embryo development.

In summary, maternal TRF supplementation exerts a multifaceted protective mechanism against asthma-induced reproductive stress by improving mitochondrial fusion, thereby creating a favourable intracellular environment that improves embryonic viability. These findings highlight the mitochondrion as a key therapeutic target through which TRF confers its developmental benefits.

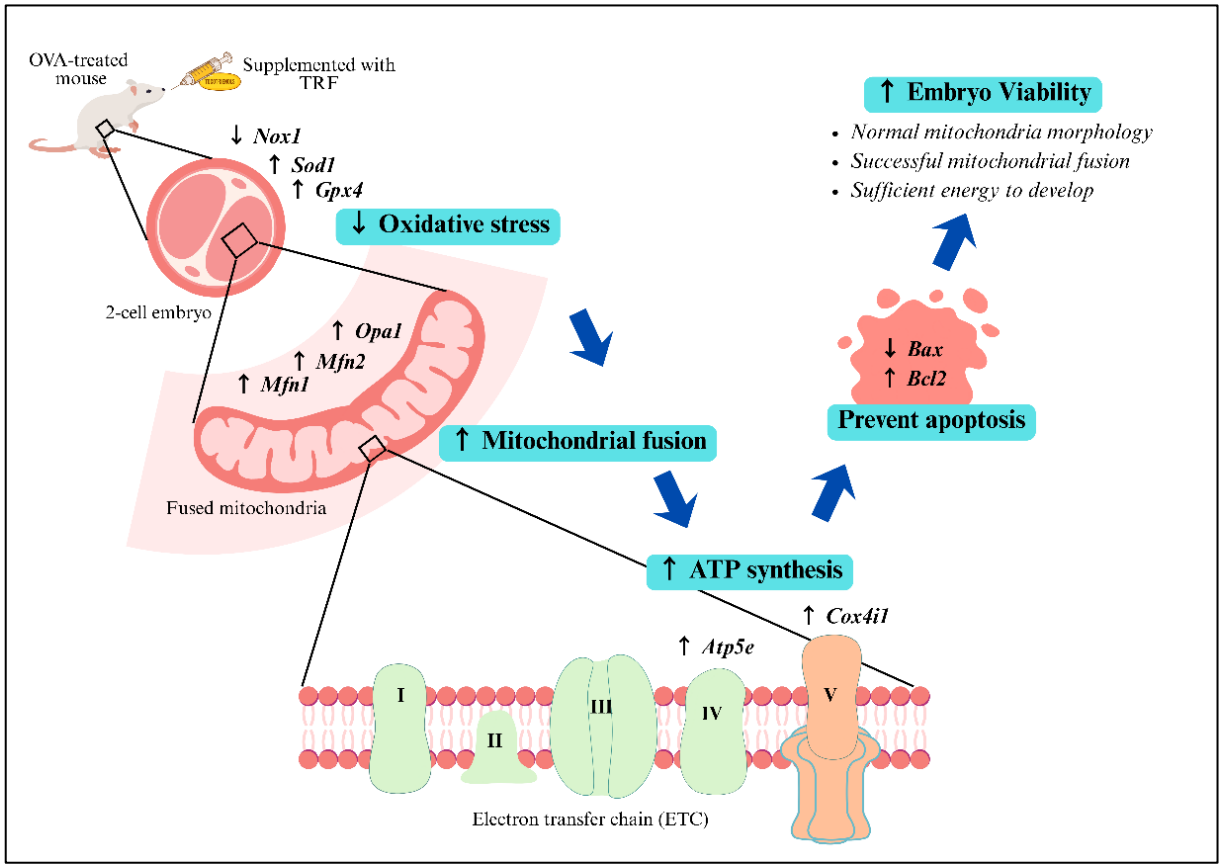


Figure 5.2 Mechanistic Insights: Role of Tocotrienol-rich Fraction in Enhancing Mitochondrial Fusion Which Improves Embryo Viability
This figure demonstrates the mechanistic insights on the role of maternal tocotrienol-rich fraction (TRF) supplementation enhanced mitochondrial fusion in 2-cell embryos. Besides, enhanced mitochondrial fusion was influenced by reduced oxidative stress. This improvement supports increased expression of electron transport chain components enhancing ATP synthesis. The resulting increase in cellular energy availability coupled with reduced apoptosis had contributed to improved embryo viability.

5.7.3 Tocotrienol-rich Fraction Supplementation Promotes Mitochondria-lipid Droplets Interaction Which Supports Embryo Viability

At the 2-cell stage of mammalian embryonic development, ZGA represents a pivotal transition in which the regulation of gene expression shifts from maternally derived transcripts to the embryo's own genome (Svoboda, 2018). This developmental milestone is highly energy-demanding requiring substantial ATP production to support chromatin remodelling, transcriptional activation, RNA processing and protein synthesis (Kaneko, 2016; H. Zhang et al., 2021).

Mechanistically, embryos at this stage rely primarily on mitochondrial β-oxidation of fatty acids as the main ATP-generating pathway, since glycolytic activity remains limited and not yet fully established (Chi et al., 2020; Kaneko, 2016). Lipid droplets act as critical energy reservoirs by storing triacylglycerols that can be hydrolyzed to release fatty acids such as Acetyl-CoA which is important for tricarboxylic acid (TCA) cycle upon demand (T. Li et al., 2023). The close spatial and functional coupling between mitochondria and lipid droplets forming peridroplet mitochondria is reported to facilitate the direct transfer of fatty acids (Acetyl-CoA) into mitochondrial matrix for mitochondrial β-oxidation pathways (L. Cui & Liu, 2020). This tight integration minimizes diffusion barriers, enhances metabolic efficiency and ensures rapid ATP generation required to sustain ZGA (Figure 5.3).

Under maternal allergic asthma, elevated oxidative stress and pro-inflammatory cytokines compromise mitochondrial integrity and reduce the number of peridroplet mitochondria. This disruption impairs fatty acid mobilization and oxidation efficiency leading to an energy deficit at the 2-cell stage. Consequently, inadequate ATP levels may hinder energy-intensive processes such as chromatin remodelling and transcriptional activation resulting in delayed or incomplete ZGA and abnormal embryonic development (Acin-Perez et al., 2023; Ning et al., 2023).

TRF supplementation appears to counteract these adverse effects by protecting mitochondrial membranes from oxidative damage stimulating mitochondrial bioenergetics and enhancing peridroplet mitochondria formation. Through restoration of efficient fatty acid oxidation, TRF supports sufficient ATP generation necessary for successful ZGA, thereby maintaining early embryonic viability. These findings elucidate TRF's potential as a therapeutic antioxidant capable of alleviating maternal

inflammation–induced metabolic dysfunction and improving developmental outcomes during this highly sensitive stage of preimplantation development.

The viability of 2-cell embryos which depends critically on robust energy production and metabolic integrity determines their capacity to develop successfully into hatched blastocysts (May-Panloup et al., 2021). Optimal mitochondria-lipid droplets interactions under healthy conditions support ATP generation required for cellular functions including cell division, differentiation and genome activation (Benador et al., 2018). Conversely, disruptions in these interactions such as those induced by maternal allergic asthma impair energy metabolism and compromise embryo viability which further diminishing the likelihood of progression to the blastocyst stage.

By restoring mitochondria–lipid droplets coupling, TRF supplementation not only enhanced 2-cell embryo viability but also improved subsequent developmental competence which increases the successful of blastocyst formation and hatching. Nevertheless, further studies employing molecular and bioenergetics assessments are required to confirm the direct impact of mitochondrial–lipid dynamics on embryo viability and long-term developmental potential.

This study used TEM and had elucidated the ultrastructural relationships between mitochondria and lipid droplets in 2-cell embryos. While TEM provides valuable morphological evidence of organelle interactions, it did not directly reveal the molecular mechanisms or dynamic metabolic processes underlying lipid utilization and mitochondrial regulation. Hence, the mechanistic interpretations drawn remain inferred from morphological observations.

To advance these findings, future investigations integrating molecular and functional analyses such as transcriptomic and proteomic profiling, live-cell metabolic imaging and targeted assays for fatty acid oxidation and mitochondrial bioenergetics are essential. Such studies would help delineate the specific signalling pathways and regulatory networks modulated by maternal allergic asthma and TRF supplementation, thereby deepening our understanding of mitochondria–lipid droplets crosstalk and validating TRF’s protective role in sustaining early embryogenesis.

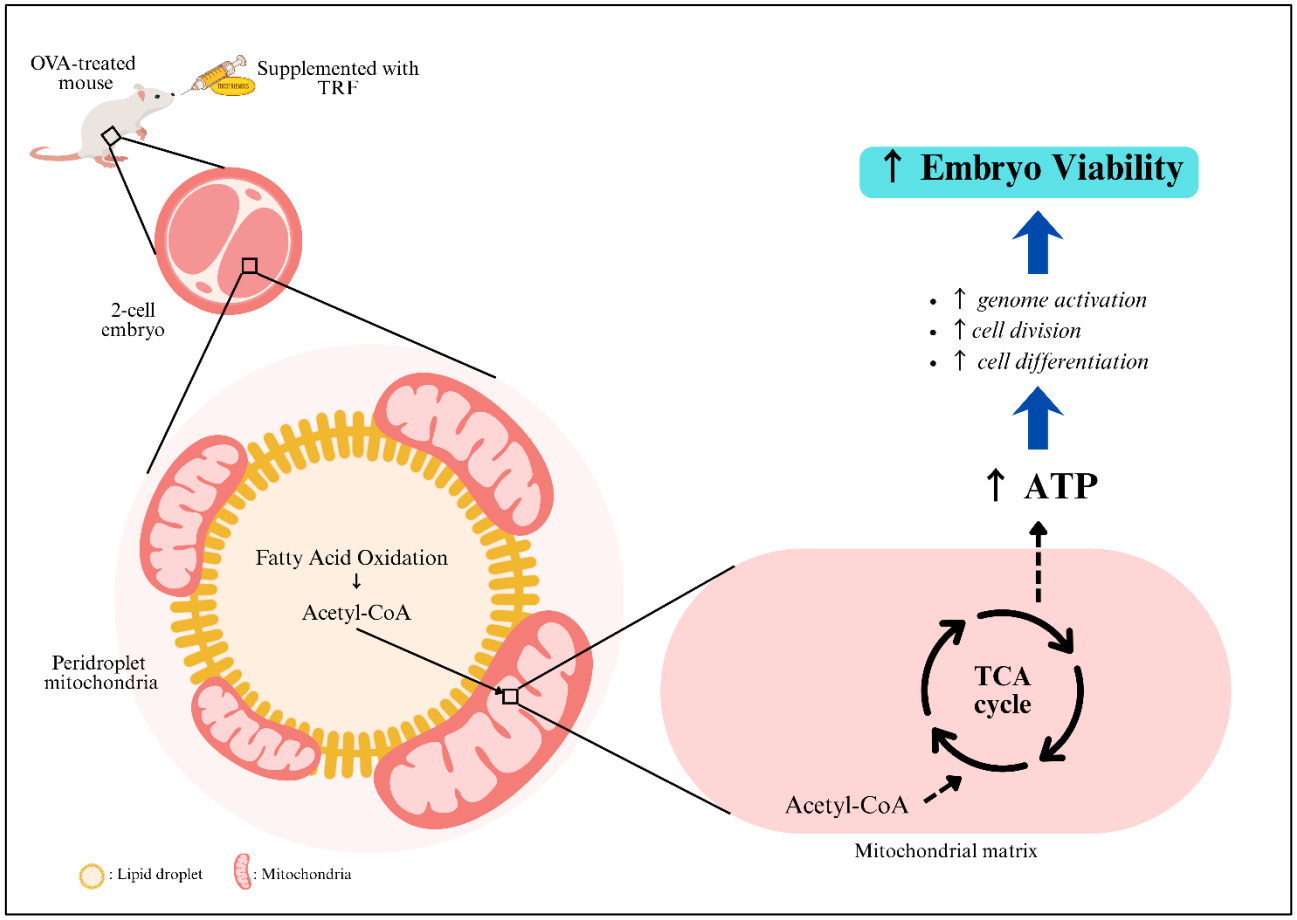


Figure 5.3 Mechanistic Insights: Role of Tocotrienol-rich Fraction in Promoting Mitochondria-lipid Droplets Interactions Which Supports Embryo Viability

This figure demonstrates the mechanistic insights on the role of maternal tocotrienol-rich fraction (TRF) supplementation promoted mitochondria-lipid droplets interaction in 2-cell embryos. TRF promotes fatty acid oxidation in peridroplet mitochondria, generating acetyl-CoA that enters the TCA cycle to increase ATP production. The elevated ATP supply supports genome activation, cell division, and cell differentiation, thereby improving embryo viability.

5.8 Implication of Findings

The findings of this study carry significant implications for clinical practice. Maternal allergic asthma during early pregnancy can markedly impair embryonic viability due to systemic inflammation and oxidative stress. These findings highlight the critical importance of careful monitoring and management of allergic asthma in pregnant patients particularly during the preimplantation and early embryonic stages to preserve foetal viability and normal development (Tamayo et al., 2022). The observed dose-dependent detrimental effects of allergic asthma on maternal systemic health and embryo quality further suggest that tighter control of maternal asthma severity may reduce the risk of developmental arrest or abnormal embryo morphology.

Importantly, TRF supplementation demonstrated protective potential in mitigating the adverse effects of maternal allergic asthma. TRF attenuated inflammation by reducing Th2-skewed cytokines, minimized oxidative DNA damage and enhanced mitochondrial physiology and gene regulation essential for early embryonic development. These findings indicate that TRF or similar antioxidant and anti-inflammatory compounds could serve as supportive interventions for asthmatic pregnant patients to improve reproductive and developmental outcomes (Cook-Mills et al., 2022). At the molecular level, TRF-mediated suppression of pro-oxidant and pro-apoptotic gene expression alongside the upregulation of mitochondrial and antioxidant defence genes provides valuable mechanistic insights and potential therapeutic targets for future drug development aimed at protecting embryos from maternal immune-mediated injury.

Clinically, these results advocate for personalized prenatal care protocols that incorporate immune and oxidative stress assessments in asthmatic patients as well as consideration of dietary or pharmacological antioxidant supplementation such as TRF to optimize embryo quality, implantation potential and pregnancy success. Finally, the protective outcomes associated with TRF supplementation highlight its potential as a nutraceutical intervention to improve reproductive outcomes under conditions of oxidative and inflammatory stress. These findings also contribute to the DOHaD framework emphasizing that maintaining maternal redox homeostasis during the peri-conceptional period is vital for ensuring long-term offspring health and developmental programming.

In summary, this study informs clinical practice by emphasizing the need for early intervention in maternal asthma, the potential utility of TRF supplementation for embryonic protection, and the importance of molecular and redox monitoring to guide targeted therapeutic strategies aimed at improving pregnancy outcomes in the context of maternal allergic asthma.

Table 5.1
Summary of Clinical Implications of Maternal Tocotrienol-rich Fraction Supplementation in Allergic Asthma

Key Aspect	**Findings / Implications**	**Clinical Relevance / Impact**
Effect of maternal allergic asthma	Induces systemic inflammation and oxidative stress, impairing preimplantation embryonic development.	Highlights need for strict monitoring and management of asthma during early pregnancy to protect embryo viability.
Dose-dependent effects	Severity of asthma correlates with deterioration in maternal systemic health and embryo quality.	Suggests that tighter asthma control may prevent developmental arrest and abnormal embryo morphology.
TRF supplementation effects	Reduces Th2-skewed cytokines (IL-4, IgE), oxidative DNA damage; enhances mitochondrial and developmental gene function.	Indicates TRF as a potential therapeutic intervention to mitigate asthma-induced embryonic damage.
Molecular mechanisms	Downregulation of pro-oxidant and pro-apoptotic genes; upregulation of mitochondrial and antioxidant genes.	Provides mechanistic targets for developing drugs to protect embryos from immune-mediated injury.
Overall implications	TRF supplementation offers protective, nutraceutical potential under oxidative and inflammatory stress.	Promotes improved reproductive outcomes and aligns with precision maternal–foetal health strategies.

This table summarizes the key clinical implications of maternal allergic asthma and the protective effects of TRF supplementation. Maternal asthma-induced inflammation and oxidative stress impair embryonic development, while TRF mitigates these effects by modulating immune responses, reducing oxidative damage, and enhancing mitochondrial function. The findings highlight the need for early asthma management, oxidative stress monitoring, and antioxidant support in personalized prenatal care, consistent with the DOHaD concept emphasizing optimal maternal redox balance for long-term offspring health.

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion and Recommendations

In summary, this study provides comprehensive mechanistic insight into how maternal allergic asthma impairs embryo viability and how tocotrienol-rich fraction (TRF) supplementation mitigates these adverse effects. Ovalbumin (OVA)-induced allergic asthma was shown to disrupt maternal immune balance and redox homeostasis as evidenced by elevated interleukin (IL)-4, immunoglobulin (Ig)-E, and 8 hydroxy-2′-deoxyguanosine (8-OHdG) levels alongside reduced total antioxidant capacity (TAOC). These systemic alterations translated into compromised embryonic morphology and delayed developmental progression highlighting the vulnerability of early embryogenesis to maternal inflammatory and oxidative stress environments.

Maternal TRF supplementation conferred significant protection against these deleterious effects by attenuating T-helper (Th)-2-mediated inflammation reducing oxidative DNA damage and partially restoring antioxidant defence capacity. At the embryonic level, TRF enhanced developmental competence, improved morphological quality and normalized ultrastructural features including mitochondrial abundance, fusion, and lipid–mitochondria interactions essential for efficient energy metabolism.

Molecular analyses further revealed that TRF exerts its protective action through coordinated regulation of oxidative, mitochondrial and apoptotic gene networks by upregulating *Sod1*, *Gpx4*, *Mfn1*, *Mfn2*, *Opa1*, *Atp5e*, *Cox4i1* and *Bcl2* while suppressing *Nox1* and *Bax* expression. Collectively, these findings establish TRF as a potent maternal dietary intervention capable of restoring cellular homeostasis, optimizing mitochondrial bioenergetics, and promoting embryo viability in inflammatory conditions such as allergic asthma.

Overall, this research underscores the critical interplay between maternal systemic health and early embryonic development while positioning TRF as a promising therapeutic strategy for improving reproductive outcomes under oxidative and inflammatory stress.

Despite the promising outcomes observed, several limitations of this study should be acknowledged. First, endocrine assessments were restricted to the early post-

fertilisation period (1.5 d.p.c) during the preimplantation stage. Although this window is critical for embryonic competence, it does not capture potential endocrine fluctuations during the pre- and post-implantation phases where embryo–uterine crosstalk, decidualisation and early placentation become increasingly important. As a result, later systemic adaptations or disruptions arising from maternal allergic inflammation may have been underestimated.

In addition, while maternal TRF supplementation demonstrated clear *in vivo* protective effects in reducing systemic inflammation and oxidative stress, the current study design can be revisited to clarify whether these improvements were mediated indirectly through modulation of the maternal immune–endocrine environment or directly via embryonic uptake of TRF. Since preimplantation embryos are highly sensitive to changes in maternal metabolic and inflammatory status, improvements in mitochondrial integrity and developmental kinetics may reflect secondary maternal effects rather than direct embryonic antioxidant action. The absence of complementary *in vitro* embryo culture experiments supplemented with TRF including the lack of tissue-specific TRF quantification limits definitive mechanistic interpretation.

Furthermore, embryonic ultrastructural evaluation was primarily qualitative and centred on mitochondrial morphology which aligns with the mechanistic focus on oxidative stress but restricts broader quantitative assessment of other organelles and cellular stress markers. The relatively limited biological replicates may also reduce statistical power to detect subtle structural differences. Additionally, although OVA-induced allergic asthma model represents an acute Th2-driven inflammatory condition, it may not fully replicate the chronic and heterogeneous nature of human maternal asthma. Therefore, while the findings provide important mechanistic insights, extrapolation to clinical settings should be approached cautiously.

Therefore, as recommendations, future studies should aim on the longitudinal endocrine evaluations beyond the early post-fertilization window are warranted to elucidate the evolving maternal-foetal cross-talk and endocrine milieu under allergic asthmatic conditions with TRF intervention. Mechanistic studies employing *in vitro* embryo culture systems and molecular approaches should be prioritised to delineate the direct versus indirect (maternal-mediated) effects of TRF at the embryonic cellular and transcriptomic levels enabling targeted identification of key signalling pathways modulated by antioxidant therapy. Given the observed restoration of mitochondrial abundance and dynamics, future research should focus in particular on mitochondrial

pathways regulating fusion (not limited to *Mfn1*, *Mfn2*, *Opa1*) and ATP synthesis (not limited to *Atp5e* and *Cox4i1*) to clarify how TRF modulates mitochondrial bioenergetics and quality control during early embryogenesis.

Besides, more detailed ultrastructural studies are important to uncover the mechanistic underpinnings of TRF's enhancement of mitochondrial fusion, cristae integrity and energy metabolism which are critical for embryonic developmental competence under inflammatory stress. Additionally, comprehensive evaluation of the long-term phenotypic and epigenetic effects of prenatal TRF exposure including embryo transfer, implantation, foetal development and postnatal outcomes are critical to ensure sustained benefits and safety. Expanding experimental models to cover diverse maternal immune dysregulation and stress will strengthen the translational potential of TRF as an adjunctive therapy for mitigating inflammation-induced reproductive dysfunction. These approaches will enhance the development of targeted antioxidant interventions to protect early embryonic development and later stage of pregnancy due to adverse maternal environments.

In addition, future studies should also aim to build upon the current findings by exploring both mechanistic depth and translational relevance. Functional assays quantifying mitochondrial respiration, ATP production and reactive oxygen species generation at the single-embryo level would provide direct evidence of mitochondrial restoration. At the molecular level, further research should focus on delineating the regulatory pathways involved in TRF's protective effects such as the Nrf2, PGC-1α and mitophagy signalling axes. These future directions will deepen understanding of maternal–embryo interactions under stress and refine TRF's potential as a therapeutic intervention in reproductive medicine.

Lastly, this study demonstrates that maternal antioxidant intervention with TRF can remediate both systemic and embryonic consequences. Hence, the novelty of this study lies primarily in identifying TRF as an effective maternal nutraceutical with multifaceted protective actions on embryo viability. For the first time, TRF is shown to confer cross-level protection by modulating maternal immune and redox balance, preserving embryonic morphology and ultrastructure, and regulating key molecular pathways involved in oxidative stress, mitochondrial fusion, and apoptosis.

This integrated mechanistic framework underscores TRF's role in promoting optimal energy metabolism, genomic stability, and developmental progression during the earliest stages of embryogenesis. By demonstrating TRF's capacity to counteract

maternal inflammatory and oxidative challenges at multiple biological levels, this study positions TRF as a promising preventive nutraceutical intervention for improving reproductive outcomes and protecting embryonic development under compromised maternal conditions.

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APPENDICES

APPENDIX A

List of Chemicals and Reagents

Table A1

Chemicals and Reagents	Manufacturer
Ovalbumin (albumin) from Dried Egg White, crude	Tokyo Chemical Industry, Japan
Aluminum hydroxide	Sigma-Aldrich, USA
Phosphate buffered saline	Sigma-Aldrich, USA
Ilium ketamil	Troy Animal Healthcare, Australia
Ilium xylazil	Troy Animal Healthcare, Australia
Folligon ® Pregnant mare serum gonadotropin (PMSG)	Merck Animal Health, Canada
Chorulon ® Chorionic gonadotropin (hCG)	Merck Animal Health, Canada
Tocotrienol Rich Fraction (Gold-Tri.ETM70)	Sime Darby, Malaysia
Palm Olein stripped with vitamin E	Malaysia Palm Oil Board, Malaysia
M2 medium	Sigma-Aldrich, USA
M16 medium	Sigma-Aldrich, USA
Paraffin oil	Sigma-Aldrich, USA
Interleukin-4 ELISA kit	Sigma-Aldrich, USA
Immunoglobulin-E ELISA kit	Elabscience, China
8-hydroxy-2'-deoxyguanosine ELISA kit	Elabscience, China
Total antioxidant capacity ELISA kit	Elabscience, China
Progesterone ELISA kit	Elabscience, China
Estrogen ELISA kit	Elabscience, China
0.2 M sodium cacodylate buffer	Agar Scientific, UK
2.5 % glutaraldehyde solution	Agar Scientific, UK
1 % osmium tetroxide solution	Agar Scientific, UK
Acetone	Agar Scientific, UK
Milipore-grade water	Agar Scientific, UK
Agar 100 resin	Agar Scientific, UK
DDSA	Agar Scientific, UK
MNA	Agar Scientific, UK
BDMA	Agar Scientific, UK
Poly-L-Lysine	Agar Scientific, UK
Uranyl acetate	Agar Scientific, UK
Lead citrate	Agar Scientific, UK
Sodium hydroxide (NaOH) pellet	Agar Scientific, UK
Acid Tyrode	Fluidigm, USA
Resuspension buffer	Fluidigm, USA
D3 Assay Design Oligonucleotide Primer	Fluidigm, USA
Lysis enhancer	Fluidigm, USA
DNase I	Fluidigm, USA
DNase buffer	Fluidigm, USA
25mM EDTA	Fluidigm, USA
CellsDirext 2× Reaction	Fluidigm, USA

SuperScript III RT/Platinum Taq	Fluidigm, USA
Exonuclease I (20 U μL^{-1})	Fluidigm, USA
Exonuclease buffer	Fluidigm, USA
Nuclease-free water	Fluidigm, USA
Assay loading reagent	Fluidigm, USA
DNA suspension buffer	Fluidigm, USA
2× SsoFast EvaGreen Supermix (low ROX)	Fluidigm, USA
20× DNA-binding dye	Fluidigm, USA

APPENDIX B

List of Laboratory Equipment and Apparatus

Table B1

Equipment and Apparatus	Manufacturer
Serum collection tube	Thermo Fisher Scientific, Norway
Vortex mixer	Stuart, Chicago, USA
Micropippete	Eppendorf, Hamburg, Denmark
Gavaging needle, 20G	Eppendorf, Hamburg, Denmark
Needle, 25G	Eppendorf, Hamburg, Denmark
Blunt needle, 32G	Eppendorf, Hamburg, Denmark
1 mL syringe	Thermo Fisher Scientific, Norway
Microcentrifuge	Eppendorf, Hamburg, Germany
Microcentrifuge tube	Thermo Fisher Scientific, Norway
35 mm petri dish	Sigma-Aldrich, USA
100 mm petri dish	Sigma-Aldrich, USA
Stereo microscope	Leica Zoom 2000, Z45 V, Germany
Inverted microscope	Leica DM IRB, Germany
Transmission electron microscope (TECNAI G2)	FEI, USA
Laminar flow hood	Esco, Oregon, USA
Microplate reader (Victor X5)	Perkin Elmer, USA
CO_2 incubator	Binder CB 170, Germany
12-well plate	Eppendorf, Germany
Fume hood	Esco, Oregon, USA
Beam capsule	Eppendorf, Germany
Oven	Laboroptik, UK
Ultramicrotome	Leica EM UC7, Germany
Diamond knife	DiATOME, Switzerland
200-mesh copper frid	Sigma-Aldrich, USA
Nuclease-free PCR tube	Eppendorf, Germany
EZ-squeeze plastic pipette	EZ-Range, USA
Fluidigm machine	Fluidigm, USA
Flex Six^{TM} IFC	Fluidigm, USA
192.24 IFC	Fluidigm, USA

APPENDIX C

Preparation Protocols for Hormonal Solutions

C.1 Preparation of Pregnant Mare Serum Gonadotrophin (PMSG)

Folligon® (Merck Animal Health, Canada), containing 1000 IU of lyophilised PMSG, was used. The contents of one vial were reconstituted using the manufacturer's supplied solvent. To achieve a final concentration of 5 IU per 0.1 mL, the reconstituted solution was further diluted with 20 mL of sterile sodium chloride (NaCl) solution (0.9%). The solution was aliquoted into sterile microtubes and stored at –20 °C. Aliquots were thawed at room temperature prior to use.

C.2 Preparation of Human Chorionic Gonadotrophin (hCG)

Chorulon® (Merck Animal Health, Canada), containing 5000 IU of lyophilised hCG, was reconstituted using the supplied solvent. To obtain a final concentration of 5 IU per 0.1 mL, the solution was diluted with 100 mL of sterile NaCl solution (0.9%). The final solution was aliquoted and stored at –20 °C in sterile microtubes. Aliquots were thawed at room temperature immediately before administration.

APPENDIX D

Preparation and Handling of Tocotrienol-rich Fraction and Palm Olein

D.1 Preparation of Tocotrienol-Rich Fraction (TRF)
TRF was provided by Sime Darby Research in collaboration with the Malaysian Palm Oil Board (MPOB). A working concentration of 60 mg/kg body weight was used, based on published antioxidant efficacy studies. TRF was stored in amber vials at 4 °C to prevent oxidative degradation. Each week, fresh TRF working solutions were prepared based on the most recent recorded body weight of the mice. Before administration, the solution was brought to room temperature.

D.2 Preparation of Palm Olein (PO) Vehicle
Palm olein (PO), stripped of vitamin E and tocotrienols, was used as the vehicle control in the POVA group. It was handled and stored in the same manner as TRF to ensure consistency across experimental groups. The volume administered matched that of TRF, adjusted according to each animal's weight.

D.3 Oral Gavage Procedure
Oral administration was carried out using a 1 mL syringe fitted with a stainless-steel feeding needle. Dosing was performed daily between 9:00 and 10:00 AM to minimise circadian variation. The gavage volume was calculated based on individual body weight (typically 0.1 mL per 10 g body weight). Mice were gently restrained during administration to minimise distress.

APPENDIX E

IL-4 and IgE ELISA Procedure (Sandwich-ELISA)

E.1 Preparation of Working Solutions

Standards (IL-4 & IgE):

- Reconstitute standard with 1.0 mL of diluent to make 2000 pg/mL stock.
- Prepare serial dilutions: 2000, 1000, 500, 250, 125, 62.5, 31.25, 0 pg/mL using 1:1 dilution with diluent.

Biotinylated Detection Antibody:

- Dilute 100× stock to 1× using a 1:99 ratio with diluent.

HRP Conjugate:

- Dilute 100× stock to 1× using a 1:99 ratio with conjugate diluent.

Wash Buffer:

- Dilute 30 mL of concentrated wash buffer in 720 mL distilled water (final volume: 750 mL).
- Warm to 40 °C in water bath to avoid crystallization.

E.2 Assay Procedure (Sandwich-ELISA)

1. Use 96-well plates pre-coated with anti-IL-4 or anti-IgE antibodies.

2. Add 100 µL of each standard or sample (in duplicate) to appropriate wells.

3. Incubate at 37 °C for 1.5 hours with plate sealer.

4. Discard solution, add100 µL of biotinylated detection antibody to each well.

5. Incubate at 37 °C for 1 hour.

6. Wash plate 3 times:

- Add 350 µL wash buffer per well, soak 1–2 minutes, decant and pat dry.

7. Add 100 µL HRP conjugate to each well.

8. Incubate at 37 °C for 30 minutes.

9. Wash plate 5 times using the same method.

10. Add 90 µL substrate reagent (protect from light), incubate 15 minutes at 37 °C.

11. Add 50 µL stop solution in the same order.

12. Read absorbance at 450 nm using Victor X5 microplate reader.

E.3 Result Calculation

1. Average the OD readings for each standard and sample.

2. Subtract average OD of 0 pg/mL standard from all values.

3. Plot standard curve (x-axis: concentration, y-axis: corrected OD).

4. Interpolate sample concentration from the curve.

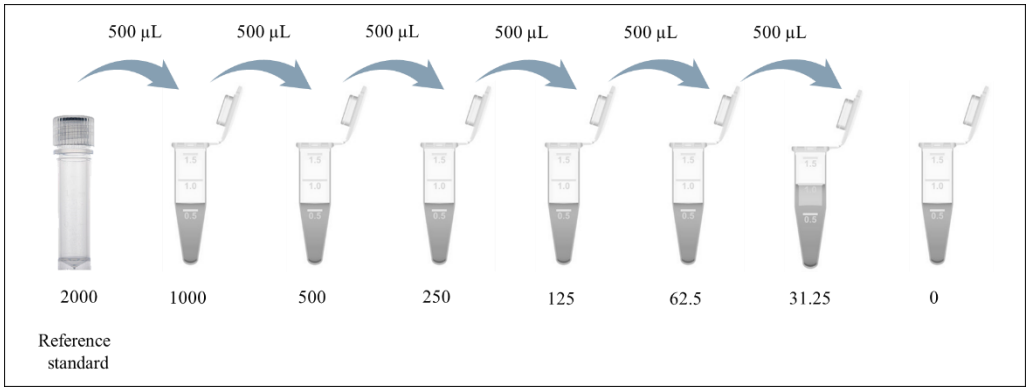

Figure E.1 Serial concentration dilution for IL-4 and Ig-E

APPENDIX F

8-OHdG ELISA Procedure (Competitive-ELISA)

F.1 Preparation of Working Solutions

Standard (8-OHdG):

- Reconstitute with 1.0 mL diluent to make 100 ng/mL stock solution.
- Prepare serial dilutions: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0 ng/mL.
- Dilute 1:1 using 500 µL steps in 1.5 mL tubes.

Biotinylated Detection Antibody:

- Dilute 100× stock to 1× using 1:99 ratio with antibody diluent.
- Use 50 µL per well.

HRP Conjugate:

- Dilute 100× stock to 1× using a 1:99 ratio with conjugate diluent.

Wash Buffer:

- Dilute 30 mL of concentrated wash buffer in 720 mL distilled water (final volume: 750 mL).
- Warm to 40 °C in water bath to avoid crystallization.

F.2 Assay Procedure (Competitive-ELISA)

1. Use 96-well plate pre-coated with 8-OHdG.

2. Add 50 µL of standards and samples (in duplicate) into respective wells.

3. Immediately add 50 µL of biotinylated detection antibody to each well.

4. Seal and incubate at 37 °C for 45 minutes.

5. Wash plate 3 times:

- Add 350 µL wash buffer, soak 1–2 minutes, decant and pat dry.

6. Add 100 µL HRP conjugate to each well.

7. Incubate at 37 °C for 30 minutes.

8. Wash plate 5 times using the same method.

9. Add 90 µL substrate reagent (avoid light), incubate 15 minutes at 37 °C.

10. Add 50 µL stop solution in the same order as substrate reagent.

11. Read absorbance at 450 nm using Victor X5 microplate reader.

F.3 Result Calculation

1. Average OD values for each standard and sample.

2. Plot standard curve (x-axis: concentration, y-axis: corrected OD)

3. Determine sample concentration by interpolating from the curve.

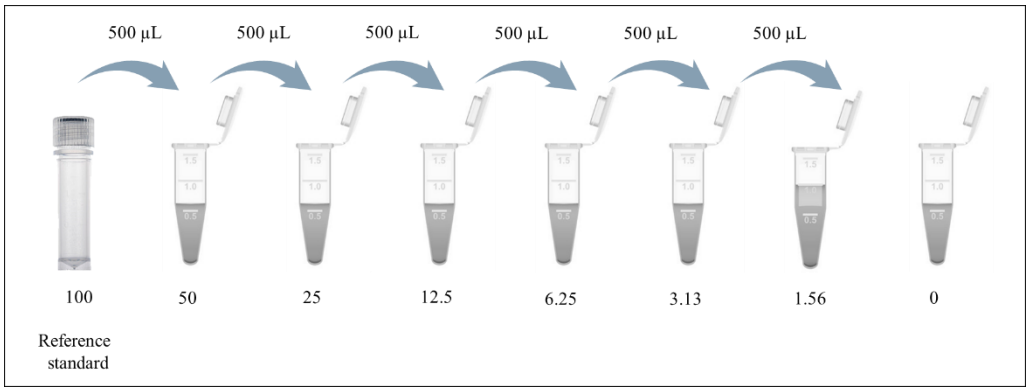

Figure F.1 Serial concentration dilution for 8-OHdG

APPENDIX G

Total Antioxidant Capacity (TAOC) – FRAP Method

G.1 Preparation of Working Solutions

FRAP Working Solution:

- Mix reagents in ratio: 10× Buffer: 1× TPTZ: 1× Substrate.
- Mix thoroughly and protect from light.

100 mM $FeSO_4$ Solution:

- Dissolve 27.8 mg $FeSO_4 \cdot 7H_2O$ in 1 mL double distilled water.

TAOC Standards:

- Dilute 100 mM $FeSO_4$ to prepare standards at: 0, 0.3, 0.6, 0.9, 1.2, 1.8, 2.1, 2.5 mmol/L.

G.2 Assay Procedure (FRAP Method)

1. Add 5 µL of each standard and sample into designated wells.

2. Add 180 µL of FRAP working solution into each well.

3. Incubate the 96-well plate at 37 °C for 5 minutes.

4. Measure absorbance at 595 nm using Victor X5 Microplate Reader.

G.3 Result Calculation

1. Plot standard curve in Excel (x-axis: concentration, y-axis: OD reading

2. Use formula to calculate TAOC:

$$T - AOC\ (mmoL) = \left[\left(OD_{sample} - OD_{blank}\right) - \ std\ curve's\ intercept\right] \div \text{std curve's slope} \times Dilution\ factor$$

APPENDIX H

P_4 and E_2 ELISA Procedure (Competitive-ELISA)

H.1 Preparation of Working Solutions for Progesterone and Estrogen Standards

Progesterone Standard (ng/mL)

- Add1.0 mL Reference Standard & Sample Diluent into Reference Standard.
- Invert gently for 10 minutes to make 20 ng/mL working solution.
- Prepare serial dilutions: 20, 10, 5, 2.5, 1.25, 0.62, 0.31, 0 ng/mL
- Add 500 µL diluent into 7 Eppendorf tubes (1.5 mL).
- Perform 1:1 serial dilution as follows:
    - 500 µL of 20 ng/mL → 1st tube = 10 ng/mL
    - 500 µL of 10 ng/mL → 2nd tube = 5 ng/mL
    - Repeat to get: 2.5, 1.25, 0.62, 0.31 ng/mL
    - Last tube remains undiluted = 0 ng/mL

Estrogen Standard (pg/mL)

- Add1.0 mL Reference Standard & Sample Diluent into Reference Standard.
- Invert gently for 10 minutes to make 600 pg/mL working solution.
- Prepare serial dilutions: 600, 300, 150, 75, 37.5, 18.75, 9.375, 0 pg/mL
- Add 500 µL diluent into 7 Eppendorf tubes (1.5 mL).
- Perform 1:1 serial dilution as follows:
    - 500 µL of 600 pg/mL → 1st tube = 300 pg/mL
    - 500 µL of 300 pg/mL → 2nd tube = 150 pg/mL
    - Repeat to get: 75, 37.5, 18.75, 9.375 pg/mL
    - Last tube remains undiluted = 0 pg/mL

Biotinylated Detection Antibody:

- Dilute 100× stock to 1× using a 1:99 ratio with diluent.

HRP Conjugate:

- Dilute 100× stock to 1× using a 1:99 ratio with conjugate diluent.

Wash Buffer:

- Dilute 30 mL of concentrated wash buffer in 720 mL distilled water (final volume: 750 mL).
- Warm to 40 °C in water bath to avoid crystallization.

H.2 Assay Procedure (Competitive-ELISA)

1. Use 96-well plate pre-coated with 8-OHdG.

2. Add 50 µL of standards and samples (in duplicate) into respective wells.

3. Immediately add 50 µL of biotinylated detection antibody to each well.

4. Seal and incubate at 37 °C for 45 minutes.

5. Wash plate 3 times:

- Add 350 µL wash buffer, soak 1–2 minutes, decant and pat dry.

6. Add 100 µL HRP conjugate to each well.

7. Incubate at 37 °C for 30 minutes.

8. Wash plate 5 times using the same method.

9. Add 90 µL substrate reagent (avoid light), incubate 15 minutes at 37 °C.

10. Add 50 µL stop solution in the same order as substrate reagent.

11. Read absorbance at 450 nm using Victor X5 microplate reader.

H.3 Result Calculation

1. Average OD values for each standard and sample.

2. Plot standard curve (x-axis: concentration, y-axis: corrected OD)

3. Determine sample concentration by interpolating from the curve.

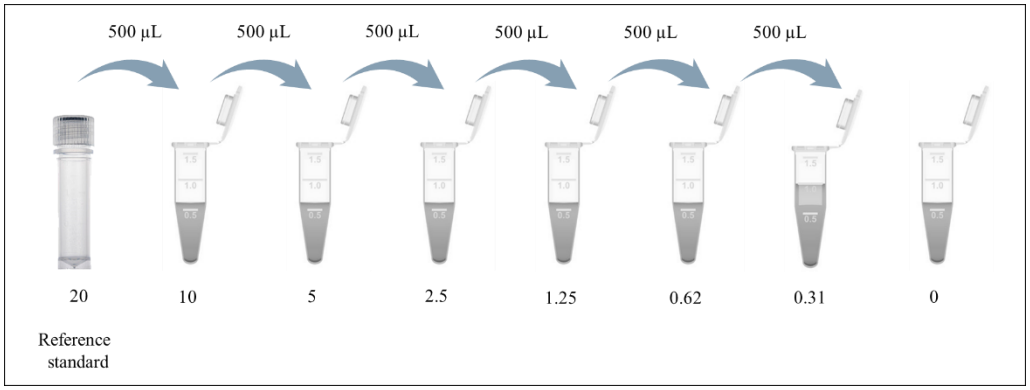

Figure H.1 Serial concentration dilution for progesterone

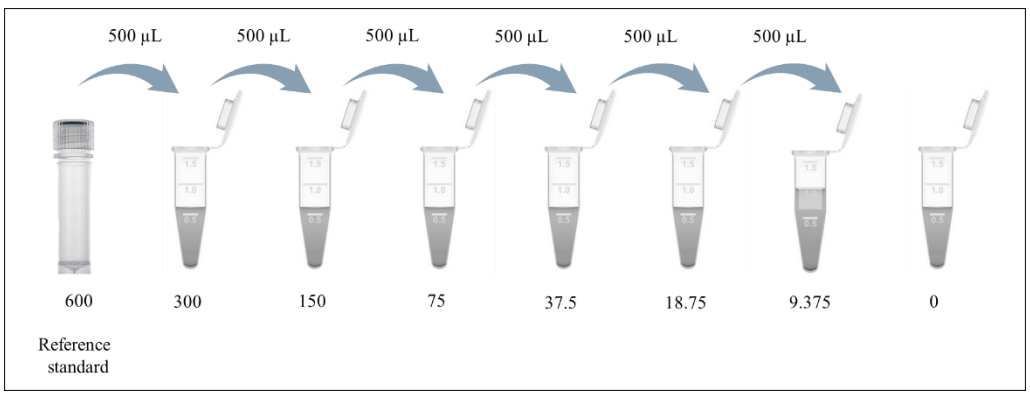

Figure H.2 Serial concentration dilution for estrogen

APPENDIX I

Observation of Morphology and Developmental Potential of Embryos

ASEBIR's morphological scoring system outlined by Saiz et al., (2018)

a) Stage-specific characteristics: For cleavage embryos (2-, and 8-cell), the term ''stage- specific'' was used to unify and define the cellular symmetry of the embryos (Prados et al., 2012). Stage-specified embryo was considered when the size of the blastomeres at each stage is consistent with their cleavage cycle (assessment time). Non-stage-specific embryos was considered when the size of the blastomeres at each stage were incompatible with their cleavage cycle. The size of blastomeres can be classified into even or uneven size. Uneven blastomere was observed when two blastomeres were unequal in size. The uneven size of blastomeres was identified when there was >25% difference in the diameter size of the larger blastomere compared to the smallest blastomere.

b) Compaction activity: Fully compaction morula was defined when the embryo appeared as a compact mass. Partial compaction was defined when blastomeres begun to compact but individual blastomeres can still be identified.

c) Degree of expansion of the blastocoel: The degree of expansion was classified into fully or partial. If the blastocoel cavity made up majority of the blastocyst, it indicated fully expansion. If only half or less than half of blastocyst was made up of blastocoel cavity, it indicated partial expansion.

d) Characteristics of TE and ICM: TE was assessed by their total number, shape, and degree of cohesion lining the layer of cells. The number was classified either many, not many and few cells. The shape of TE was observed either they were homogenous, non-homogenous or degenerated between each TE. For the ICM, the assessment was based on the shape of the ICM where the ICM must be oval and compacted.

e) Degree of fragmentation: Fragmentation was defined as the presence of anucleate structures from blastomeric origin (Prados et al., 2012). The degree of fragmentation was presented as the percentage of the total cytoplasmic volume. The fragmentation was classified into 4 degrees of fragmentation; ≤ 10 %, > 10 % to 25 %, > 25 % to 35% and > 35% based on Saiz et al., (2018). The embryos were considered abnormal of the fragmentation degree was > 25 %.

APPENDIX J

Preparation of Chemicals for Transmission Electron Microscopy (TEM)

Sodium Cacodylate Buffer: The purchased 0.2 M sodium cacodylate buffer (Agar Scientific, UK) was readily to be used and was stored at 4 °C. The buffer was used to maintain the pH between 7.2 – 7.4 during fixation to avoid morphological artifacts if pH decrease.

2.5 % Glutaraldehyde: The purchased glutaraldehyde (Agar Scientific, UK) was at 25 % concentration. However, the concentration to be used was 2.5 %. To prepare 100 mL of 2.5 % glutaraldehyde stock solution, 10 mL of 25 % glutaraldehyde was diluted in 50 mL sodium cacodylate buffer. Then, 40 mL distilled water was added to volume up until 100 mL. The stock solution was stored at 4 °C.

1 % Osmium Tetroxide: The purchased osmium tetroxide was (Agar Scientific, UK) was at 4 % concentration. However, as the embryos size less than 0.5 mm, usage of 1 % osmium tetroxide was adequate. To prepare 10 mL of 1 % osmium tetroxide stock solution, 2.5 mL of 4 % osmium tetroxide was diluted in 7.5 mL distilled water. The stock solution was stored in dark Schott bottle at 4 °C.

Serial Dehydration of Acetone: The serial dehydration of acetone was prepared as 35 %, 50 %, 75 %, 95 % and 100 %. The purchased acetone was at 100 % concentration. Briefly, the concentration was diluted with Millipore pure water as shown in Table J.1.

Resin Mixture: The purchased Agar 100 Resin Kit (Agar Scientific, UK) consisted of Agar 100 Resin, Dodecenyl Succinic Anhydride (DDSA), Methyl Nadic Anhydride (MNA) and Benzyl Dimethylamine (BDMA). To prepare resin mixture, 10 mL Agar 100 Resin with 6 mL DDSA, 5.5 mL MNA and 0.5 mL BDMA was mixed in a plastic container. The mixture was then stirred thoroughly by using autoclaved wooden stick and left for few minutes until formed bubbles subsides. Absence of bubbles in the mixture ensures thorough and proper infiltration into the embryos. Figure J.2

Uranyl Acetate: The purchased uranyl acetate (Agar Scientific, UK) was readily to be used and was stored at 4 °C.

Lead Citrate: The preparation of lead citrate was conducted under the fume hood. To prepare the stock solution, 1.33 g lead nitrate (Agar Scientific, UK) and 1.76 g sodium citrate (Agar Scientific, UK) were added into 50 mL volumetric flask containing 30 mL CO_2-free double-distilled water. The flask was then shaken vigorously several times for over a period of 30 minutes until a milky white suspension was produced. The solution was then stirred on a stir plate until remaining crystals (lead nitrate and/or sodium citrate) were dissolved. While stirring, 5 – 8 mL 1M NaOH was added to the solution to change milky white suspension to clear colour. Next, the pH of the solution was tested by using pH meter before the solution was left to cool down at room temperature. The pH ranged 12.0 ± 0.1 was recommended. Once the pH was verified, CO_2-free water was added to volume up until 50 mL. Lastly, the flask was sealed and stored. Prior usage, the solution was centrifuged at 5000 × g for 10 minutes.

Table J.1 Preparation of Serial dehydration of acetone

Serial dehydration of acetone (%)	Volume of acetone (mL)	Volume of pure water (mL)
100	100	0
95	95	5
75	75	25
50	50	50
35	35	65

Table J.2 Preparation of resin mixture

Resin mixture	Volume (mL)
Agar 100 Resin	10
Dodecenyl Succinic Anhydride (DDSA)	6
Methyl Nadic Anhydride (MNA)	5.5
Benzyl Dimethylamine (BDMA)	0.5
Total volume	20

APPENDIX K

Table K.1: Mean Relative Volume (Vv) of Selected Organelles (%) in 2-cell Embryos

Relative volume (Vv) of organelle (%)	SHAM	PBS	OVA3	POVA	TOVA
Total organelles	36.25 ± 0.210 ns-*	36.52 ± 5.851	11.12 ± 2.047 ***	16.40 ± 1.571 **, ns-&	24.57 ± 0.404 ns-*, &, ns-$
Total mitochondria	21.54 ± 1.560 ns-*	20.65 ± 1.053	7.33 ± 3.266 **	7.52 ± 0.565 **, ns-&	15.59 ± 1.811 ns-*, ns-&, ns-$
Mitochondrial clusters	19.25 ± 1.617 ns-*	16.70 ± 0.877	0.646 ± 0.333 ****	5.81 ± 0.521 **, ns-&	13.12 ± 2.161 ns-*, &&&, $
Lipid droplet	6.22 ± 0.567 ns-*	5.95 ± 1.114	1.05 ± 0.658 ns-*	6.54 ± 1.885 ns-*, &	2.64 ± 0.191 ns-*, ns-&, ns-$
Peridroplet mitochondria	10.12 ± 1.379 ns-*	7.48 ± 0.477	0.48 ± 0.480 **	3.53 ± 1.156 ns-*, ns-&	4.64 ± 0.353 ns-*, &, ns-$

This table presents the mean relative volume (%) of total organelles and their subtypes (total mitochondria, mitochondrial clusters, lipid droplets, and peridroplet mitochondria) in 2-cell embryos obtained from different experimental groups: SHAM (untreated), PBS (control), OVA3 (asthmatic), POVA (Palm Olein + OVA), and TOVA (TRF + OVA). The data are expressed as mean ± SEM. Symbols indicate statistical comparisons. *: compared to the PBS group. &: compared to the OVA3 group. $: compared to the POVA group. ns: not significant

APPENDIX L

Indexed Publication [1]

Wafriy C.I, Kamsani Y.S, Nor-Ashikin M.N.K, Nasir N.A.A, Hanafiah M. (2020). Ovalbumin Causes Impairment of Preimplantation Embryonic Growth in Asthma-induced Mice. Journal of Reproductive Immunology. https://doi.org/10.1016/j.jri.2020.103240

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Ovalbumin causes impairment of preimplantation embryonic growth in asthma-induced mice

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ARTICLE INFO

Keywords:
Ovalbumin
Asthma
Inflammation
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ABSTRACT

Insufficient experimental studies have reported the effect of ovalbumin (OVA) as an allergen towards embryonic growth in asthma mouse model. The impact of 10 μg/200 μL OVA on maternal inflammatory and oxidative stress (OS) responses, and preimplantation embryonic development was investigated in this study. We first established OVA-induced asthma mouse model, and following superovulation, mated the females and challenged them with Methacholine (Mch) test. Upon embryo retrieval, only those with the highest implantation potential were cultured *in vitro*. Significant reduction in the number of embryos at each preimplantation stage was noted in the treated group. Uneven sized blastomeres at 2-, 4- and 8-cell stages were also evident in this group. Embryo fragmentation was significant at only 2-, 4- and 8-cell stages. We also found that OVA tended to raise maternal inflammatory and OS biomarker levels as well as to cause inappropriate levels of pregnancy hormones progesterone (P_4) and estrogen (E_2) although insignificant. The combined results indicate that 10 μg/200 μL OVA had altered both quality and quantity of the embryos in asthma mouse model although its effect on pregnancy hormones, inflammatory and OS responses were non-pathological.

1. Introduction

Asthma is a chronic disease where inflammation occurs in the airways causing it to become hyper-responsive with symptoms of wheezing, dyspnea, chest tightness and coughing (Mims, 2015). In Malaysia, mortality rate due to asthma is 1.29 % (Ministry of Health Malaysia, 2018). This respiratory condition is caused by factors such as allergies, tobacco smoking, environmental factors, obesity, stress or genetic (Brazier, 2020) among many others. Among types of asthma (including allergic, non-allergic or occupational); allergic asthma may be triggered by allergens such as pollen, dust or pet dander.

In animal model of asthma, ovalbumin (OVA), a protein component in egg white is commonly used to mimic allergic asthma due to its association with T-helper type 2 (Th 2) immune response. Activation of Th 2 immune response promotes production of Th 2 cytokines namely interleukin (IL)-4, IL-5 and IL-13 which play critical role in asthma exacerbation (Casaro et al., 2019). IL-4 stimulates the release of immunoglobulin (Ig)-E and promotes mucus secretion in the airways. IL-5 on the other hand causes inflammatory cells (mainly eosinophil) to infiltrate, which induces airway hyper-responsiveness (Kim D.I et al., 2019; Kim S.-B. et al., 2019).

Presently, little is known about crosslink between OVA and oxidative stress (OS). However, literature indicated that inflammatory process is a key mediator behind OVA-induced OS effects. Inflammatory process is known to associate with OS and reduced cellular antioxidant capacity. The theory being overproduced free radicals impair cell membrane fatty acids and proteins, and compensate their functions is evident in the literature. In terms of cellular organelle integrity, access of free radicals to the DNA leads to mutation, single- or double strand breaks. This may be a predisposing factor for various genetic disorders.

Evidence of inflammatory cells invasion into the respiratory airways and the resulting lipid peroxidation in female BALB/c mice induced by

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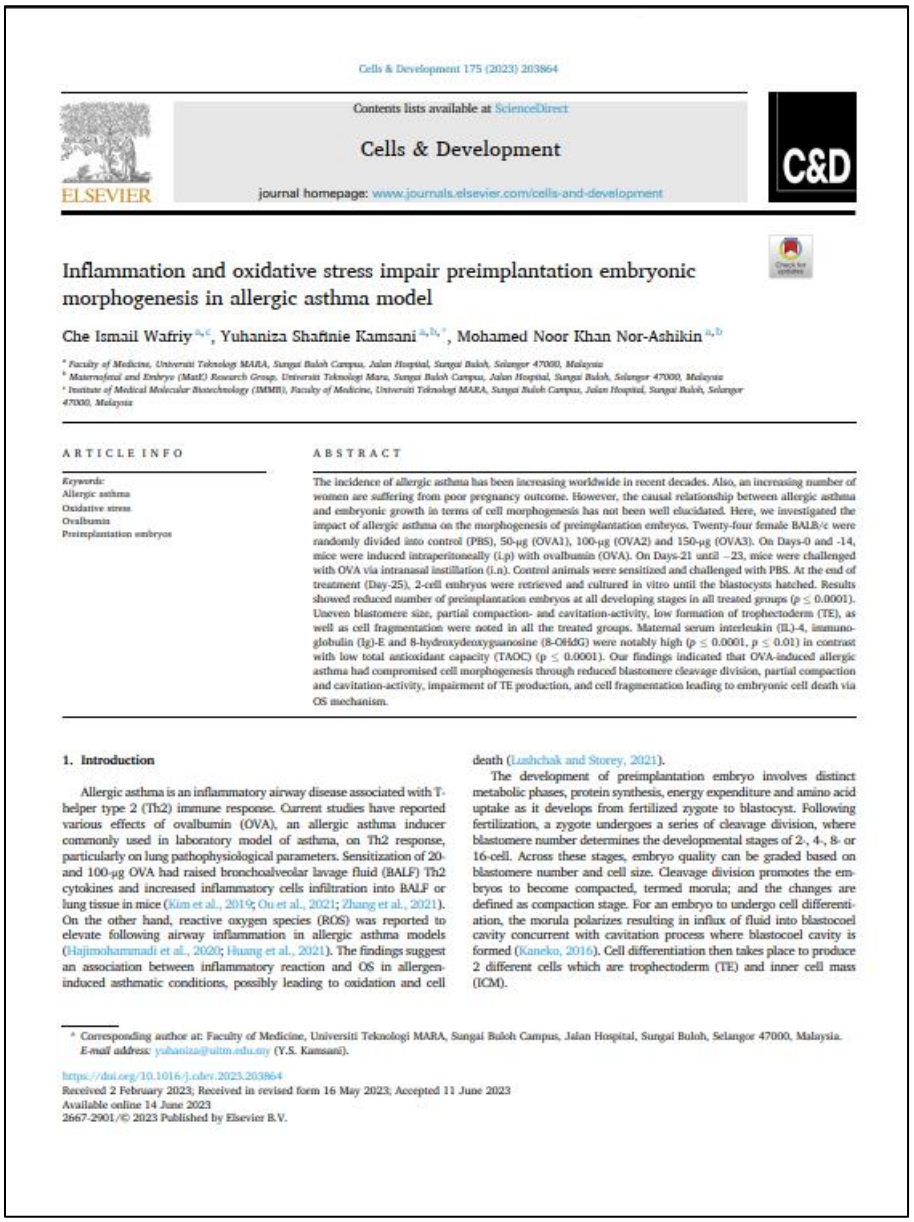
APPENDIX M

Indexed Publication [2]

Wafriy C.I, Kamsani Y.S, Nor-Ashikin M.N.K. (2023). Inflammation and Oxidative Stress Impair Preimplantation Embryonic Morphogenesis in Allergic Asthma Model. Cells & Development. https://doi.org/10.1016/j.cdev.2023.203864



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Inflammation and oxidative stress impair preimplantation embryonic morphogenesis in allergic asthma model

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ARTICLE INFO

Keywords:
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Ovalbumin
Preimplantation embryos

ABSTRACT

The incidence of allergic asthma has been increasing worldwide in recent decades. Also, an increasing number of women are suffering from poor pregnancy outcome. However, the causal relationship between allergic asthma and embryonic growth in terms of cell morphogenesis has not been well elucidated. Here, we investigated the impact of allergic asthma on the morphogenesis of preimplantation embryos. Twenty-four female BALB/c were randomly divided into control (PBS), 50-μg (OVA1), 100-μg (OVA2) and 150-μg (OVA3). On Days-0 and -14, mice were induced intraperitoneally (i.p) with ovalbumin (OVA). On Days-21 until −23, mice were challenged with OVA via intranasal instillation (i.n). Control animals were sensitized and challenged with PBS. At the end of treatment (Day-25), 2-cell embryos were retrieved and cultured in vitro until the blastocysts hatched. Results showed reduced number of preimplantation embryos at all developing stages in all treated groups ($p \leq 0.0001$). Uneven blastomere size, partial compaction- and cavitation-activity, low formation of trophectoderm (TE), as well as cell fragmentation were noted in all the treated groups. Maternal serum interleukin (IL)-4, immunoglobulin (Ig)-E and 8-hydroxydeoxyguanosine (8-OHdG) were notably high ($p \leq 0.0001$, $p \leq 0.01$) in contrast with low total antioxidant capacity (TAOC) ($p \leq 0.0001$). Our findings indicated that OVA-induced allergic asthma had compromised cell morphogenesis through reduced blastomere cleavage division, partial compaction and cavitation-activity, impairment of TE production, and cell fragmentation leading to embryonic cell death via OS mechanism.

1. Introduction

Allergic asthma is an inflammatory airway disease associated with T-helper type 2 (Th2) immune response. Current studies have reported various effects of ovalbumin (OVA), an allergic asthma inducer commonly used in laboratory model of asthma, on Th2 response, particularly on lung pathophysiological parameters. Sensitization of 20- and 100-μg OVA had raised bronchoalveolar lavage fluid (BALF) Th2 cytokines and increased inflammatory cells infiltration into BALF or lung tissue in mice (Kim et al., 2019; Ou et al., 2021; Zhang et al., 2021). On the other hand, reactive oxygen species (ROS) was reported to elevate following airway inflammation in allergic asthma models (Hajimohammadi et al., 2020; Huang et al., 2021). The findings suggest an association between inflammatory reaction and OS in allergen-induced asthmatic conditions, possibly leading to oxidation and cell death (Lushchak and Storey, 2021).

The development of preimplantation embryo involves distinct metabolic phases, protein synthesis, energy expenditure and amino acid uptake as it develops from fertilized zygote to blastocyst. Following fertilization, a zygote undergoes a series of cleavage division, where blastomere number determines the developmental stages of 2-, 4-, 8- or 16-cell. Across these stages, embryo quality can be graded based on blastomere number and cell size. Cleavage division promotes the embryos to become compacted, termed morula; and the changes are defined as compaction stage. For an embryo to undergo cell differentiation, the morula polarizes resulting in influx of fluid into blastocoel cavity concurrent with cavitation process where blastocoel cavity is formed (Kaneko, 2016). Cell differentiation then takes place to produce 2 different cells which are trophectoderm (TE) and inner cell mass (ICM).

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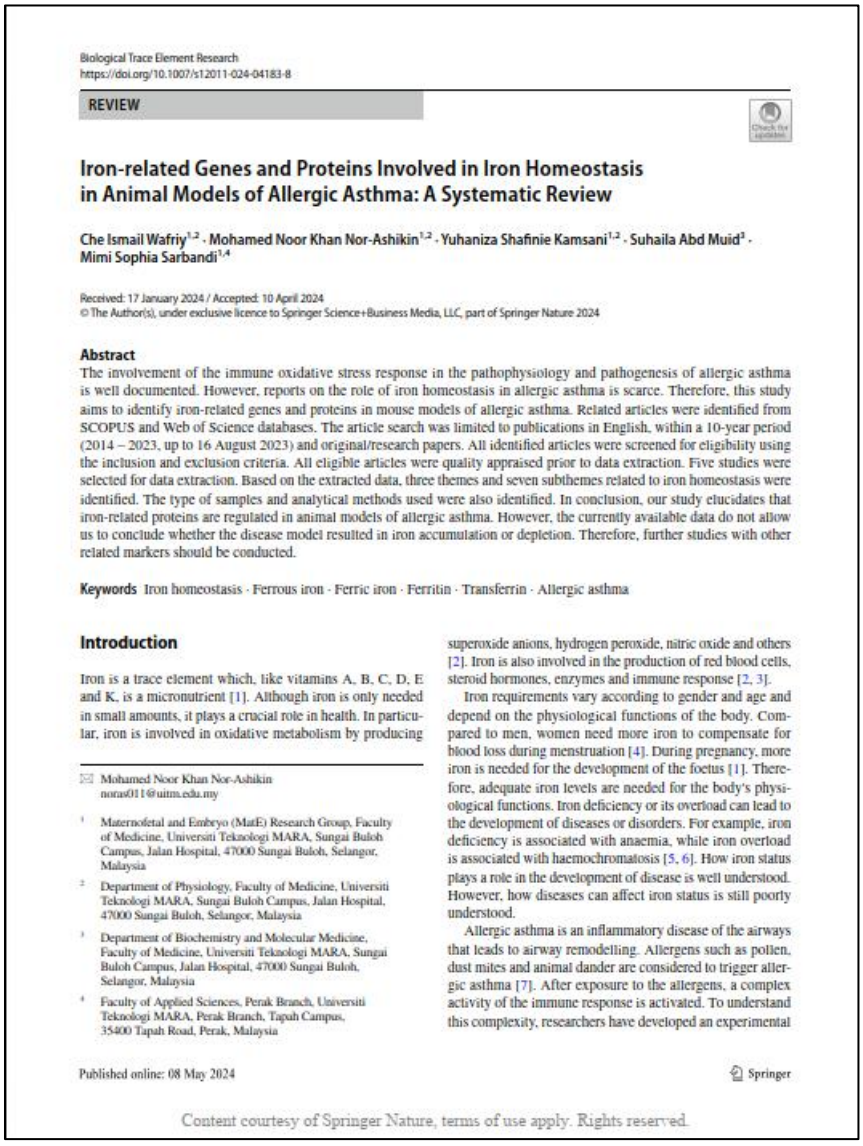
APPENDIX N

Indexed Publication [3]

Wafriy C.I, Nor-Ashikin M.N.K, Kamsani Y.S, Muid S.A, Sarbandi M.S. (2024). Iron-related Proteins and Genes Involved in Iron Homeostasis in Animal Models of Allergic Asthma: A systematic review. Biological Trace Element Research. https://doi.org/10.1007/s12011-024-04183-8



Biological Trace Element Research
https://doi.org/10.1007/s12011-024-04183-8

REVIEW

Iron-related Genes and Proteins Involved in Iron Homeostasis in Animal Models of Allergic Asthma: A Systematic Review

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Abstract
The involvement of the immune oxidative stress response in the pathophysiology and pathogenesis of allergic asthma is well documented. However, reports on the role of iron homeostasis in allergic asthma is scarce. Therefore, this study aims to identify iron-related genes and proteins in mouse models of allergic asthma. Related articles were identified from SCOPUS and Web of Science databases. The article search was limited to publications in English, within a 10-year period (2014 – 2023, up to 16 August 2023) and original/research papers. All identified articles were screened for eligibility using the inclusion and exclusion criteria. All eligible articles were quality appraised prior to data extraction. Five studies were selected for data extraction. Based on the extracted data, three themes and seven subthemes related to iron homeostasis were identified. The type of samples and analytical methods used were also identified. In conclusion, our study elucidates that iron-related proteins are regulated in animal models of allergic asthma. However, the currently available data do not allow us to conclude whether the disease model resulted in iron accumulation or depletion. Therefore, further studies with other related markers should be conducted.

Keywords Iron homeostasis · Ferrous iron · Ferric iron · Ferritin · Transferrin · Allergic asthma

Introduction

Iron is a trace element which, like vitamins A, B, C, D, E and K, is a micronutrient [1]. Although iron is only needed in small amounts, it plays a crucial role in health. In particular, iron is involved in oxidative metabolism by producing superoxide anions, hydrogen peroxide, nitric oxide and others [2]. Iron is also involved in the production of red blood cells, steroid hormones, enzymes and immune response [2, 3].

Iron requirements vary according to gender and age and depend on the physiological functions of the body. Compared to men, women need more iron to compensate for blood loss during menstruation [4]. During pregnancy, more iron is needed for the development of the foetus [1]. Therefore, adequate iron levels are needed for the body's physiological functions. Iron deficiency or its overload can lead to the development of diseases or disorders. For example, iron deficiency is associated with anaemia, while iron overload is associated with haemochromatosis [5, 6]. How iron status plays a role in the development of disease is well understood. However, how diseases can affect iron status is still poorly understood.

Allergic asthma is an inflammatory disease of the airways that leads to airway remodelling. Allergens such as pollen, dust mites and animal dander are considered to trigger allergic asthma [7]. After exposure to the allergens, a complex activity of the immune response is activated. To understand this complexity, researchers have developed an experimental

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APPENDIX O

Certificate of Award

- First Runner-up in 3MT Competition 2022

APPENDIX P

Certificate of Award

- Top 2 Oral Presenter in Graduate Research Symposium 2021

APPENDIX Q

Certificate of Award

- Best Oral Presenter (Gold Award) in 2nd International Conference on Reproductive Science & Medicine, Embryology & Andrology (ICRSMEA) 2025

UNIVERSITI MALAYA

2nd International Conference of Reproductive Science & Medicine, Embryology & Andrology (ICRSMEA)

24th-25th April 2025

Organized by

Department of Physiology, Faculty of Medicine
Universiti Malaya, Kuala Lumpur, Malaysia.

Presented to

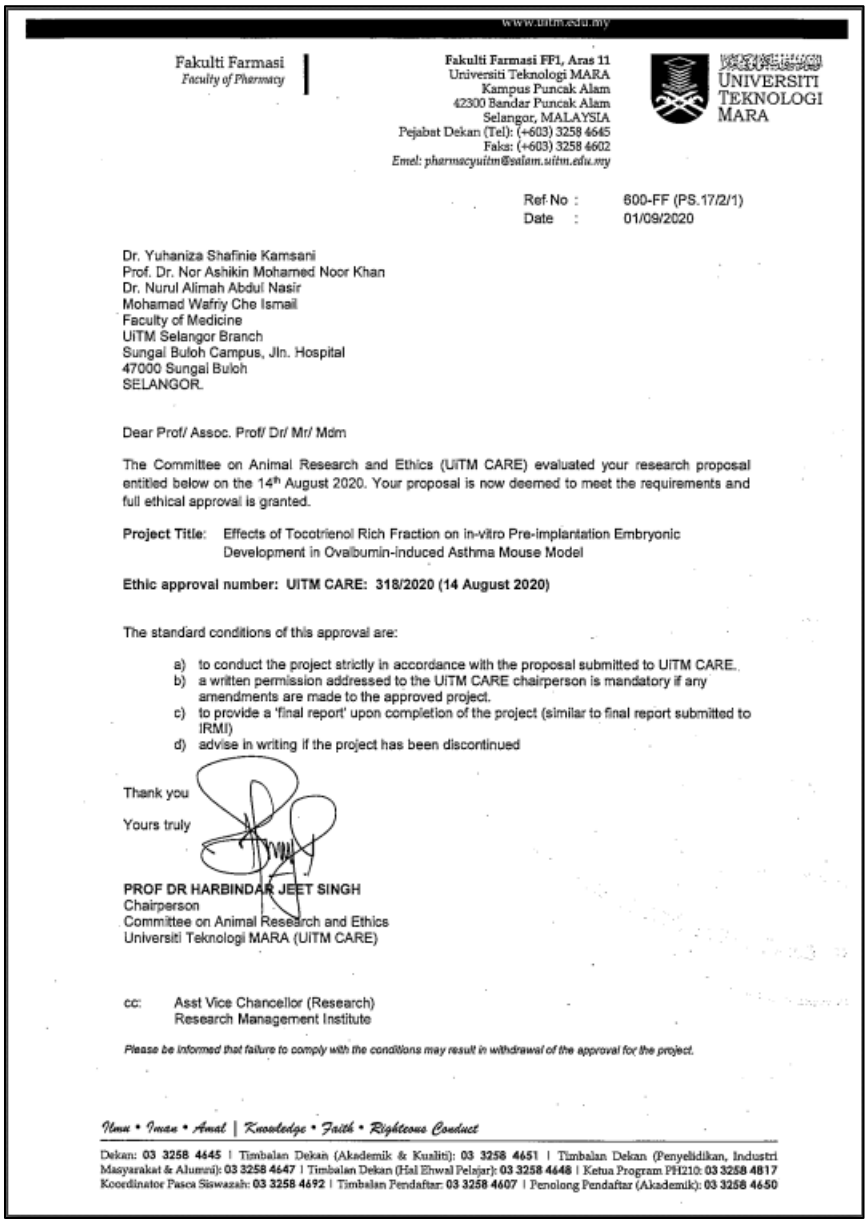
Mohamad Wafriy Che Ismail

Best Oral Presentation
Gold Award

ICRSMEA 2025 CHAIRPERSON
Prof Dr Naguib bin Salleh
Head of Department of Physiology Faculty of Medicine, Universiti Malaya,
Kuala Lumpur, malaysia.

APPENDIX R

Animal Ethics Approval Letter



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UNIVERSITI TEKNOLOGI MARA

Ref No : 600-FF (PS.17/2/1)
Date : 01/09/2020

Dr. Yuhaniza Shafinie Kamsani
Prof. Dr. Nor Ashikin Mohamed Noor Khan
Dr. Nurul Alimah Abdul Nasir
Mohamad Wafriy Che Ismail
Faculty of Medicine
UiTM Selangor Branch
Sungai Buloh Campus, Jln. Hospital
47000 Sungai Buloh
SELANGOR.

Dear Prof/ Assoc. Prof/ Dr/ Mr/ Mdm

The Committee on Animal Research and Ethics (UiTM CARE) evaluated your research proposal entitled below on the 14th August 2020. Your proposal is now deemed to meet the requirements and full ethical approval is granted.

Project Title: Effects of Tocotrienol Rich Fraction on in-vitro Pre-implantation Embryonic Development in Ovalbumin-induced Asthma Mouse Model

Ethic approval number: UiTM CARE: 318/2020 (14 August 2020)

The standard conditions of this approval are:

a) to conduct the project strictly in accordance with the proposal submitted to UiTM CARE.
b) a written permission addressed to the UiTM CARE chairperson is mandatory if any amendments are made to the approved project.
c) to provide a 'final report' upon completion of the project (similar to final report submitted to IRMI)
d) advise in writing if the project has been discontinued

Thank you

Yours truly

PROF DR HARBINDAR JEET SINGH
Chairperson
Committee on Animal Research and Ethics
Universiti Teknologi MARA (UiTM CARE)

cc: Asst Vice Chancellor (Research)
Research Management Institute

Please be informed that failure to comply with the conditions may result in withdrawal of the approval for the project.

Ilmu • Iman • Amal | Knowledge • Faith • Righteous Conduct

APPENDIX S

Animal Handling Certificate

UNIVERSITI TEKNOLOGI MARA

FACULTY OF PHARMACY

Certificate of Participation

MOHAMAD WAFRIY CHE ISMAIL

Workshop on Laboratory Rodents (Rats and Mice) Care and Use

26th - 27th March 2019

Laboratory Animal Facility and Management (LAFAM)
Faculty of Pharmacy, UiTM Selangor, Puncak Alam Campus, Malaysia.

The workshop covered the following topics :

- Ethics in laboratory animal research
- Laboratory rodent health monitoring
- Anaesthesia and euthanasia of rodents
- Blood sampling for rodents
- Statistical analysis using SPSS
- Management of laboratory rodents
- Established standing procedures (SOP) of LAFAM
- Institutional Care and Use Form (UiTM CARE)

Practical Assessment

Restraint	PASS
Oral gavage	PASS
Anaesthesia	PASS
Blood sampling	PASS
Euthanasia	PASS
Work ethics	PASS
OVERALL RESULT	**PASS**

ASSOC. PROF. DATO' DR. S. VELLAYAN
Chairman
Workshop on Laboratory Rodents (Rats and Mice)
Care and Use
Faculty of Pharmacy
UiTM Selangor

PROF. DR. AISHAH ADAM
Dean
Faculty of Pharmacy
UiTM Selangor

AUTHOR'S PROFILE

Mohamad Wafriy bin Che Ismail was born and raised in Kelantan, Malaysia. He attended Machang Science Secondary School (SM Sains Machang), Kelantan before pursuing a Foundation in Medical Science and subsequently obtaining a Bachelor of Medical Science (Hons) from Management and Science University (MSU), Shah Alam, Malaysia. During his undergraduate studies, he was actively involved with the Reproductive Health Association promoting reproductive health awareness. He later enrolled in a Master's program (MSc) and successfully converted to Doctoral study. His PhD research focused on embryology and reproductive biology where he developed expertise in embryo handling, retrieval, culture and observation as well as protein, ultrastructural, and molecular analyses. Throughout his study, he has published his research in high-quartile scientific journals and presented his findings at both national and international conferences. His research reflects a deep interest in embryology particularly on the cellular and molecular mechanisms influencing early embryonic viability.

LIST OF PUBLICATION

Wafriy C.I, Kamsani Y.S, Nor-Ashikin M.N.K, Nasir N.A.A, Hanafiah M. (2020). Ovalbumin Causes Impairment of Preimplantation Embryonic Growth in Asthma-induced Mice. Journal of Reproductive Immunology. https://doi.org/10.1016/j.jri.2020.103240

Wafriy C.I, Kamsani Y.S, Nor-Ashikin M.N.K. (2023). Inflammation and Oxidative Stress Impair Preimplantation Embryonic Morphogenesis in Allergic Asthma Model. Cells & Development. https://doi.org/10.1016/j.cdev.2023.203864

Wafriy C.I, Nor-Ashikin M.N.K, Kamsani Y.S, Muid S.A, Sarbandi M.S. (2024). Iron-related Proteins and Genes Involved in Iron Homeostasis in Animal Models of Allergic Asthma: A systematic review. Biological Trace Element Research. https://doi.org/10.1007/s12011-024-04183-8

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Wafriy C.I, Nasir N.A.A & Kamsani Y.S (2019), Effects of Tocotrienol Rich Fraction (TRF) on in vitro Pre-implantation Embryonic Development in Asthma Mouse Model. Abstract of Postgraduate Conference (FYPPC), 14th June 2019, Capitol Hotel, Kuala Lumpur, page 1-3.

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Wafriy C.I, Nor-Ashikin M.N.K, & Kamsani Y.S (2021), Tocotrienol Rich Fraction Improves Blastomeres Symmetry and Embryo Fragmentation in Ovalbumin-induced Asthma Model. Abstract of International Postgraduate Symposium in Biology, Biotechnology and Bioengineering (IPSBIO3 2021), 27th & 28th October 2021, Institute of Systems Biology, Universiti Kebangsaan Malaysia (Online).

Wafriy C.I, Nor-Ashikin M.N.K, & Kamsani Y.S (2021), Tocotrienol Rich Fraction Supplementation Increases the Rate of Hatching Blastocyst in Ovalbumin-induced Asthma Model. Abstract of Molecular Medicine Postgraduate Students (MOMPES) 1st Scientific Symposium, 22nd November 2021, University Malaya (Online).

Wafriy C.I, Nor-Ashikin M.N.K, & Kamsani Y.S (2021), Tocotrienol Rich Fraction Promotes 2-cell Embryo Retrieval in Ovalbumin-induced Asthma Model. Abstract of Graduate Research Symposium 2021, 23rd – 25th November 2021, Taylor's

University Lakeside Campus, Sunway (Online).

Wafriy C.I, Nor-Ashikin M.N.K, & Kamsani Y.S (2022), Protective Effects of Tocotrienol Rich Fraction Against Oxidative Stress in Embryos of Asthma Mouse Models. Abstract of the 11th Congress of The Asia Pacific Initiative on Reproduction, 28th April – 1st May 2022 (Online).

Wafriy C.I, Nor-Ashikin M.N.K, Kamsani Y.S, Muid S.A & Sarbandi M.S (2023), Alteration of Embryonic Ultrastructure in Ovalbumin-induced Allergic Asthma Murine Model. Abstract of International Conference on Reproductive Science & Medicine and Embryology (ICRSME) 2023, 18th – 19th September 2023, University of Malaya, Malaysia.

Wafriy C.I, Nor-Ashikin M.N.K, Kamsani Y.S, Muid S.A & Sarbandi M.S (2023), Embryonic Oxidative Stress Altered Mitochondrial Fusion In 2-cell Embryos of Ovalbumin-induced Allergic Asthma Murine. Abstract of 2nd International Conference on Reproductive Science & Medicine, Embryology & Andrology (ICRSMEA) 2025, 24th – April 2025, University of Malaya, Malaysia (Gold award).

LIST OF AWARDS

- **Top 2 Oral Presenter** at Graduate Research Symposium 2021, Taylor's University Lakeside Campus, Sunway, 23rd -25th November 2021.
- **Second Runner-up** in 3MT Competition 2022, Faculty level and selected to represent Faculty of Medicine at UiTM level.
- **First Runner-up** in 3MT Competition 2022, University level and selected to represent UiTM at National level.
- **Best Oral Presenter (Gold Award)** at 2nd International Conference on Reproductive Science & Medicine, Embryology & Andrology (ICRSMEA) 2025, University of Malaya, 24th April 2025.