



**Interaction of *Toxoplasma gondii* with human brain
microvascular endothelial cells *in vitro***

By

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Abstract

Background: The brain is the most commonly affected organ during *Toxoplasma gondii* infection but the mechanisms utilized by this protozoan parasite for disrupting the brain's endothelial cells lining the blood–brain barrier (BBB) and moving to invade the brain are not yet understood. In the present study, we investigated the cellular pathogenicity of *T. gondii* infection in human brain microvascular endothelial cells (HBMECs), a fundamental component of the BBB.

Methods: Intracellular development of *T. gondii* tachyzoites within HBMECs was characterized by using Acridine Orange (AO) staining. The integrity of HBMECs monolayer and tight junction permeability during *T. gondii* infection were assessed using transendothelial electrical resistance (TEER). Morphological changes associated with infection was assessed by scanning electron microscopy (SEM) and transmission electron microscope (TEM). Cell viability and metabolic changes associated with infection were identified using alamar blue and nuclear magnetic resonance (^1H NMR). The changes in lipid content and fatty acid composition of the total phospholipids were evaluated using Lipid^{Tox} staining and gas chromatography (GC). The changes in the content of trace elements in response to *T. gondii* infection was assessed using inductively coupled plasma mass spectrometry (ICP-MS).

Results and Discussion: The invasion, growth, and replication of *T. gondii* tachyzoites within HBMECs are possible, with disruption of the integrity and viability of the host cell through the course of infection. AO staining of *T. gondii* tachyzoites infecting HBMECs showed a marked increase in the surface area of tachyzoites during infection, indicating that tachyzoites invade their host cell and form their own compartments (PV) in which tachyzoites proliferate with a generation time of 24 h, eventually leading to cell rupture and exit of the parasites.

A decrease was noted in the TEER of infected cells compared to uninfected controls, indicating that the invasion of the HBMECs by *T. gondii* had detectable effects on the integrity HBMECs monolayer by increased tight junction permeability.

Morphological analysis revealed that the intracellular development of the tachyzoites disruption of tight junctions HBMECs monolayer and reorganization of some organelles of the host cell, such as the mitochondria, endoplasmic reticulum, and Golgi apparatus around the Parasitophorous vacuole membrane (PVM) and remained stable throughout the growth of the tachyzoites. The tight association between the PVM and host organelles may provide lipids and other macromolecules for parasite survival, proliferation, membrane biogenesis, and energy requirement.

A marked decrease was noted in cell viability of infected cells at 48 h PI, compared to uninfected controls by alamar blue assay, indicating that growth of the parasites that cause a metabolic burden on the host cells.

Metabolite analysis of HBMECs infected with *T. gondii* revealed a drastic increase in lactate and glutamine levels, as well as a reduction in choline and myo-inositol levels with infection. A drastic increase in lactate and glutamine levels may be attributed to the fact that *T. gondii* requires energy from the host, primarily via glycolysis and glutaminolysis. It is believed that increased lactate and glutamine levels result in increased paracellular permeability. A drastic reduction in choline and myo-inositol levels suggests the use of host lipid fractions for increased membrane maintenance or parasite lipid anabolism. It is believed that increased phosphatidylcholine levels result in altered monolayer permeability.

Fatty acid analysis of HBMECs infected with *T. gondii* revealed a significant increase in C18:0 and C18:1n9c. It is believed that increased monounsaturated and

polyunsaturated fatty acids result in increased tight junction permeability via modulated occludin and ZO-1 localization.

Trace element analysis of HBMECs infected with *T. gondii* revealed a significant increase in Zn, Fe, Mg, and Cd levels, as well as a reduction in Co levels in growth medium obtained from the infected cells compared to non-infected controls. It is possible that altered trace elements levels, whether a parasite-induced or host-cell response, is important for protection against cellular oxidative stress and DNA damage during infection, and for suppressing cell apoptosis.

In conclusion, the results obtained show that HBMECs permit the invasion, growth and proliferation of *T. gondii* tachyzoites and that infection can disrupt tight junction permeability and cause multiple morphological changes in the relocation of the host cell organelles around the PV and changes in the levels of host cell metabolites and trace elements. These findings provide a more in depth understanding of how *T. gondii* replicate within the HBMECs during infection, which may lead to novel ways to prevent or treat this disease.

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List of abbreviations

$\times g$	Gravitational force
$^{\circ}\text{C}$	Degree celsius
$\mu\text{g} / \text{l}$	Microgram per liter
$\mu\text{g}/\text{ml}$	Microgram per milliliter
μm	Micrometre
ACCase	Acetyl-CoA carboxylase
Ac-CoA	Acetyl coenzyme A
ACP	Acyl carrier protein
ACs	Acetyl-CoA synthetase
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AO	Acridine orange
APCs	Antigen-presenting cells
ATP	Adenosine triphosphate
APES	3-aminopropyltriethoxysilane
b	Blank
B-lymphocyte	Bone marrow-lymphocyte
BBB	Blood brain barrier
CCD	Charge-coupled-device
cDMEM	Dulbecco's modified Eagle's medium
cm^2	Square centimeter
CNS	Central nervous system
CO_2	Carbon dioxide
Con	Control
CK	Choline kinase
CCT	Cytidylyltransferase
CDP-choline	Cytidine diphosphate-choline
C/EPT	phosphatidyl choline by choline/ ethanolamine phospho transferase
cRPMI	Roswell Park Memorial Institute medium-1640
CPK	creatine phosphokinase
D_2O	Deuterated water
DAPI	4', 6-diamino-2-phenylindole, dihydrochloride
DCs	Dendritic cells
dsDNA	Double-stranded deoxyribonucleic acid

EDTA	Trypsin-ethylene diaminetetra acetic acid
ELO	Endoplasmic reticulum elongases
EM	Electron microscopy
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
g/l	Gram per liter
FAS I	Fatty acid synthesis one
FAS II	Fatty acid synthesis two
GABA	Gama aminobutyric acid
GRA	Dense granule protein
GC	Gas chromatography
Glc	Glucose
Gln	Glutamine
Glu	Glutamate
Go	Golgi apparatus
GPI	Glycosylphosphatidylinositol
h	Hour
H	Host cell
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HBMECs	Human brain microvascular endothelial cells
HNO ₃	Nitric acid
ICP-MS	Inductively coupled plasma mass spectrometry
ICAM-1	intercellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IF	Infected
IFN γ	Interferon gamma
IFs	Intermediate filaments
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IVN	Intravacuolar network
IL	Interleukin
J	J-coupling constants
LDL	Low-density lipoprotein receptor
m	Mitochondria
mal-CoA	Malonyl-coenzyme A

MIP-1 α	macrophage inflammatory protein-1 alpha
MDCK	Madin-Darby Canine Kidney
mg	Milligrams
mg /l	Milligrams per liter
MHC class I	Major histocompatibility complex class one molecules
MHC class II	Major histocompatibility complex class two molecules
MIC	Microneme proteins
min	Minute
ml	Milliliter
mM	Millimetre
Mn	Microneme
MMPs	Matrix metalloproteinases
MTOC	Microtubule organizing center
MTs	Microtubules
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor-kappa B
NK	Natural Killer
nm	Nanometre
^1H NMR	Hydrogen-one nuclear magnetic resonance
NO	Nitrogen oxide
NOE	Nuclear Overhauser Enhancement
OPLSD-DA	Orthogonal partial least squares-discriminant analysis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCA	principal component analysis
PCs	Principal components
PCR	polymerase chain reaction
PD-10	Protein Desalting
PEP	Phosphoenolpyruvate
PFA	Paraformaldehyde
PI	post-infection
PLS-DA	partial least squares-discriminant analysis
ppm	parts per million
PRRs	pattern recognition receptors
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane

PVTN	Parasitophorous vacuole tubular network
Rb	Residual body
ROP	Rhoptry protein
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
s	Second
S.E.M	Standard error of the mean
SCT	Succinate- CoA transferase
SEM	Scanning electron microscopy
STAT-1	Signal transducer and activator of transcription 1
ssRNA	single-stranded ribonucleic acid
<i>T. gondii</i>	<i>Toxoplasma gondii</i>
T1	Longitudinal relaxation
T2	Transversal relaxation
TEER	Trans-endothelial electrical resistance
TfR	Transferrin receptor
TEM	Transmission electron microscopy
Tg	<i>Toxoplasma gondii</i> Tachyzoites
Th 1	T- helper one
Th2	T- helper two
TLRs	Toll-like receptors
T-lymphocyte	Thymus -lymphocyte
TJs	Tight junctions
TNF	Tumour necrosis factor
TSP	Trimethylsilyl propanoic acid
TPTs	Triosephosphate transporters
V/V	Volume/Volume
ZO	Zonula-occludens
α -KG	Alpha-ketoglutarate
μ l	Microliter

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1. Introduction

1.1 Overview

Toxoplasma gondii (*T. gondii*) is an intracellular protozoan parasite that invades and proliferates within nucleated cells of almost all warm-blooded animals and humans. *T. gondii* infection can result in the disease toxoplasmosis, which manifests clinical signs that are most clearly evident in humans and farm animals. Sheep with toxoplasmosis can develop a wide range of clinical signs, including reproductive failure, abortion or stillbirth. In human, toxoplasmosis commonly causes birth defects, including hydrocephalus (abnormal accumulation of cerebrospinal fluid in the brain), intracerebral calcification, convulsions, and retinitis. However, *T. gondii* infection is generally latent and asymptomatic, often remaining within the brain and other body tissues throughout life (Dubey and Beattie, 1988).

One of the most important clinical consequences of *T. gondii* infection is the damage caused to the brain, the most common site where *T. gondii* cysts are found (Steininger et al., 2003; Carruthers and Suzuki, 2007). One of the principal steps in the pathogenesis of cerebral toxoplasmosis is the breach of the blood–brain barrier (BBB), which is comprised of microvascular endothelial cells (Däubener et al., 2001), which regulates substance entry into neuronal tissue (Elsheikha and Khan, 2010; Schäfer et al., 2011). The ability of intracellular pathogens to infect the brain's microvascular endothelial cells is key to their ability to cause brain damage (Däubener et al., 2001).

1.2 The role of blood brain barrier in protection against CNS infection

To illustrate the process of brain invasion by neuropathogenic microbes, it is important to be aware of the BBB, the defensive interface in the brain that prevents microbial invasion. The BBB is a single monolayer of endothelial cells lining the inner surfaces of the capillaries to form a protective barrier around the brain, and its main function is to maintain the homeostasis of the brain environment (Abbott et al., 2010).

The endothelial cells fit tightly into each other due to adhesive forces, tight junctions with high electrical resistance and a relatively low number of pinocytotic vesicles to protect the brain from microbial invasion and restrict the passage of large or hydrophilic molecules (Pardridge, 1999). The adhesive junctions are composed of vinculin–cadherin proteins that interact with the actin cytoskeleton via catenins to provide the structural support for the development of tight junctions. The tight junction is composed of occludin, claudin, junctional adhesion molecule, and endothelial cell-selective adhesion molecules that interact with their counterparts in the neighbouring endothelial cells. The cytoplasmic tails of these proteins link to the cell cytoskeleton via several accessory proteins, including members of zonula-occludens (ZO)-1, -2, and -3 (Figure 1.1). In addition to the tight junctions, the brain microvascular endothelial cells are supported by other cellular structures such as astrocytes, microglia, pericytes, neurons and the basement membrane to form a functional neurovascular unit. These support systems are accompanied by a large profusion of mitochondria that are responsible for the generation of energy required for the active transport system (Abbott et al., 2006).

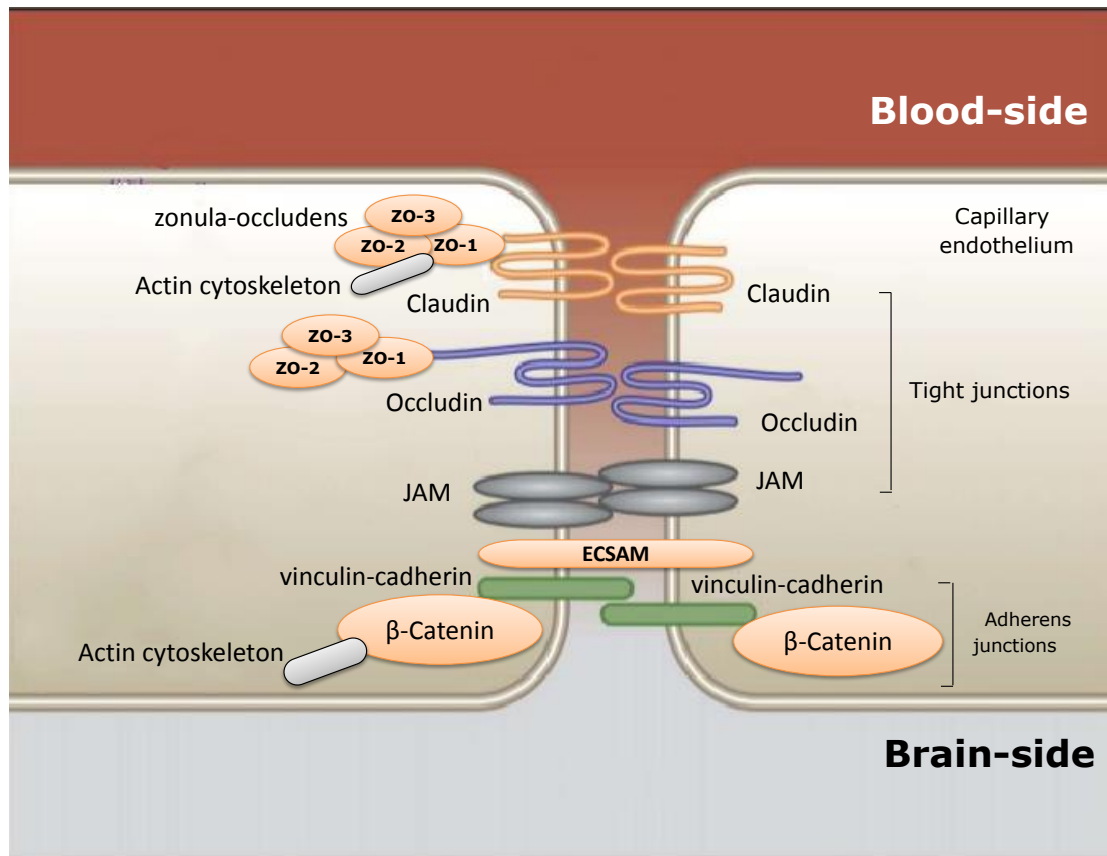


Figure 1.1: Schematic representation of two endothelial cells exhibiting tight junctions. The tight junctions are composed of claudin, occludin, junctional adhesion molecule (JAM), and endothelial cell-selective adhesion molecules (ECSAM) that interact with their counterparts on the adjacent endothelial cells. The cytoplasmic tails of these proteins interact with the actin cytoskeleton via zonula-occludens (ZO). Vinculin-cadherins of the adherens junctions provide structural attachment between the cells, and are necessary for formation of tight junctions. The adhesive junctions are composed of vinculin-cadherin proteins that interact with the actin cytoskeleton via catenins.

1.3 Pathogens known to cause CNS infection

A wide range of pathogens are known to infect the central nervous system (CNS), including viral, bacterial, parasitic and fungal (Table 1.1). Some of the causative agents of CNS infection affect the membranes covering the brain and result in meningitis. Others affect the brain itself, resulting in encephalitis. An infection that affects both the meninges and brain tissue is called meningoencephalitis (Kennedy, 2004).

Table 1.1: Important pathogens in encephalitis and meningitis

Bacteria	Virus
<i>Neisseria meningitidis</i>	<i>Human immunodeficiency virus</i>
<i>Escherichia coli</i>	<i>Tick-borne encephalitis virus</i>
<i>Streptococcus pneumoniae</i>	<i>Enteroviruses</i>
<i>Listeria monocytogenes</i>	<i>Herpes viruses</i>
<i>Mycobacterium tuberculosis</i>	<i>Rabies virus</i>
<i>Treponema pallidum</i>	<i>John Cunningham virus</i>
<i>Bacillus anthracis</i>	<i>West Nile virus</i>
<i>Staphylococcus aureus</i>	<i>Measles virus</i>
<i>Borrelia burgdorferi</i>	
Helminths	Protozoa
<i>Schistosoma mansoni</i>	<i>Toxoplasma gondii</i>
<i>Taenia solium</i>	<i>Trypanosoma brucei</i>
<i>Echinococcus granulosus</i>	<i>Entamoeba histolytica</i>
<i>Toxocara canis</i>	<i>Balamuthia mandrillaris</i>
<i>Trichinella spiralis</i>	<i>Plasmodium falciparum</i>
Fungi	
<i>Candida albicans</i>	
<i>Cryptococcus neoformans</i>	
<i>Aspergillus species</i>	

Modified from Kennedy,(2004).

1.4. Mechanisms of crossing the blood brain barrier

The strategies used by neuropathogenic protozoa to breach the BBB include the following: (i) transcellular route, by producing endothelial cell damage as in the case of *Trypanosoma brucei*, (ii) paracellular route, by disrupting the tight junctions as in the case of *Plasmodium falciparum* and *Balamuthia mandrillaris*, and (iii) via infected leukocytes (i.e. 'Trojan horse' mechanism). The 'Trojan horse' mechanism has been suggested for *Toxoplasma gondii*, in fact, even paracellular penetration of the BBB has been demonstrated for this organism (Kim, 2008) (Figure 1.2)

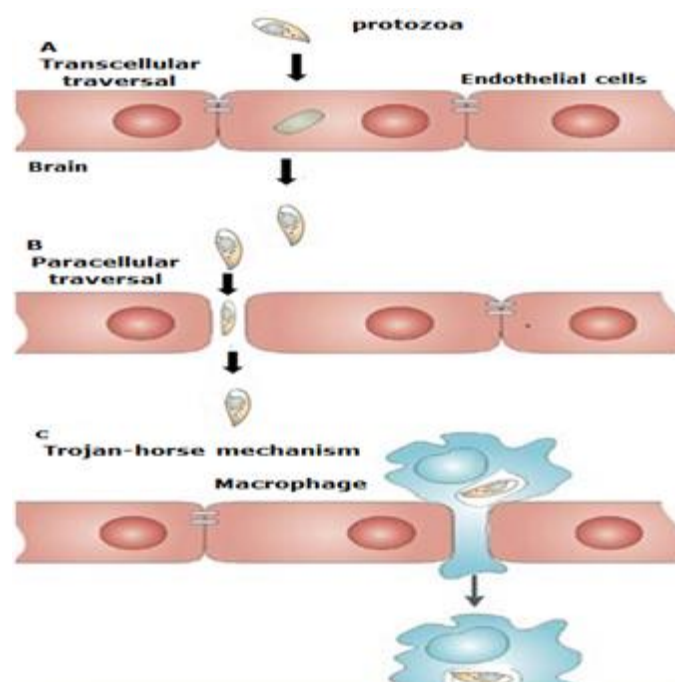


Figure 1.2: Schematic depiction of microorganism transmigration of the endothelium at the BBB via transcellular, paracellular, and infected macrophages (the so-called Trojan-horse mechanism) routes. Modified from Kim (2008).

The mechanism of the BBB disruption may occur through two pathways: the transcellular or the paracellular pathways. The paracellular route is usually the main route providing increased endothelial barrier permeability. The mechanism of the BBB disruption associated with paracellular entry of microbes include alterations in

the matrix metalloproteinases (MMPs), phosphorylation of tight junction proteins, disruption of the basal lamina and disruption of the actin cytoskeleton. In the absence of this mechanism, indirect effects of microbes on the immune system are possibly caused due to barrier disruption (Spindler & Hsu, 2012).

MMPs are zinc-dependent enzymes with proteolytic activity, which they exert on the extracellular matrix molecules in the basal laminae of the BBB, including laminin, type IV collagen and fibronectin. MMP activity induced under pathological conditions causes BBB disruption after basement membrane degradation and the cleavage of the tight junction proteins occludin and claudin-5 (Bauer et al., 2010; Feng et al., 2011).

Increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) also correlate with the altered expression of tight junction protein and phosphorylation, which stimulates increased MMPs activity and increases the permeability of the BBB. Neuropathogenic protozoa can directly increase ROS and RNS through stimulation of intracellular signaling by protozoa components. The host inflammatory response to protozoa infection can generate ROS and RNS, which in turn stimulate the production of inflammatory cytokines and MMP secretion by the endothelial cells of the BBB. Thus, protozoa infections of the CNS have the potential to cause BBB disruption by inducing oxidative damage which alters MMP activity (Mayhan 2001; Rao 2008; Hamada et al., 2013).

Tight junction proteins can also be disrupted by inducing alterations in the actin cytoskeleton within the cells, resulting in paracellular permeability changes (Stamatovic et al., 2008; Lei et al., 2005). ROS play a role in the alterations of the actin cytoskeleton within the cells by the induction of the small GTPase RhoA, PI3 kinase and protein kinase B (PKB/Akt) signaling pathways. Furthermore, the tight junction proteins ZO and claudin-5 are phosphoproteins, and changes in their phosphorylation alters the paracellular permeability (Stamatovic et al., 2008).

1.5 BBB model

The human brain microvascular endothelial cells (HBMECs) were used in this research project as an *in vitro* BBB model, as these cells represent the main constituent of the BBB and are responsible for maintaining barrier tightness and regulation of brain function by directly controlling the uptake of nutrients and oxygen. Two widely used methods for studying the BBB are (i) cell culture models using the brain endothelial cells and (ii) isolated microvessels. The cell culture model is more popular owing to ease of use and cost effectiveness as compared to that for isolated microvessels. The brain endothelial cells can also be co-cultured with astrocyte- or astroglial-conditioned media, in which the brain endothelial cells and astrocytes are grown on the opposite sides of gelatin-coated tissue culture inserts with 3- μ m pores to permit the penetration of astrocyte cells in order to insert and establish contact with the endothelial cells.

The endothelial cells possess the advantage of expressing several tight junction proteins (TJPs) such as occludin, claudins and ZO (ZO-1, ZO-2, ZO-3) displayed *in vivo* (Kniesel and Wolburg, 2000; Wolburg and Lippoldt, 2002). Functionally, HBMECs form much tighter capillary endothelium than the peripheral endothelial cells (Abbott, 2002). Because microvessels are isolated directly from the brain, they express all of the transporters displayed *in vivo*. However, their disadvantage is that the preparation process is laborious, requires animals and has a shorter lifespan *in vitro*. Nonetheless, *in vitro* BBB models are of key importance for facilitating our understanding of the BBB functions in physiological and pathological scenarios (Helms et al., 2016). In the present work, we used a cell monolayer model to study the structural and metabolic changes because of the intrinsic simplicity of this monolayer model. Moreover, the monolayer model takes 3 to 4 days to reach steady-state transendothelial electrical resistance and is relatively easy to construct. Although these observations were out of scope of the present

study, they should be validated in more sophisticated dynamic models and *in vivo* before reaching a conclusion.

1.6 The model organism

T. gondii was used in this research project for the following reasons: (i) it can cause severe neurological diseases, like encephalitis (McAllister, 2005), (ii) it is used as a model for the apicomplexan parasites that cause important human diseases, including malaria and cryptosporidiosis, and (iii) it is a significant veterinary parasite associated with an elevated cost due to disease, abortion or vaccination in animal farming (Scott et al., 2007).

T. gondii belongs to the phylum Apicomplexa, a parasite in the class of conoidasida, and is the only species in the *Toxoplasma* genus. The taxonomy of *T. gondii* is given below (Nicolle and Manceaux, 1908).

Domain: Eukaryota

Kingdom: Chromalveolata

Super phylum: Alveolata

Phylum: Apicomplexa

Class: Conoidasida

Subclass: Coccidiasina

Order: Eucoccidiorida

Family: Sarcocystidae

Subfamily: Toxoplasmatinae

Genus: *Toxoplasma*

Species: *Toxoplasma gondii*

Most *T. gondii* strains have been isolated from human and animals with toxoplasmic encephalitis. This species is classified into three clonal lineages: i) type I strain (RH strain), which is hyper-virulent in mice and humans; infection with even low doses

of the type I strain causes enormous parasite growth. ii) type II strain (Pru strain), which is non-virulent in mice and virulent in humans and is present mostly in Europe and North America. The type III strain (VEG strain) is non-virulent in mice, virulent in animals, and also virulent in humans, albeit to a lesser degree (Blader and Saeij, 2009).

The main differences between the three types of *T. gondii* strains (type I, II and III) are outlined in Table 1.2.

Table 1.2: Differences among the three major strains of *T. gondii*

Strain	Virulence	Cysts formation	Acute phase (Tachyzoites)	Chronic phase (Tachyzoites and Bradyzoites)
Type I (e.g.: RH)	+ + +	-	+	-
Type II (e.g.: Pru)	+ +	+	+	+
Type III (e.g.: VEG)	+	+	+	+

Type I strain (RH strain) was used because it is a virulent genotype, does not form cysts and because infection with even low doses of this strain can cause a productive infection, which allows the study and characterization of the effect of the parasite on host cells *in vitro*.

1.7 Life cycle of *T. gondii*

The life cycle of *T. gondii* encompasses three distinct forms: tachyzoites, bradyzoites and sporozoites. The tachyzoite is the rapidly replicating form of the parasite, found during the acute phase of toxoplasmosis. It has a typical crescent shape with a conoidal anterior end and a rounded posterior end (Figure 1.3). It is approximately 2-3 μm in width and 6-8 μm in length. The tachyzoite contains various organelles and inclusion bodies (Frenkel, 1973). The anterior part contains components typical for the members of Apicomplexa, which include the conoid, an essential component of the parasite to aid motility and penetration into a host cell, and secretory organelles: micronemes, rhoptries and dense granules that they can play a crucial role in parasitic invasion and intracellular growth. Another typical apicomplexan organelle found in the tachyzoite is the apicoplast: a plastid organelle similar to plant chloroplasts. It contains its own DNA as a circular genome. The apicoplast creates proteins and is responsible for fatty acid biosynthesis and lipid metabolism (McFadden, 2011). Tachyzoites have no locomotor organelles for motility (flagella, cilia or pseudopodia); however, they are able to glide (Chiappino et al., 1984).

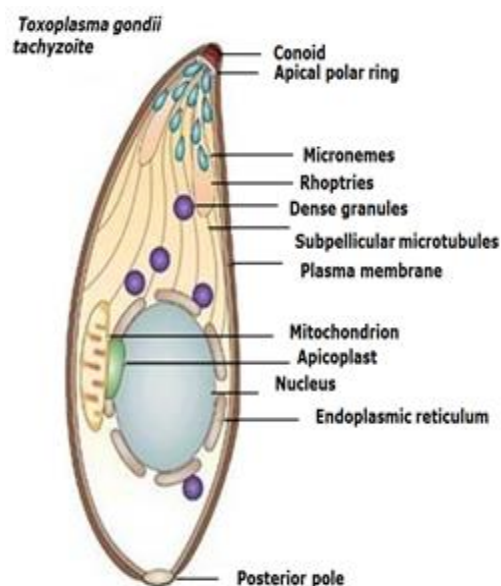


Figure 1.3: Ultra-structure of a tachyzoite of *T. gondii*. Adapted from Baum et al. (2006).

The bradyzoites are the slowly replicating form of the parasite, present during the chronic phase of toxoplasmosis. They are approximately 1-3 μm wide and 5-8.5 μm long. Bradyzoites are found encysted in the host target tissue. The tissue cysts differ in size as they grow slowly and can remain within the host for several years. The tissue cysts containing the bradyzoites are more prevalent in the neural, ocular and muscular tissues. The tissue cysts in the brain tissue are spherical and smaller (Figure 1.4) than those in the intramuscular cysts, which are elongated and up to 100 μm in length (Dubey et al., 1998). The transformation of tachyzoites to bradyzoites is induced by several stimuli triggered by the immune response of the host (Tomavo, 2001). It has been reported that, in HBMECs, the immune response mediator gamma interferon ($\text{IFN-}\gamma$) induces a strong induction of indoleamine 2,3-dioxygenase (IDO) activity in the cells, resulting in the breakdown of tryptophan (Däubener et al., 2001). Starvation for amino acids like tryptophan and arginine possibly slows the parasite growth and triggers the cyst formation (Fox et al., 2004). $\text{IFN-}\gamma$ also induces the production of ROS and nitric oxide (NO), which may trigger bradyzoite differentiation (Bohne et al., 1994).

Other cytokines have been associated with the promotion of bradyzoite differentiation, such as tumor necrosis factor (TNF). In murine macrophages, TNF cooperates with IFN- γ to facilitate the conversion to bradyzoites (Bohne et al., 1994). Another study reported that interleukin-6 (IL-6) stimulates the formation of bradyzoites *in vitro* in the human fibroblasts (Weiss et al., 1995).

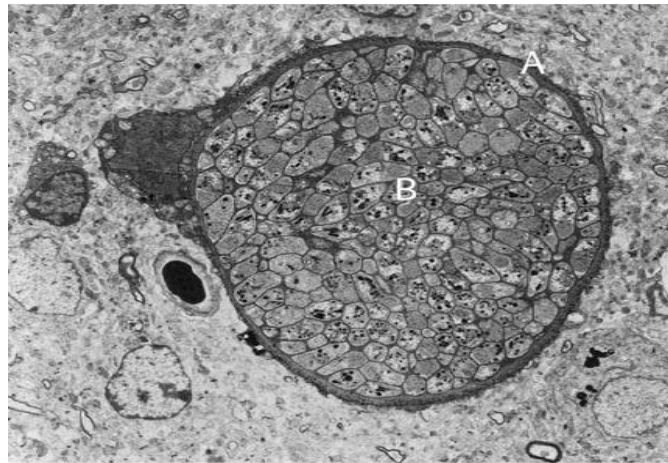


Figure 1.4: Transmission electron micrograph of a tissue cyst containing bradyzoites in the brain of a mouse infected with *T. gondii*. A: cyst wall; B: bradyzoites. Adapted from Baldelli (1983).

Sporozoites represent the phase of the parasite present in the sporulated oocysts and measure approximately 6-8 μm length and 2 μm in width (Figure 1.5).

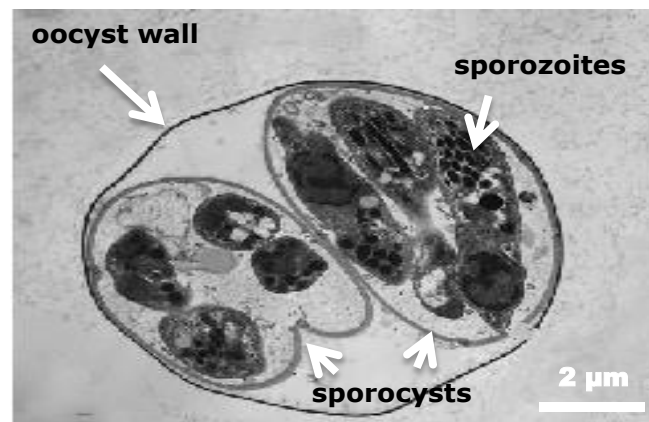


Figure 1.5: Transmission electron micrograph of a sporulated oocyst (large arrow) containing two sporocysts (arrowheads). Three sporozoites are visible in one of the sporocysts (small arrows). Adapted from Dubey et al. (1998).

Almost all cats shed oocysts after ingestion of *T. gondii* tachyzoites, bradyzoites or sporozoites, within different time periods, depending on the form ingested. As cats are more infected by bradyzoites than oocysts, the ingestion of these tissue cysts will result in the shedding of the oocysts in nearly all cats, within periods varying from 3-10 days. After ingesting the oocysts, fewer than 50% of cats will shed the oocysts and the maximum period will be 18 days or longer (Dubey, 2001; Dubey, 2006).

The life cycle is initiated immediately following the ingestion of the tissue cysts, with the rupture of the cyst wall by the proteolytic enzymes in the digestive tract. The bradyzoites are liberated and replicate non-sexually within the epithelium of the small intestine to form merozoites (Dubey and Frenkel, 1972).

The sexual cycle is initiated 48 h after ingestion of *T. gondii* tissue cysts in the cat. The mechanism by which the gamonts are created is yet to be understood; the merozoites possibly induce gamete formation. Microgamonts and macrogamonts develop in the small intestine, particularly in the ileum. The male microgametes are

created by several nuclear divisions during the process of microgametogenesis and fertilize the mature female macrogametes to form a zygote (Dubey and Frenkel, 1972; Speer and Dubey, 2005).

The zygote evolves into an oocyst and is liberated after destroying the infected epithelial cell (Ferguson et al., 1979b). Once the oocysts are shed in the cat's faeces, they are seen to contain a single mass, the sporoblast, measuring 10 x 12 μm . The oocysts shed sporulate for 1-5 days, depending on the surrounding environmental conditions (Dubey and Frenkel, 1972; Ferguson et al., 1979c; Ferguson et al., 1979d). Sporulated oocysts measure 11 x 13 μm and contain two subspherical to ellipsoidal sporocysts. Each sporocyst is 6 x 8 μm in diameter and divides by endodyogeny into 4 sporozoites (Dubey and Frenkel, 1972; Ferguson et al., 1979a). Oocysts are infectious only after sporulation. They may continue to be infectious for more than a year (Frenkel et al., 1975).

After ingestion of infected meat (tissue cysts) or sporulated oocysts by the intermediate or final host, the bradyzoites or sporozoites first get transformed to tachyzoites. Tachyzoites get distributed in tissues of the host. Once the specific immune response is produced by the host, the parasites are transformed from the tachyzoite to the bradyzoite phase and encyst in various tissues (Dubey, 1998a). When the tissue cysts are digested, bradyzoites return to the intestines to begin the sexual cycle. The duration of oocyst formation here is still unknown (Freyre et al., 1989; Dubey et al., 1996) (Figure 1.6).

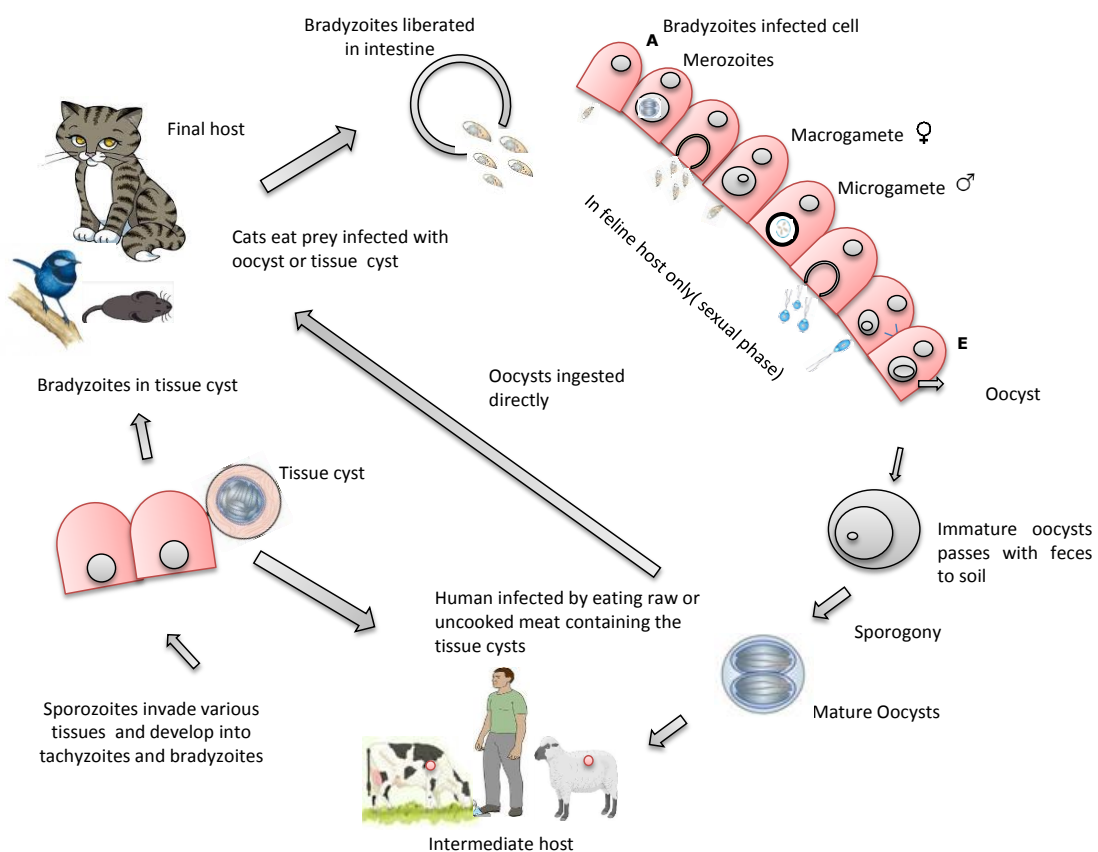


Figure 1.6: Life cycle of *T. gondii*. Sexual replication takes place in cats, resulting in oocyst shedding. In the environment, the oocysts sporulate by sporogony to form sporozoites. The sporozoites induce an asexual replication cycle following oral ingestion by the intermediate and final hosts. A-E refers to stages of development within the final host enterocytes.

1.8 Tachyzoite invasion, intracellular replication and egression.

Cell invasion by the tachyzoites has been studied intensely and many of its mechanisms have been elucidated in great detail. At first, the apical end of the tachyzoite makes contact with the host cell surface. When the conoid penetrates into the host cell, a parasitophorous vacuole (PV) is formed. Within the PV, the parasite proliferates with a generation time of 24 h to form rosettes, eventually leading to cell rupture and exit of the parasites (Kafsack et al., 2007). The invasion of a cell by tachyzoites is an active, oriented, and specific process mediated by sequential protein secretion from granular organelles. These include microneme proteins, which are secreted from the microneme organelle to allow the parasite to glide and adhere to its host cell. A second wave of secretion originates from the rhoptry organelle, to allow the parasite to enter its host cell and form the PV. A third secretion originates from dense granules to modify parasitophorous vacuole membrane (PVM), forming a protective compartment and exchange site in which the parasite proliferates asexually by repeated endodyogeny (Bonhomme et al., 1992; Carruthers and Sibley, 1997) (Figure 1.7).

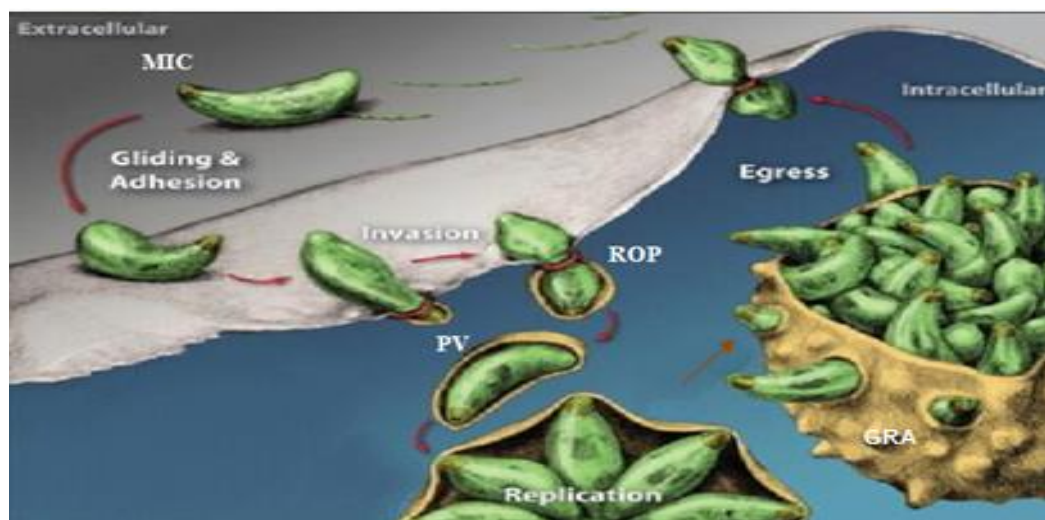


Figure 1.7: Intracellular replication cycle of *T. gondii*. Gliding motility and adhesion to the host cell membrane are mediated by the secretion of microneme proteins (MIC) from the apical end of the tachyzoite. At the point of attack, penetration into the host cell includes the secretion of rhoptry proteins (ROP; depicted as a red ring) and formation of the PV. PV growth and modulation is mediated by the liberation of the dense granule proteins (GRA). Modified from Oliver et al. (2009).

T. gondii rely on the gliding motility for their host cell invasion and egress. This mode of locomotion is mediated by the interactions between secretory adhesins, a myosin motor, actin cytoskeleton and proteases. During invasion, microneme proteins (MICs) and rhoptry neck proteins (RONs) are discharged to the apical end of the tachyzoite. Some proteins, such as MIC-2, bind to the host cell receptors such as intercellular adhesion molecule-1 (ICAM-1), whereas others are involved in the moving junction formation. These complexes distribute toward the posterior end of the tachyzoite via a physical connection to the parasite actomyosin system and are eventually released from the tachyzoite surface by the action of tachyzoite proteases (Keeley & Soldati, 2004). Both actin and myosin are localized beneath the plasma membrane in the tachyzoite, where they combine to produce the force necessary for invasion and egress, a process triggered by the elevated calcium levels (Sibley, 2010).

1.9 The mechanism of reduced apoptosis in *T. gondii*-infected cells

Induction of apoptosis is a host response to infection with a variety of viruses, bacteria and eukaryotic pathogens (Morimoto and Bonavida 1992; Takizawa et al., 1993). In some respects, it is a natural body defence mechanism where the death of the infected cell is usually associated with the death of the infecting agent and with pathogen clearance. The mechanism of apoptosis in mammalian cells includes the (i) recruitment and activation of pro-caspase-9 by the apoptosome, resulting in cleavages and activation of caspase-3 and -7 and (ii) the release of cytochrome c from mitochondria to the host cell cytoplasm by Bcl-2 family members. However, parasites actively arrest host cell apoptosis to their advantage to keep their host alive in order to sustain their proliferation. The mechanism of reduced apoptosis in *T. gondii*-infected cells is complex and depends on the parasite strains and host cell types being infected. Previous studies have reported that parasites arrest the host cell apoptosis at multiple levels, such as by (i) directly

interfering with the caspase processing and function (Vutova et al., 2007), (ii) inhibiting the release of cytochrome c from the mitochondria to the host cell cytoplasm (Keller et al., 2006), (iii) affecting the balance of pro- and anti-apoptotic BCL-2 such as Bax and Bak (Carmen et al., 2006), and (iv) by blocking the upstream signalling pathways (Molestina et al., 2003). It has also been reported that *T. gondii* proteins are involved in the modulation of host signalling pathways and the arrest of host cell apoptosis such as by modulation of Janus kinase/signal transducer and activator of transcription via the action of rhoptry protein 16 (Saeij et al., 2007), modulation of mitogen-activated protein kinase via the action of rhoptry protein 38 and dense granule protein 24 (Peixoto et al., 2010; Braun et al., 2013), modulation of tumor protein 53 via the action of dense granule protein 16 (Bougdour et al., 2013), and modulation of nuclear factor kappa (NF- κ B) via the action of dense granule protein 15 (Rosowski et al., 2011).

1.10 Toxoplasmosis in animals

1.10.1 Feline species

As described earlier, cats are the definitive hosts for *T. gondii* and they mainly acquire the infection by ingestion of infected prey or by eating meat containing the tissue cysts.

A wide variety of clinical signs are possible in felines, such as fever, anorexia, abdominal pain, joint pain, and ocular and neurological diseases resulting in blindness, seizures and loss of control over urination and defecation. In the most serious cases of feline toxoplasmosis, pulmonary lesions resulting in rapidly developing pneumonia are the most common finding and often the cause of death (Dubey and Carpenter, 1993).

The outcome of toxoplasmosis in cats depends upon a wide variety of factors, including the age of the cat, the phase of the parasite, and the route of infection. Bradyzoites are much more infectious to cats compared to oocysts: the

transmission of *T. gondii* is most infectious when cats ingest the tissue cysts. Oral infection by oocysts from the *T. gondii* type III (VEG) and type I (TgCkAr23) strains are non-pathogenic for cats (Dubey, 1996; Dubey, 2006).

When cats are inoculated with tachyzoites, bradyzoites or oocysts, they can develop serious forms of the disease and even die. Immunodeficiency, resulting from an infection with the feline immunodeficiency virus or for instance after therapy with corticosteroids, can significantly exaggerate the clinical signs (Dubey and Frenkel, 1974; Davidson et al., 1993; Levy et al., 2004).

Infection during pregnancy can result in the congenital infection of the kittens, which can cause severe toxoplasmosis leading to anorexia, hypothermia (drop in body temperature), uveitis (inflammation of the middle layer of the eye), and sudden death. kittens can die from an infection and transplacental transfer of antibodies have been found to have no impact on the outcome of the disease (Sato et al., 1993; Dubey et al., 1995). After a primary infection, a large number of oocysts are produced between one and three weeks. The number of oocysts shed naturally by an infected cat is still unknown. However, as described in the experimental infections, it can reach up to millions: cats fed with infected ham meat shed around 25 to 810 million of oocysts (Dubey et al., 2002).

It is still not known whether naturally infected cats are able to re-shed oocysts. A study revealed that re-infection of surviving cats with the ME-49 strain 39 days later did not cause the re-shedding of oocysts. However, re-infection of cats six years later after a primary infection with the P89 strain induced the re-shedding of oocysts in 4 of the 9 cats studied (Dubey, 1995). Certain conditions could render the cats more susceptible to re-shedding. For instance, *T. gondii* infection in cats infected with *Cystoisospora felis* prevented re-shedding of *T. gondii* oocysts. However, when the cats were secondarily infected with *C. felis*, re-shedding was induced. No re-shedding was revealed when the experiment was conducted with

Cystoisospora rivolta, the other feline infecting *Cystoisospora species* (Chessum, 1972; Dubey, 1976; Dubey, 1978).

A feline immunodeficiency virus disease in cats chronically infected with *T. gondii* neither induces re-activation of toxoplasmosis nor re-shedding of the oocysts (Lappin et al., 1996). However, immunosuppression, induced by high doses of corticosteroids (such as methylprednisolone acetate or dexamethasone) can provoke re-shedding of the oocysts (Dubey and Frenkel, 1974; Malmasi et al., 2009).

Sporulated oocysts are highly resistant to environmental conditions and can be present in the soil, water, animal feed and vegetables. They can stay contagious for up to 18 months at temperatures between -20°C and +35°C (Frenkel et al., 1975) and remain contagious in water, at temperatures from 10°C to 20°C for 200 days, although the degree of infectivity reduces dramatically for temperatures higher than 30°C; they are killed at temperatures of 60°C (Dubey, 1998b). At room temperature or at 4°C oocysts can be sporulated and they can stay viable in seawater for several months (Lindsay and Dubey, 2009). They are very resistant to disinfectants (like sodium hydrochlorite, chlorine or ozone treatment of water); however, they are sensitive to ultraviolet radiation, based on the dose 1,000 mJ/cm² (Wainwright et al., 2007a; Wainwright et al., 2007b). Non-sporulated oocysts are less resistant to high temperatures than sporulated oocysts as they die when they are exposed to 37°C for 24 h (Dubey et al., 1970).

Although the consumption of raw or undercooked meat is considered the major route of infection for humans, oocysts in soil, water or vegetables are likely to contribute towards the transmission of toxoplasmosis to vegetarians. Several investigations have related drinking untreated water to seropositivity in humans (Jones and Dubey, 2010).

1.10.2 Livestock

As most warm-blooded animals act as the intermediate hosts of *T. gondii*, livestock animals are a potential risk for the transmission of the disease to humans. When consuming raw or undercooked meat and even viscera, bradyzoites can be transmitted via tissue cysts. Such cysts are readily infectious and are mostly located in brain tissue and muscle (Dubey et al., 1998).

Eating raw or undercooked meat has been associated with *T. gondii* seropositivity in many studies and is viewed as the major route of infection in humans in many countries (Cook et al., 2000; Tenter et al., 2000; Kijlstra and Jongert, 2008; Dubey, 2009a). A nationwide prevalence survey conducted by Halos et al. (2010) revealed that around 5.04% of all sheep carcasses consumed in France contain viable *T. gondii*. Other studies have also revealed the risk related to consuming ovine meat (Berger et al., 2009; Dubey, 2009a). Not only meat from sheep poses a risk for human *T. gondii* infection, but also goat, pork, chicken and probably even beef have been found to harbour viable parasites (Dubey et al., 2005; Dubey et al., 2011; Kijlstra et al., 2004; Opsteegh et al., 2011).

Tissue cysts are relatively resistant and this resistance is affected by several agents: *T. gondii* tissue cysts survive in chilled carcasses for more than 21 days; this is typically longer than the shelf life (Dubey et al., 1990). Freezing meat for 3 days at a minimum of -12°C effectively kills tissue cysts. However, it requires a longer freezing time for a large piece of meat, before the deeper located *T. gondii* cysts are frozen (Dubey, 2000). Heating meat products to an internal temperature of 67°C or more is an active way of elimination of tissue cysts (Dubey, 2000). However, the survival of tissue cysts at lower temperatures based upon the period of exposure to heat is as follows: in laboratory experiments, the tissue cysts stayed viable if heated to 60°C and 50°C for around 4 min and 10 min, respectively (Dubey et al., 1990). The duration of heating necessary to kill the bradyzoites in the meat will depend upon the thickness of the meat and the homogeneity of the

cooking method; microwave cooking is, in this regard, inefficient for killing *T. gondii* tissue cysts (Lunden and Uggla, 1992).

The impact of treatment of meat on the survival of *T. gondii* tissue cysts depends upon many factors: the chemicals used, their concentration and the treatment procedure itself. For instance, tissue cysts were eliminated in 6% NaCl at temperatures between 4°C and 20°C under laboratory conditions, but survival continued at lower salt concentrations for several weeks (Dubey, 1997). Brain homogenates of infected mice remained partially infectious when exposed to 3% NaCl and presserved at 4°C for 7 days (Jamra et al., 1991). Hill et al. (2004) tested the effect of salt and other chemical solutions on immediately inoculated tissues from experimentally infected mice (brain) and pigs (pork loins) and preserved at 4°C; it was found that 2% sodium chloride and potassium or sodium lactate at 1.4% or more controlled the transmission of *T. gondii* to cats.

1.11 Toxoplasmosis in humans

Toxoplasmosis presents with an acute illness, including one or more of the following symptoms: dysfunction of the CNS, encephalitis, lymphadenopathy (swollen lymph nodes) and ocular disease. Congenital infections can also cause severe toxoplasmosis resulting in hydrocephalus, microcephalus, intracerebral calcification, convulsions and cerebral retardation (Aiello and Mays 1988).

1.11.1 Toxoplasmosis in immunocompetent individuals

For most immunocompetent individuals, a *T. gondii* infection is nearly asymptomatic. Most of the recurring symptoms are flu-like illnesses associated with lethargy, headaches, excessive sweating, slight fever, muscle and joint pains. Such clinical symptoms are often not diagnosed to result from a *T. gondii* infection (Ho-Yen, 2001). Once the patient acquires immunity, the clinical symptoms disappear and the individual is protected life-long against re-infection. However, some

symptoms following re-infection have been detected, probably due to significant strain variations (Abdul-Fattah et al., 1992).

The severity of the toxoplasmosis can be associated with the virulence of the infectious strain. More severe forms are not uncommon in patients with immunodeficiency; a serious infection with a fatal outcome is quite possible. Some of the most prevalent clinical symptoms include lymphadenopathy and ocular toxoplasmosis (Ho-Yen, 2001). Ocular toxoplasmosis can evolve a long time after an acquired *T. gondii* infection postnatally, even when no preceding clinical symptoms are apparent or ocular lesions are detected. This may be because of the reactivation of the persistent tissue cysts in the retina or in non-ocular areas, with the later spread of the parasites to the eye. Patients with ocular toxoplasmosis face a life time risk of reappearance of the disease (Holland, 2003).

1.11.2 Toxoplasmosis in individuals with immunodeficiency

Despite the emergence of antibodies as the indicator that an individual has acquired immunity against *T. gondii*, the real protection against the parasite occurs through an adequate T-lymphocyte response. If T-lymphocytes are impaired, then toxoplasmosis will cause severe damage (Suzuki and Remington, 1988; Gazzinelli et al., 1991; Montoya et al., 1996). The T-lymphocyte in particular gets altered in acquired immuno deficiency syndrome (AIDS) patients. The reactivation of *T. gondii* tissue cysts is one of the major causes of death in AIDS patients (Luft and Remington, 1992; Israelski et al., 1993). Nowadays, the availability of highly active antiviral therapy has contributed much towards the reduction of the incidence of *toxoplasma* encephalitis (Maschke et al., 2000).

Some medical states require immunosuppressive therapy, such as some kinds of cancer and organ transplantation. The combination with toxoplasmosis often results in very high morbidity and mortality rates (Israelski and Remington, 1993a;

Israelski and Remington, 1993b; Slavin et al., 1994). The clinical consequences are similar in all types of immunosuppression. The organs affected include the brain, spinal cord, eyes, lungs and occasionally some infections affect the heart, bone marrow, bladder, rhinopharynx, skin, liver, lymph nodes, pericardium, muscle, and pancreas (Rabaud et al., 1996). In most cases, reactivation of the tissue cysts from an earlier acquired toxoplasmosis is the main cause, except in the case of organ transplantation from a seropositive donor to a seronegative patient (Derouin and Pelloux, 2008).

1.11.2.1 Cerebral toxoplasmosis

Cerebral forms of toxoplasmosis are more common in patients with immunodeficiency. The cerebral lesions caused by *T. gondii* initiate at a focus of inflammation of the brain, in the parietal and frontal lobes and at the corticomedullary junction, basal ganglia, thalamus, and pituitary gland. The disease spreads to the parenchymal abscesses with necrosis and surrounding inflammation. Tachyzoites are usually found in the periphery of the lesions (Skiest, 2002).

1.11.2.2 Ocular toxoplasmosis

This is the less frequent form of toxoplasmosis in patients with immunodeficiency and may be present with or without related encephalitis (Rabaud et al., 1994).

1.11.2.3 Pulmonary toxoplasmosis

Pulmonary toxoplasmosis seems to occur mainly in patients with advanced immunodeficiency. The clinical symptoms are fever, cough, and acute dyspnea (shortness of breath). The radiological findings mostly reveal bilateral diffuse interstitial infiltrates (Pomero and Filice, 1992). In most cases, the clinical symptoms progress rapidly, leading to septic shock followed by patient death. Although scarce, the mortality rate among individuals with immunodeficiency with pulmonary toxoplasmosis is high (Lucet et al., 1993; Rabaud et al., 1996).

1.11.3 Congenital toxoplasmosis

A primary infection with *T. gondii* during pregnancy can result in congenital toxoplasmosis, even when the mother experiences no symptoms. However, around 60-70% of babies born from mothers infected during pregnancy are healthy. The likelihood of infection reaching the foetus increases with the gestational age. A study done on 603 confirmed maternal *T. gondii* infections showed the presence of a transmission rate of 6% during the first trimester, and 72% during the third trimester. However, the risk for severe clinical signs is much higher if the foetus is infected during early pregnancy. A late maternal infection results in mild to subclinical disease and 85% of the live infants with congenital toxoplasmosis appear normal at birth (Remington and Klein, 1995; Dunn et al., 1999).

The foetus becomes infected as the parasite crosses the placental barrier. The placenta is infected during the acute stage of the disease in the mother, rarely from reactivation of an infection before pregnancy. Once infected, the placenta becomes a source of infection for the foetus. As the infection to the foetus occurs via the placenta, testing for it at birth (by bio-assay and/or PCR) is an efficient means for the early diagnosis of congenital toxoplasmosis, if the baby is born without clinical signs (Robert-Gangeneuve et al., 2010).

Early infection of the foetus can cause abortion, stillbirth or live infants with severe defects. Those are the outcomes of a fully developed case of the disease affecting the CNS including hydrocephalus, meningoencephalitis, intracranial calcifications, and chorioretinitis. This can be related to the clinical symptoms of a general infection: hepatosplenomegaly, jaundice, rash, anemia, myocarditis, pericarditis, and pleura effusion (Ambroise-Thomas and Petersen, 2000; Dubey, 2009b).

Congenital toxoplasmosis can lead to various clinical appearances in the foetus. Ocular toxoplasmosis is the most common appearance of prenatal toxoplasmosis

including microphthalmia, cataracts, strabismus and even total blindness. In most cases, these are the outcomes of *T. gondii* multiplication in the retina. Ocular toxoplasmosis resulting from a congenital toxoplasmosis can be evident at birth, as active lesions or as healed scars, although may only become clinically evident after months or years following birth (Holland, 2003).

1.12 Immune response to *T. gondii*

1.12.1 Innate immune response

The first line of host defence against *T. gondii* infection is through the innate immune response, which is activated within a few hours of infection. There are three major components used by the host, namely, antigen-presenting cells (APCs), pattern recognition receptors (PRRs) and cytokines (Waree, 2008). The main function of APCs is the engulfment of foreign particles through phagocytosis as well as the production of cytokines such as interferons. In addition, they play a role in the breakdown of the pathogen to clear the infection. Interferons produced from these cells can stimulate the dendritic cells (DCs), resulting in the enhancement of antigen presentation to CD4⁺ and CD8⁺ T-cells, which further promote the adaptive immune response (Kimberly, 2010). *T. gondii* infection can be recognized by the presence of some PRRs such as toll-like receptors (TLRs). These receptors recognize pathogen-associated molecular patterns (PAMPs). Of the 13 TLRs known, TLR-2, TLR-4 and TLR-11 have been shown to play an important role in the recognition of *T. gondii*-derived antigens. Signalling by these receptors leads to the production of cytokines from infected host cells, including IFN- γ , TNF- α and macrophage inflammatory protein-1 alpha (MIP-1 α) released by natural killer (NK) cells (Kimberly, 2010). These cytokines have strong antiparasitic activity and can kill parasite-infected cells, thereby limiting the parasitic replication.

Although the host organism develops antiparasitic responses in order to defeat the emerging parasites following infection, *T. gondii* can avoid or subvert these responses by evolving various immune evasion strategies to replicate efficiently. This process involves the binding of *T. gondii* proteins to various components of the innate immune system, leading to their inhibition. For example, activation of transcription factors such as signal transducer activator of transcription-1 (STAT-1) blocks the production of TNF- α .

1.12.2 Adaptive immune response

The adaptive immune response represents the second line of host defence against *T. gondii*. It consists of a cellular response, which is represented by T-lymphocytes, and a humoral response represented by specific antibodies produced by B-lymphocytes.

1.12.2.1 Cellular immune response

During infection, CD4⁺ and CD8⁺ T-lymphocytes may be induced. APCs (DCs, macrophages and monocytes) express the major histocompatibility complex class-II (MHC-II) on their surfaces. When the parasite is engulfed, a lysosome-containing digestive enzymes combines with the phagosome to process the antigens. The processed antigens then combine with the MHC-II proteins to form an MHC-II-antigenic peptide complex, which is presented on the surface of the APC. Helper T-cells (CD4⁺) recognize the displayed antigen and bind to the complex. These cells are divided into T helper-1 (Th1) and Th2. Th2 cells produce cytokines such as IL-4, -5, -6 and -10, which further promote the humoral response (Lindberg and Frenkel, 1977). In contrast, Th1 cells produce IL-2 and IFN- γ , which promote the development of cellular immunity via stimulation of CD8⁺ T-lymphocytes. Some parasite peptides are expressed on MHC-I of APCs. In this case, peptides are transported to the endoplasmic reticulum (ER), where they associate with MHC-I; then, this peptide-MHC-I complex is transported via the Golgi apparatus to the

plasma membrane. CD8⁺ cells (cytotoxic T-cells) recognise this complex as abnormal and activate these cells, eventually leading to the lysis of the parasite-infected cells (Brown and McLeod, 1990).

1.12.2.2 Humoral immune response (antibody response)

Humoral (antibody-based) immunity is a specific type of immune response that helps eliminate disease and prevent reinfection in hosts who have been previously exposed to the same strain (Filisetti & Candolfi, 2004). Antibodies against *T. gondii* have been shown to increase host resistance to *T. gondii* infections. The antibodies bind directly to the *T. gondii* antigens and inhibit parasite attachment and entry. Antibodies against *T. gondii* are not completely protective, but may limit the spread of infection (Filisetti & Candolfi, 2004).

The main antibody types secreted after *T. gondii* infection are the mucosal antibodies immunoglobulin A (IgA), the circulatory antibodies IgM and IgG, all of which play a major role in *T. gondii* neutralization and clearance (Filisetti & Candolfi, 2004).

1. 13 Role of host lipids in *T. gondii* invasion, growth, and proliferation.

Although several varieties of strategies have been developed by intracellular parasites to invade and replicate within the host cells, increasing evidence suggests that most parasites depend on host lipids for invasion and then survival (Isabelle, 2006). In most cases, intracellular parasites exploit lipid rafts that are enriched in cholesterol and sphingolipids, which are believed to act as a platform through which the intracellular parasites gain entry to the host cells (Goldston et al., 2012). However, in some cases, intracellular parasites either synthesize enzymes that may target the host lipids or take advantage of the host's lipid-metabolizing enzymes; these enzymes are involved in the release of arachidonic acid C20:4n6 from the phospholipids of the host cell membrane, whereby they alter the fluidity of the membrane and facilitate invasion by tachyzoites (Li et al., 2008).

Lipids are key to the growth and proliferation of *T. gondii* as an intracellular protozoan parasite. It has been found that *T. gondii* gains access to various host metabolites (<1,300 Da) via diffusion through small pores in the PVM (Schwab et al., 1994). *T. gondii* incorporates low-density lipoprotein-derived cholesterol from the host endolysosomes (Coppens et al., 2000). Host mitochondria, ER and Golgi apparatus are also recruited efficiently to the cytosolic face of the PV soon after invasion, allowing *T. gondii* to siphon host energy and lipids such as lipoic acid, choline, and serine (Sinai et al., 1997). In addition to nutrient acquisition from the host cytosol, *T. gondii* facilitates its intracellular proliferation by actively modifying the PVM. Dense granules (GRA) are secretory organelle generated by the parasite and contain several proteins, which participate in the modification of the PVM. The purpose of this modification has not yet been established.

Electron micrographs of the PV show membrane-like material within the PVM and close to the posterior end of the parasites early in the invasion process (Sibley et

al., 1995). A complex of secreted GRA2, GRA4 and GRA6 proteins interacts with this material and are seemingly involved in its reorganization (Labruyere et al., 1999). Later in the infection process, similar thin tubules fill the lumen of the PV to form an intravacuolar network (IVN) (Magno et al., 2005). While electron micrographs suggested that the IVN might be primarily parasitic in origin (Sibley et al., 1995), no direct biochemical analysis has been reported and its origin is unknown. Furthermore, host ER proteins also appear to have access to the vacuolar space and, in particular, host glucose-6-phosphatase has been found in the posterior membrane-like material seen in the nascent PV (Melo & De Souza, 1997). This observation suggests that there may be a flow of lipids between the host ER and the PVM, but gives no hint of the relative contributions that host and parasite lipids might each represent within the IVN.

Lipid metabolism has been studied in intracellular parasites. Coppens et al. (2000) have reported that most of the intracellular parasites either lacks or have incomplete *de novo* lipid synthesis. However, some of these intracellular parasites can synthesize complex lipids by slaving precursors such as choline, serine, and ethanolamine from the host cell. These intracellular parasites exploit the host lipids for survival, proliferation, membrane biogenesis, and for energy requirements. Coppens (2006) has shown that a variety of host lipids are exchanged across the PVM, including fatty acids, phospholipids and cholesterol, some of which are further metabolized by the parasite to ensure its survival and proliferation.

Fatty acids

The *T. gondii* apicoplast contains enzymes for the fatty acid synthetic type-II pathway. *T. gondii* also contains the fatty acid synthetic type-I pathway and fatty acyl-elongases, whose enzymes reside in the mitochondria and ER, respectively. All fatty acid synthetic type-I and -II pathways as well as fatty acyl-elongases are complementary to satisfying the parasite metabolism. Host-free tachyzoites can

uptake selected exogenous fatty acids (Tomavo et al., 1989; Quittnat et al., 2004). Extracellular parasites incorporate palmitic acid (C16:0) or myristic acid (C14:0) into glycosylphosphatidylinositol (GPI) anchors, such as that of surface antigen-1 (Tomavo et al., 1989). After uptake of butyric acid C4:0 by *T. gondii*, the lipid is anabolized into phosphatidylcholine (Charron and Sibley, 2002).

Glycerolipids

Triacylglycerol is synthesized *de novo* through the acyl-CoA:diacylglycerolacyl transferase-mediated pathway in the *T. gondii* ER (Quittnat et al., 2004). These glycerolipids are stored in *T. gondii* lipid bodies. *T. gondii* apicoplasts contain the complete pathway to synthesize galactosyl glycerolipids from fatty acids and glycerol-phosphate (Marechal et al., 2002). Quittnat et al. (2004) used fluorescent lipid 1-stearoyl-2-[1-14C]arachidonyl-sn-glycerol and found that it was internalized by extracellular parasites and converted into triacylglycerol.

Aminoglycerophospholipids

Quantitative measurements of the phospholipid profile of *T. gondii* revealed that phosphatidylcholine is the most abundant phospholipid, accounting for about 75% of the total phospholipids (Gupta et al., 2005). The next most abundant phospholipids are phosphatidylethanolamine (10%), phosphatidylinositol (7.5%), phosphatidylserine (6%) and phosphatidic acids (1%). *T. gondii* contains enzymes to synthesize all these phospholipids via the Kennedy pathways (Vance and Vance, 2004). Alternatively, *T. gondii* can take up their head precursors such as choline, serine, and ethanolamine from the host cells for their incorporation into complex phospholipids (Charron and Sibley, 2002; Gupta et al., 2005). Charron and Sibley (2002) used fluorescent lipid [oleic-1-14C] L- α -phosphatidic acid and found that only parasites inside the host cells could further metabolize the scavenged lipid into phosphatidylcholine.

Sterol lipids

No anabolic pathway has been identified for any sterol lipids in *T. gondii*. Nevertheless, their membranes contain cholesterol that is mainly derived from low-density lipoprotein (LDL) host cells (Coppens et al., 2000; Portugal et al., 2008). The ability of a subsequent enzymatic conversion of cholesterol into other sterol molecules, has not been found in *T. gondii* (Roberts et al., 2003). Quantification of the lipid profile of *T. gondii* revealed a relatively low cholesterol:phospholipid ratio (Foussard et al., 1991). Interestingly, the rhoptry, the content of which is liberated upon parasite invasion, contains high levels of free cholesterol (Coppens and Vielemeyer, 2005). The *T. gondii* ER synthesizes cholesteryl esters by acyl-CoA:cholesterolacyl transferases. These lipid esters are the most abundant neutral lipids of parasite lipid bodies (Nishikawa et al., 2005).

Sphingolipids

Glycosphingolipids are synthesized from serine and palmitoyl-CoA *de novo* via the 3-ketosphinganine pathway, with ceramide as an intermediate (Azzouz et al., 2002; Sonda et al., 2005). Sphingomyelin has been found in the plasma membrane in relatively low amounts (Gallois et al., 1988). However, GPIs are present as membrane anchors for a large number of plasma membrane proteins (Tomavo et al., 1992; Striepen et al., 1997; Zinecker et al., 2001). Their biosynthetic pathway is initiated in the *T. gondii* ER with the transfer of N-acetylglucosamine to phosphatidylinositol (Wichroski and Ward, 2003).

Isoprenoid

T. gondii can take up isoprenoids from the medium to produce prenylated proteins (Ibrahim et al., 2001; Ling et al., 2005). A xylulose pathway present in the *T. gondii* apicoplast may also contribute to the production of farnesyl and geranylgeranyl molecules. The PVM is a membrane bilayer that is formed rapidly *de novo* at the time of invasion (Suss-Toby et al., 1996). The origin of the PVM derives both from the host plasma membrane and an apical secretory organelle that is

liberated into the host cytosol upon tachyzoite entry (Hakansson et al., 2001). The host cell membrane lipids such as cholesterol and gangliosides are incorporated into the PVM (Coppens and Joiner, 2003; Charron and Sibley, 2004). However, the discharge of GRA into the PV during parasite residence within the host cell is probably accompanied by modifications in the PVM lipid composition. Apicoplast-derived lipids may also form components of the PVM, which may explain the delayed-death phenotype of *T. gondii* following antibiotic disruption of apicoplast biogenesis (Coppens, 2006).

1.14 Hypothesis

Survival of intracellular protozoan parasite *T. gondii* typically depends on host cells for their nutrient needs to grow and proliferate. As the infection progresses, the cellular stress increases; however, host cells develop a variety of strategies to compensate for the metabolic alterations and maintain cell viability, while the intracellular *T. gondii* utilises various mechanisms to ensure growth and proliferation. To what extent the intracellular protozoan *T. gondii* alters metabolism of the host cell during the intracellular parasitism, and the consequences of this disturbance, is unknown. HBMECs have been previously used to study the strategies used by neuropathogenic protozoa to breach the BBB. However, *T. gondii* proliferation within HBMECs and the impact the infection on the structural features and metabolic processes have not yet been investigated.

1.15 Aim and objectives

1.15.1 Aim

This study aimed to examine structural and functional changes in HBMECs following infection by *T. gondii*.

1.15.2 Objectives

Primary cultures of HBMECs were used as a cellular model to address the following objectives:

1. To assess the integrity and viability of HBMECs during the intracellular development of *T. gondii*
2. To characterize the morphological changes in HBMECs following an infection by *T. gondii*.
3. To identify the molecular metabolic changes in HBMECs in response to *T. gondii* infection.

4. To evaluate the changes in fatty acid compositions of the total phospholipids in HBMECs in response to *T. gondii* infection.
5. To assess the changes in the content of trace elements within HBMECs in response to the infection.

2. General materials and methods

2.1 Introduction

The general materials used and methods followed in this study are presented in the following chapters. Specific methods have been presented in the appropriate chapters thereafter.

2.2 Human brain microvascular endothelial cells (HBMECs)

HBMECs at passage 16 were kindly provided by Professor Naveed A. Khan (Aga Khan University). The cell line was used as a cellular model for experimental toxoplasmosis to (i) assess the cell viability and integrity, (ii) characterize the intracellular development of the tachyzoites, (iii) identify the molecular metabolic changes, (iv) study changes in fatty acid compositions of the total phospholipids, and (v) study changes in the level of trace elements in these cells in response to infection. The cells were cultured in Roswell Park Memorial Institute medium-1640 (cRPMI) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 1% vitamin, and 1% antibiotic-antimycotic solution (10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per ml). On reaching confluence, the cells were harvested with trypsin-ethylene diaminetetra acetic acid (EDTA) and passed in a sub-cultivation ratio of 1:3 into new culture flasks with fresh cRPMI medium. The HBMECs were grown at 37°C in a humidified atmosphere of 5% CO₂ (Elsheikha et al., 2013). The number of viable cells present in the medium was quantified by using a hemocytometer under a light microscope, following staining with trypan blue solution (Figure 2.1).

All media, FBS, tissue culture supplements, and antibiotics were purchased from Invitrogen Co. (GIBCO™; Grand Island, NY, USA).

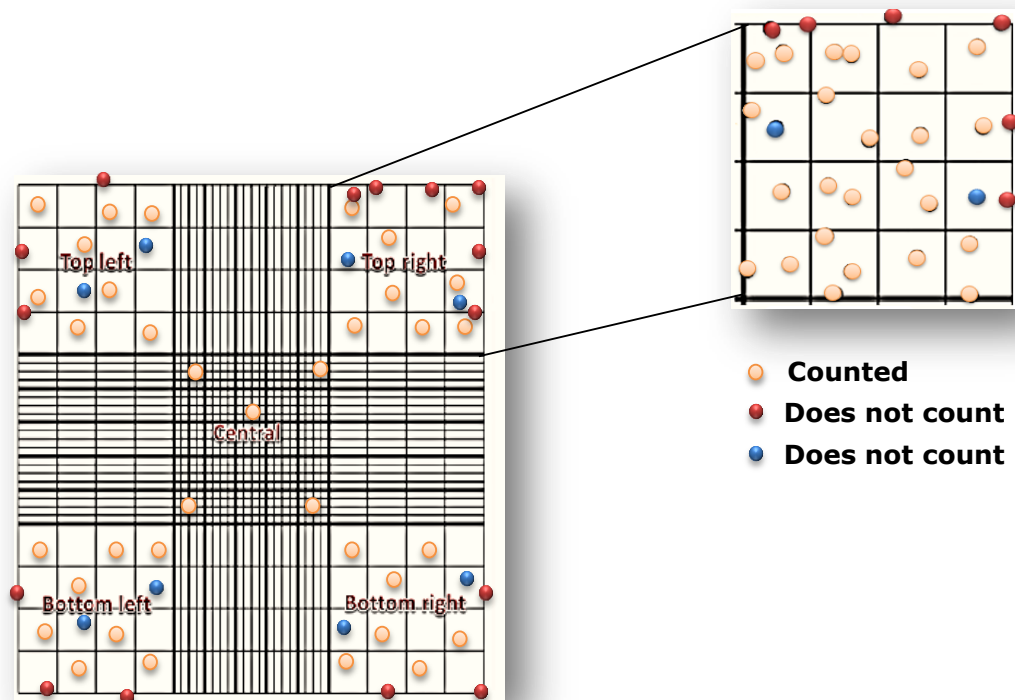


Figure 2.1: Schematic view of a haemocytometer chamber under the light microscope. The cells were counted in five labelled squares: top left, top right, bottom left, bottom right, and the centre square. The cells located on the edges were excluded from two sides only (indicated by red circles). Dead cells (indicated by blue circles) were also excluded.

2.3 Maintenance and purification of *T. gondii* tachyzoites

2.3.1 *T. gondii* tachyzoite propagation

T. gondii tachyzoites (RH strain) were kindly provided by Professor Lee Innes (Moredun Research Institute, Scotland). They were propagated by continuous passage on Madin-Darby Canine Kidney (MDCK) cell cultures in complete Dulbecco's modified Eagle's medium (cDMEM), supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 1% antibiotic-antimycotic solution at 37°C under a humidified atmosphere of 5% CO₂.

2.3.2 Tachyzoite purification

To purify *T. gondii* tachyzoites, the parasites were collected from their feeder cell cultures and eluted through Protein Desalting (PD-10) Columns filled with Sephadex (Elsheikha et al., 2006). Briefly, the parasite suspension was washed thrice in sterile phosphate-buffered saline (PBS) (8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH₂PO₄, and 1.15 g/l Na₂HPO₄) by centrifugation at 1500 ×g for 5 min at 4°C. The pellet obtained was resuspended in 2.5 ml PBS and applied to a Desalting Column (Amersham Biosciences, Piscataway, NJ, USA), previously equilibrated with 25 ml PBS. The parasites were then eluted with 3.5 ml of the buffer used in the equilibration of the column (Figure 2.2). Then, the purified parasites were centrifuged at 800 ×g, re-suspended in fresh medium, and quantified by using a hemocytometer. The final volume of suspension was adjusted with cRPMI medium to achieve a ratio of 2:1 parasite:host cell for subsequent infection experiments.

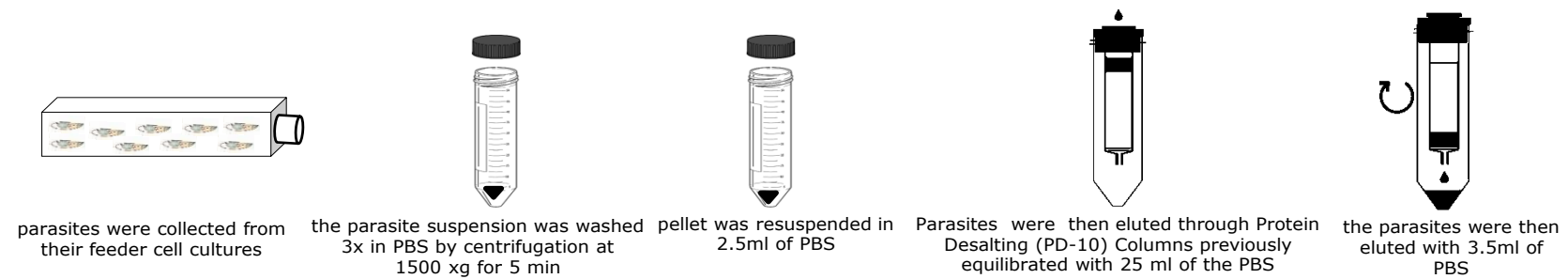


Figure 2.2: Purification of tachyzoite from their feeder cells. The tachyzoite suspension was washed thrice in phosphate buffered saline (PBS) by centrifugation at 1500 xg for 5 min at 4°C. The pellet obtained was resuspended in 2.5 ml of PBS and applied to a desalting column previously equilibrated with 25 ml of the PBS. The parasites were then eluted with 3.5 ml of the buffer used in the equilibration of the column.

2.4 Infection of HBMECs with *T. gondii* tachyzoites

To infect HBMECs with *T. gondii* tachyzoites, the cells were grown in cRPMI medium. Once a confluent monolayer of the cells was formed (~24 h), the tachyzoites were added in a host:parasite ratio of 1:2. Uninfected controls were inoculated with an equal volume of medium without tachyzoites. After 3 h from the beginning of incubation, the media from the culture flask or plate were discarded and fresh cRPMI was added to remove unbound parasites and cellular debris.

3. Development of *T. gondii* tachyzoites within HBMECs *in vitro*

3.1 Introduction

T. gondii is a protozoan parasite that has significant public health and economic importance owing to its ability to cause neurological and ocular diseases, as well as reproductive disorders in humans and animals (Dubey and Beattie, 1988). It invades and proliferates within mammalian cells (Tenter et al., 2000) and upon entry, tachyzoites form their own compartments (PV) (Laliberté & Carruthers, 2008).

In vitro cultures have been used to study the intracellular development of parasites, host–parasite interactions, screening for potential chemotherapeutic agents (Leepin et al., 2008), and for performing genetic manipulations of parasites (Radke et al., 2000; Vinayak et al., 2014). *T. gondii* has been successfully cultured in various animal cell lines, such as monkey fibroblast cells (VERO cells) (Saadatnia et al., 2010), bovine endothelial cells (Taubert et al., 2006), cat and dog fibroblast cells (Lei et al., 2005), cat kidney cells (Lüder et al., 1999), rat astrocytes (Oura et al., 1993), and ovine fibroblast cells (Zhang et al., 2010). Human cells have also been used, including fibroblast cells (Pfefferkorn and Guyre, 1984), retinal vascular endothelial cells (Zamora et al., 2008), and brain microvascular endothelial cells (Däubener et al., 2001). *T. gondii* is known to infect a wide range of mammalian cells including HBMECs (Däubener et al., 2001) and, upon entry, form its own compartment (PV). Within the PV, the parasite proliferates with a generation time of 24 h to form rosettes, eventually leading to cell rupture and the spread of the tachyzoites to infect a neighbouring cell (Laliberté & Carruthers, 2008).

Previous studies on the ultrastructural analysis of the initial stages of entry of *T. gondii* showed that tachyzoites were in close contact with macrophage cells. The process of entry into macrophage cells by tachyzoites of *T. gondii* is initiated by the parasite's anterior end making contact with the host cell. As entry progresses, pseudopodia of the macrophage cells surround the parasite and it forms the PV once inside the macrophage (Toulah et al., 2011). The mechanism of entry of *T. gondii* into Hela cells was also previously investigated to observe the ultrastructural changes associated with intracellular parasitism. Entry into Hela cells by tachyzoites is initiated by the apical end of the tachyzoite making contact with the host cell and, upon entry, forming its own compartment (Toulah et al., 2011). The ultrastructural analysis of tachyzoites in VERO cells revealed that there were changes in the host cell organelles, as well as their spatial distribution and alteration in response to infection. In uninfected cells the mitochondria are elongated structures which extend from the nuclear region toward the cell membrane. After parasite invasion mitochondria become shorter and tend to concentrate around the PVM. The host ER and Golgi apparatus also concentrate around, and even establish physical contact with, the PVM (De Melo et al., 1992; Romano et al., 2013).

Thus far, interaction of intracellular protozoan parasites with mammalian cells has been shown to result in structural changes in affected target cells (De Melo et al., 1992; Romano et al., 2013). To date, no information is available on the interaction of *T. gondii* with HBMECs. In the present study, we investigated the hypothesis that invasion, growth, and replication of tachyzoites within HBMECs can disrupt the cell viability and integrity of host target cells, involving changes in the morphology of target cells during the course of infection. Primary cultures of HBMECs were used as a cellular model for experimental toxoplasmosis studies to (i) investigate the intracellular development of the tachyzoites, and (ii) assess the cell viability and integrity of HBMECs during the course of infection.

3.2 Materials and methods

3.2.1 Acridine Orange (AO) staining

AO is a nucleic acid-binding stain that is used to identify intracellular parasites by fluorescence microscopy. This assay is based on the permeation of AO (3-N, 3-N, 6-N, 6-N-tetramethyl acridine-3, and 6-diamine) into cells and parasite membranes, emitting green fluorescence when bound to double-stranded deoxyribonucleic acid (dsDNA) and red fluorescence when bound to single-stranded ribonucleic acid (ssRNA) (Darzynkiewicz, 1990). The intracellular parasites were identified from their characteristic shapes and dimensions. AO staining was performed as per the manufacturer's instructions [Sigma-Aldrich Chemicals, Munich, Germany]. Briefly, the HBMECs were cultured in 6-well cell plates (Corning Incorporated, USA) on coverslips at a concentration of 1×10^5 /ml in cRPMI medium. Once a confluent monolayer of the cells was formed (~24 h), the tachyzoites were added in a host:parasite ratio of 1:2 as described in section 2.4. The coverslips were removed from the wells at 6, 24, and 48 h post-infection (PI), washed twice in PBS, fixed in 4% paraformaldehyde for 30 min, stained with 10% AO for 10 min, rewashed twice in PBS, and then mounted on to a microscope slide. Coverslips were observed under a Leica DM 5000B epifluorescence microscope equipped with a charge-coupled-device (CCD) camera (Figure 3.1). This assay was performed in triplicate, and the data represent the mean and standard error of the mean (S.E.M).

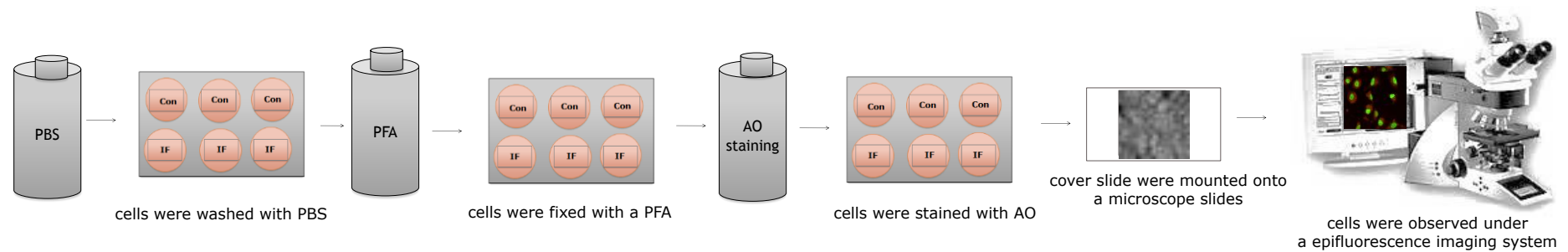


Figure 3.1: Schematic illustration of AO staining assay. HBMECs were seeded in 6-well plate on cover slides. When the cells became confluent (~24 h), the tachyzoites were added in a host:parasite ratio of 1:2. Then, the culture medium was removed from the wells. The cells were then washed twice in PBS, fixed in 4% paraformaldehyde (PFA), stained with 10% AO, rewashed twice in PBS, and mounted onto a microscope slide. Intracellular parasites were observed under a Leica DM 5000B epifluorescence imaging system. Con: control, IF: infected.

3.2.2 Alamar blue assay

Alamar blue[®] is a method of determining cell viability (Rampersad, 2012). This assay is based on the reduction of blue non-fluorescent resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) to pink fluorescent resorufin by the action of mitochondrial dehydrogenase enzymes in living cells. The reaction requires the presence of an electron coupling reagent, serving as an intermediate electron acceptor (Anoopkumar-Dukie et al. 2005). Damaged and non-viable cells have lower metabolic activity and generate a lower signal (Figure 3.2).

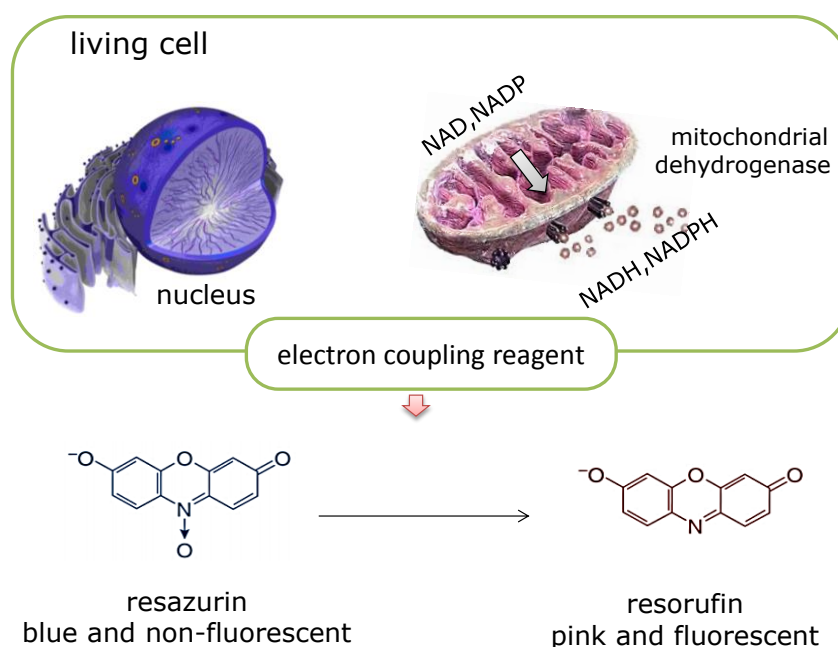


Figure 3.2: Resazurin sodium salt reduction in living cells by mitochondrial activity results in the formation of resorufin, characterized by high absorptivity at 570 nm. NAD: Nicotinamide adenine dinucleotide; NADP: Nicotinamide adenine dinucleotide phosphate.

The Alamar blue[®] assay was performed following the manufacturer's instructions [Alamar blue[®]; Cell Viability Reagent Invitrogen, UK]. Briefly, the HBMECs were seeded in 96-well plate at 5×10^3 cells/well and grown in cRPMI medium at 37°C in a humidified atmosphere of 5% CO₂. When the cells became confluent (~24 h), the tachyzoites were added in a host:parasite ratio of 1:2 as described in section 2.4. At 6, 24, and 48 h PI, 10 µl of alamar blue dye solution was added to each well. Following incubation for 4 h, the absorbance was read at 570 nm on a microplate reader (Labtech International Ltd., UK) (Figure 3.3). This assay was performed in six replicates, and the data represent the mean and standard error of the mean (S.E.M) from at least three independent experiments. *T. gondii* tachyzoites did not seem to contribute to the readings, as in a preliminary experiment up to 10,000 tachyzoites did not cause any change in the colour of the alamar blue and the results were similar to the blank reading (data not shown).

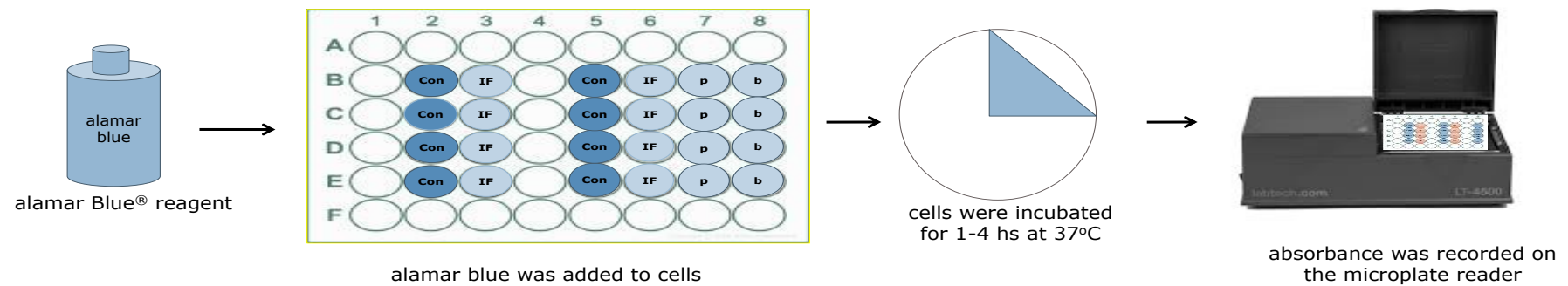


Figure 3.3: Schematic view of the alamar blue[®] assay. The HBMECs were seeded in 96-well plate. When the cells became confluent (~24 h), the tachyzoites were added in a host:parasite ratio of 1:2. Then, 10 µl of alamar blue dye solution was added to each well. Following incubation for 4 h, the absorbance was recorded on a microplate reader. Con: control, IF: infected; P: parasite; b: blank.

3.2.3 Trans-endothelial electrical resistance (TEER) assay

TEER is a quantitative technique used to measure the integrity of a cell monolayer and tight junction permeability during pathogen infection. It measures the movement of ions across the cell (transcellular) and between cells (paracellular). High electrical resistance indicates higher integrity of a cell monolayer, while low electrical resistance indicates a compromise in the cell monolayer's integrity (Wilhelm et al. 2011). The TEER assay was performed as described by Wilhelm et al. (2011). Briefly, cell cultures were grown in 24-well cell plate on cell culture inserts (0.4 μm) at 1×10^4 cells/well in cRPMI medium at 37°C in a humidified atmosphere of 5% CO_2 . When the cells became more confluent (~5 day), the tachyzoites were added in a host:parasite ratio of 1:2 as described in section 2.4. At 6, 24, and 48 h PI, TEER was measured across the cell monolayer by an EVOM resistance meter (Millicell-Electrical Resistance System, Millipore, and Bedford, USA) and expressed in Ω/cm^2 units. The electrodes of the EVOM meter were cleaned with 70% ethanol and rinsed with sterile PBS before measurements to avoid contamination of the test well. One electrode was inserted in the medium covering the HBMEC monolayer, while the other electrode was inserted in the medium on the outside of the transwell (Figure 3.4). Blank measurements were taken using a transwell culture insert with media and without cells. TEER values obtained with inserts containing media without cells were subtracted from the TEER values obtained with inserts containing cells. This assay was performed in triplicate, and the data represent the mean and standard error of the mean (S.E.M) from at least three independent experiments.

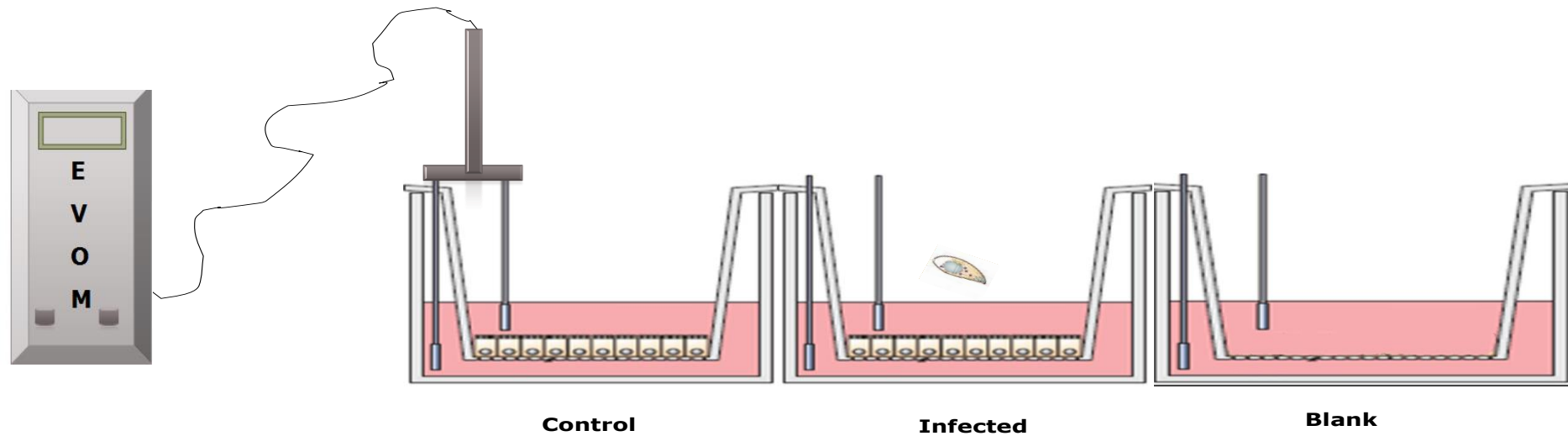


Figure 3.4: Schematic view of TEER assay. The HBMECs were seeded in 24-well plate on cell culture inserts ($0.4\ \mu\text{m}$). When the cells became confluent, the tachyzoites were added in a host: parasite ratio of 1:2. The integrity of the cell monolayer was then evaluated by measuring TEER. One electrode was inserted in the medium covering the HBMEC monolayer, while the other was inserted in the medium on the outside of the transwell.

3.2.4 Scanning and transmission electron microscopy

To characterize the disruption of tight junctions of HBMECs and reorganization of host organelles around the PV, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were applied as described by Bozzola and Russell (1992). SEM was used to describe the external morphological characteristics of tachyzoites and the mechanism of cell invasion of the tachyzoites to the host cells, while TEM was used to observe the ultrastructural changes associated with intracellular development of the tachyzoites within host cells. SEM produces an image with the help of secondary electrons and generates a three-dimensional image, while TEM projects electrons through an ultrathin slice of the sample that gives the viewer the impression of a two-dimensional image. Depending on the tool used, the samples can be magnified between approximately 10 and 100,000 times with SEM and between 500 and 500,000 times with TEM (Zhou et al., 2013). Briefly, HBMECs were grown in a 24-well plate on Thermanox Plastic Coverslips (13 mm in diameter) at a concentration of 1×10^4 /ml in cRPMI medium. Once a confluent monolayer of the cells was formed (~24 h), the tachyzoites were added in a host:parasite ratio of 1:2 as described in section 2.4. The coverslips were removed from the wells at 6, 24, and 48 h PI. These experiments were performed in triplicate.

3.2.4.1 Processing of the cells for SEM

3.2.4.1.1 Washing and fixation of the cells

The coverslip containing the cell layer was washed thrice in 0.1 M cacodylate buffer for 10 min at room temperature and fixed in EM fixative buffer (3% glutaraldehyde prepared in 0.1 M cacodylate buffer) for 30 min at room temperature. After the fixation step, the coverslips were rinsed in 0.1 M cacodylic acid buffer once for 10

min and then thrice for 20 min at 4°C and then post-fixed with 1% aqueous osmium tetroxide prepared in 0.1 M cacodylate buffer for 30 min. Thereafter, the coverslip was washed in distilled water five times for 1 min each.

3.2.4.1.2 Dehydration and coating of the cells

The coverslip was subsequently dehydrated through a graded series of ethanol dilutions by using the following protocol: dehydrated twice in 50% ethanol for 5 min, twice in 70% ethanol for 5 min, twice in 90% ethanol for 5 min, and thrice in 100% ethanol for 10 min. The coverslips were then dried using tousimis® SAMDRI® -PVT-3D critical Point dryer for 45 min, mounting on aluminum stubs, and coated with a 0.2 nm thin layer of gold-palladium in a Balzers Union SCD 040 for 15–30 min to increase specimen conductivity and prevent the build-up of high voltage charges on the specimen by conducting the charge to ground (Figure 3.5).

3.2.4.1.3 SEM imaging

The aluminum stubs were imaged on a Jeol 6060LV Digital SEM system. Photographs were taken from different areas of the aluminum stubs at a range of image magnifications.

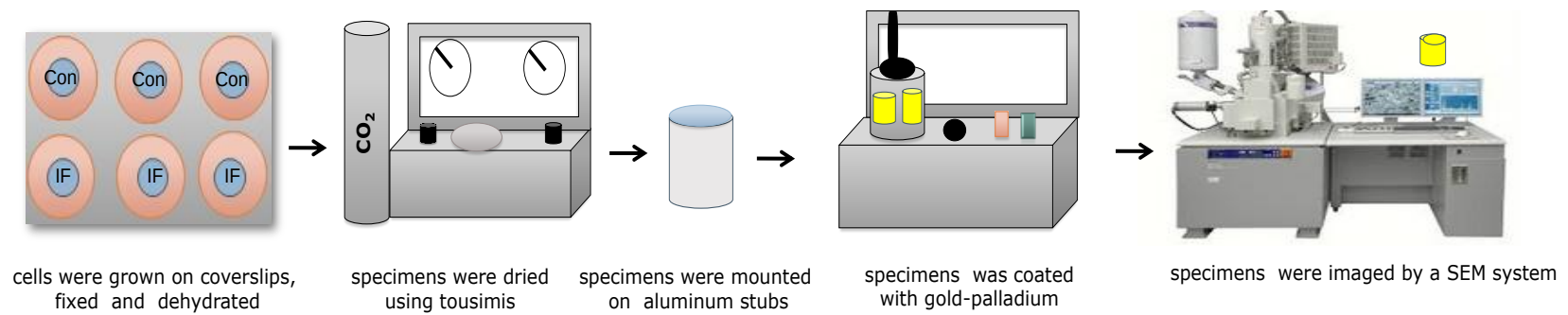


Figure 3.5: Schematic view of cell processing for SEM. HBMECs were seeded in 6-well plate on cover slips. When the cells become confluent, the tachyzoites were added in a host:parasite ratio of 1:2. The culture medium was removed from the wells. The cells were washed, fixed, dehydrated, dried using tousimis, and mounted on aluminum stubs. Specimens were then coated with gold-palladium and observed under a SEM imaging system. Con: control, IF: infected.

3.2.4.2 Processing of the cells for TEM

3.2.4.2.1 Washing and fixation of the cells

The coverslip containing the cell layer was washed thrice in 0.1 M cacodylate buffer for 10 min at room temperature and fixed in EM fixative buffer for 1 h at 4°C as described in section 3.2.4.1.1. After fixation, the coverslip was washed thrice in the same buffer for 10 min and then post-fixed with 1% aqueous osmium tetroxide prepared in 0.1 M cacodylate buffer for 30 min. Thereafter, the coverslip was washed in distilled water five times for 1 min each.

3.2.4.2.2 Dehydration and infiltration of the cells

The coverslip was subsequently dehydrated in a graded series of ethanol dilutions as described in section 3.2.4.1.2. The coverslip was then transferred to ceramic cup and immersed twice in 100% dried acetone for 5 min. The dried coverslip was then infiltrated by immersing it once in a resin:acetone mix (at a ratio of 1:3) for 30 min, once in resin:acetone mix (at a ratio of 1:1) for 1 h, and then thrice in pure resin for 1 h. After that, the coverslip was transferred to a plastic cup filled with fresh resin and incubated in a drying cabinet for 2 days at 60°C. Once the coverslip had polymerized, the block was then placed on a hotplate and the coverslip was removed by forceps, leaving the cell layer attached to the resin block. The resin block was fixed on the stage of the clamp and a layer out of the specimen was cut.

3.2.4.2.3 Slicing and staining of the cells

A piece of the resin block was placed on the chuck of a Leica EM UC6 ultramicrotome system and securely tightened. A sharp knife was fixed on the stage of the microscope and gradually advanced to the front until it almost touched the block, which was then carefully trimmed several times until the tissue was sectioned. A drop of water was placed on a 3-aminopropyltriethoxysilane (APES)-coated slide and a section was collected using a fine paintbrush and placed on the drop of water on the slide. The slide was then placed on a hotplate to flatten out and dry the section, and then it was stained with toluidine blue for 1 min on a hot

plate. After that, the slide was rinsed with 100% ethanol and distilled water, placed on the hotplate to dry, and examined under light microscope. Once the cells could be clearly observed, the area of interest was determined.

The whole chuck holder (including the block) was removed and placed into the flat holder in place of the knife holder. The surface of the block was trimmed into a trapezoid shape using a single-edged blade. The specimen was then cut thinner (75 nm). The sections were separated, flattened using chloroform vapor, and picked up on G200HH 3.05-mm copper grids, 100 HEX (TAAB) by using fine forceps. The grids were placed in a labeled Petri dish containing filter paper for 1 h at room temperature for air drying. Then, squares of parafilm slightly smaller than the staining Petri dish were prepared and attached to the bottom of the dish. By using a syringe and filter, a drop of ethanolic uranyl acetate stain and Reynold's lead citrate stain were placed on the parafilm. First, the grid was placed on the ethanolic uranyl acetate stain and incubated in the dark for 5 min at room temperature to produce the highest electron density. The grid was then retrieved from the drop with a pair of fine forceps and quickly dipped ten times in each of a series of three disposable beakers, with the first beaker containing 50% ethanol and the other two containing sterile water. Then, the grid was placed on a drop of Reynold's lead citrate stain and incubated in the dark for 5 min at room temperature to enhance the electron-scattering in the specimen. Subsequently, the grid was rinsed with sterile water, blotted on clean filter paper, and placed in a clean, labeled Petri dish (Figure 3.6).

3.2.4.2.4 TEM imaging

The grids were imaged on a Tecnai G212 Bio Twin Digital TEM system. Photographs were taken from different areas of the grid at a range of image magnifications.

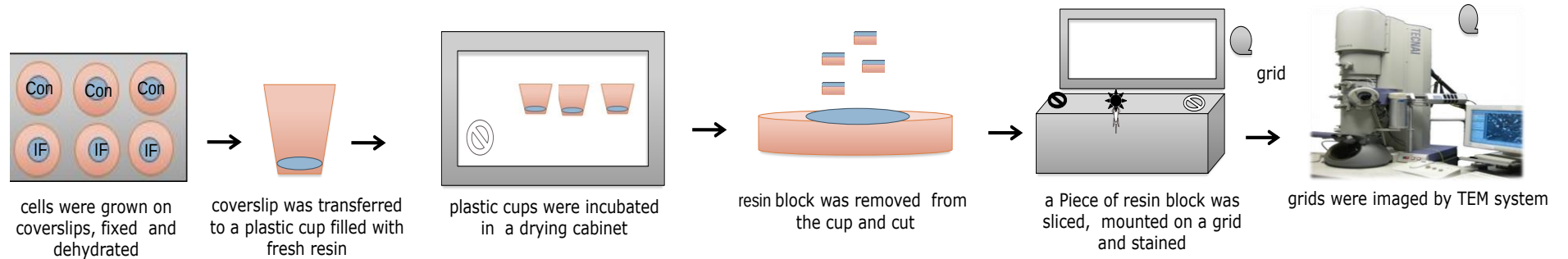


Figure 3.6: Schematic view of cell processing for TEM. HBMECs were seeded in 6-well plates on cover slips. When the cells became confluent, tachyzoites were added in a host:parasite ratio of 1:2. The culture medium was removed from the wells. The cells were washed, fixed, dehydrated, infiltrated, and polymerized by using a drying cabinet. Resin blocks were then removed from the cups and cut into small pieces. A piece of resin block was sliced, mounted on a grid, and stained. Specimens were then observed under the TEM imaging system. Con: control, IF: infected.

3.2.5 Statistical analysis

Quantification of the surface area of the tachyzoites based on AO staining was measured by using ImageJ[®] software version 15.0 (NIH, <http://rsb.info.nih.gov/ij>). The surface area of the tachyzoites between infected cells at different time points were assessed by One-way ANOVA, followed by Tukey's multiple comparison test using Graphpad Prism version 5.0 (Graph pad Software, Inc., San Diego, CA).

Differences in the integrity and cell viability between control and infected cells at different time points was assessed by two-way ANOVA, followed by Tukey's multiple comparison test using Graphpad Prism.

3.3 Results

3.3.1. AO staining

Analysis of the AO staining data revealed a marked increase in tachyzoite surface area ($F_{(2, 15)} = 67.99$, $p < 0.0001$) (Figure 3.7-3.8). Post-hoc analysis indicated that the surface area of the tachyzoites was significantly increased at 48 h compared to 6 and 24 h PI ($n = 9$ cells/group). At 6 h PI, the tachyzoites were embedded in the host cell. By 24 h PI, the proliferation of tachyzoites had occurred and numerous tachyzoites were detected intracellularly, as indicated by the increase in tachyzoite surface area. At 48 h PI, more divisions took place and larger collections of dividing tachyzoites were visible.

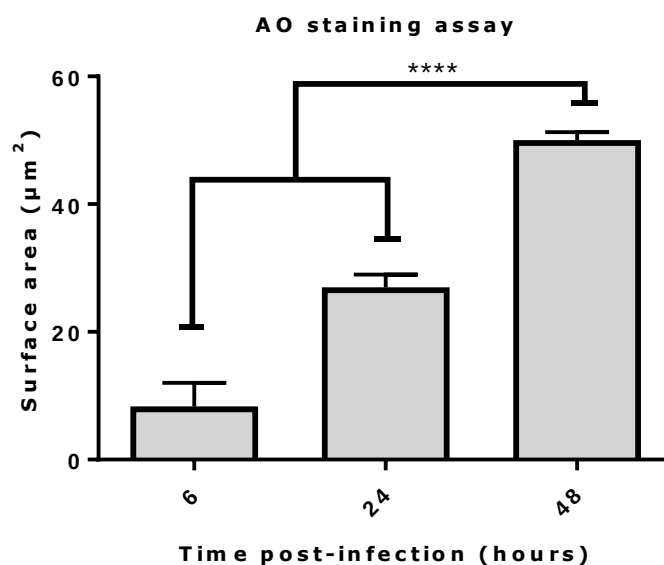


Figure 3.7: AO staining in HBMECs infected with *T. gondii* over 48 h PI. A marked increase was noted in the surface area of tachyzoites at 48 h compared to 6 and 24 h PI.

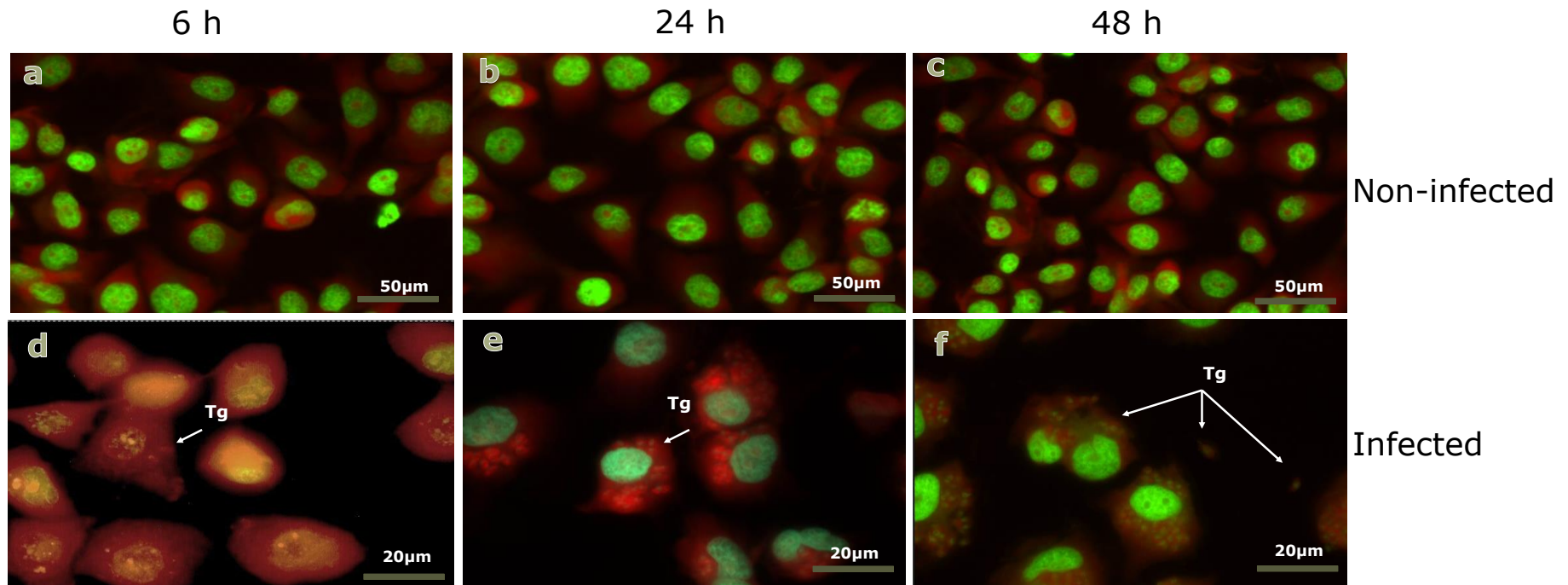


Figure 3.8: AO staining of HBMECs showing a progressive increase in the number of *T. gondii* (Tg) tachyzoites over time. HBMECs were infected with tachyzoites, followed by AO staining at different time points post-infection as indicated on the top. Cell and tachyzoite nuclei are shown in green and cytoplasm in red.

3.3.2 Alamar blue assay

Analysis of the alamar blue data revealed significant main effects of group ($F_{(1, 102)} = 15.14$, $P = 0.0002$) and time ($F_{(2, 102)} = 256.7$, $P < 0.0001$), as well as a significant group $\times$ time interaction ($F_{(2, 102)} = 9.676$, $P = 0.0001$) (Figure 3.9). Post-hoc analysis revealed that infected cells showed significantly decreased cell viability, compared to uninfected controls, at 48 h PI ($P = 0.0001$).

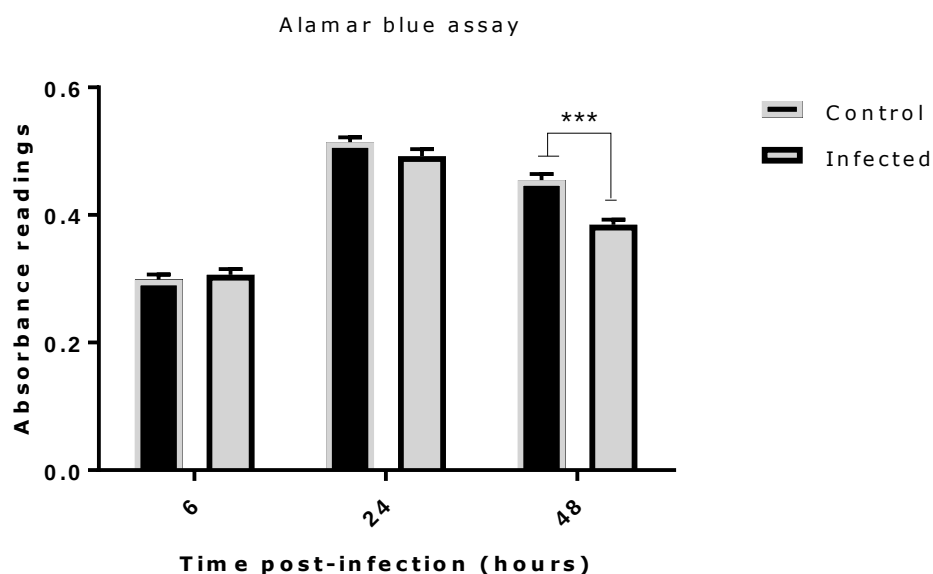


Figure 3.9: Viability of HBMECs infected and uninfected with *T. gondii* over 48 h PI, as assessed by the alamar blue assay. Infected cells showed significantly decreased metabolic activity, compared to uninfected controls, at 48 h PI.

3.3.3 TEER assay

The integrity of the HBMEC monolayer with and without infection was assessed by TEER assay. Analysis of the TEER data revealed a significant main effect of group ($F_{(1, 2)} = 6.329$, $P = 0.0271$), but no significant main effect of time ($F_{(2, 12)} = 0.3450$, $P = 0.7150$) or group $\times$ time interaction ($F_{(2, 12)} = 0.5804$, $P = 0.5747$) (Figure 3.10). Post-hoc analysis indicated that TEER was significantly decreased in infected, compared to uninfected, cells ($P = 0.0271$).



Figure 3.10: Integrity of HBMECs infected and uninfected with *T. gondii* over 48 h PI, as assessed by the TEER assay. A significant decrease in electrical resistance was noted in the infected compared to the uninfected cells.

3.3.4 SEM imaging of HBMECs infected with *T. gondii*

SEM of HBMECs infected with tachyzoites showed that tachyzoite proliferation disrupted the tight junctions (TJs) in infected cells at 24 and 48 h PI (Figure 3.11 C, D) compared to 6 h PI (Figure 3.11 B) and in non-infected cells (Figure 3.11 A).

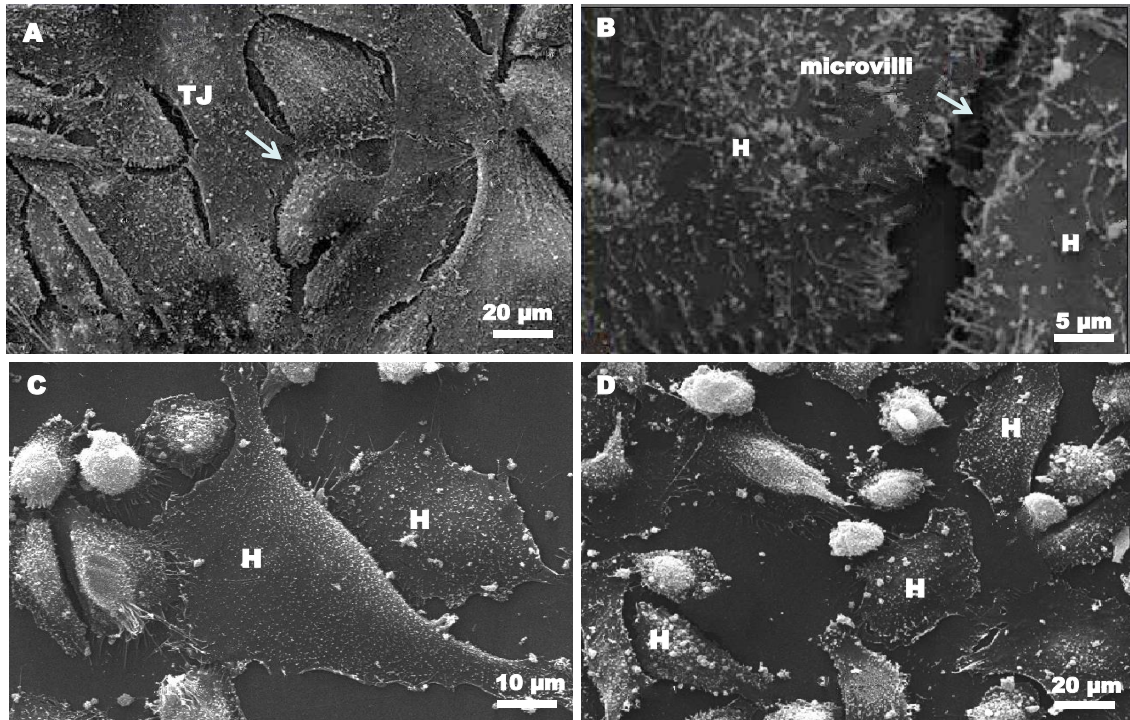


Figure 3.11: Scanning electron micrographs (SEMs) of HBMECs infected and uninfected with tachyzoites. A confluent monolayer of host cells (H) shows microvilli at the cell surface and in the region of intercellular contact (TJ) at 6 h PI (B). A large gap is evident between infected cells at 24 and 48 h PI (C,D) compared to 6 h PI (B) and non-infected ones (A).

SEM of tachyzoites in HBMECs revealed the organism to be crescent-shaped (Figure 3.12 A). The surface of the tachyzoite was finely granular and slender ridges radiated from the anterior end to the posterior end. The nucleus was an elevated round structure in the mid portion. The posterior end of the organism was smoothly curved (Figure 3.12 B). Ultrastructural analysis by SEM of the initial stages of entry showed the tachyzoites in close contact with the HBMECs at 6 h PI (Figure 3.12 C). The anterior end of the tachyzoites embedded in the host cell could be observed at 24 h PI (Figure 3.12 D). SEM of HBMECs infected with tachyzoites also showed that the tachyzoites exited from the host cell when the host cell could no longer support the growth of tachyzoites at 24 and 48 h PI (Figure 3.12E,F).

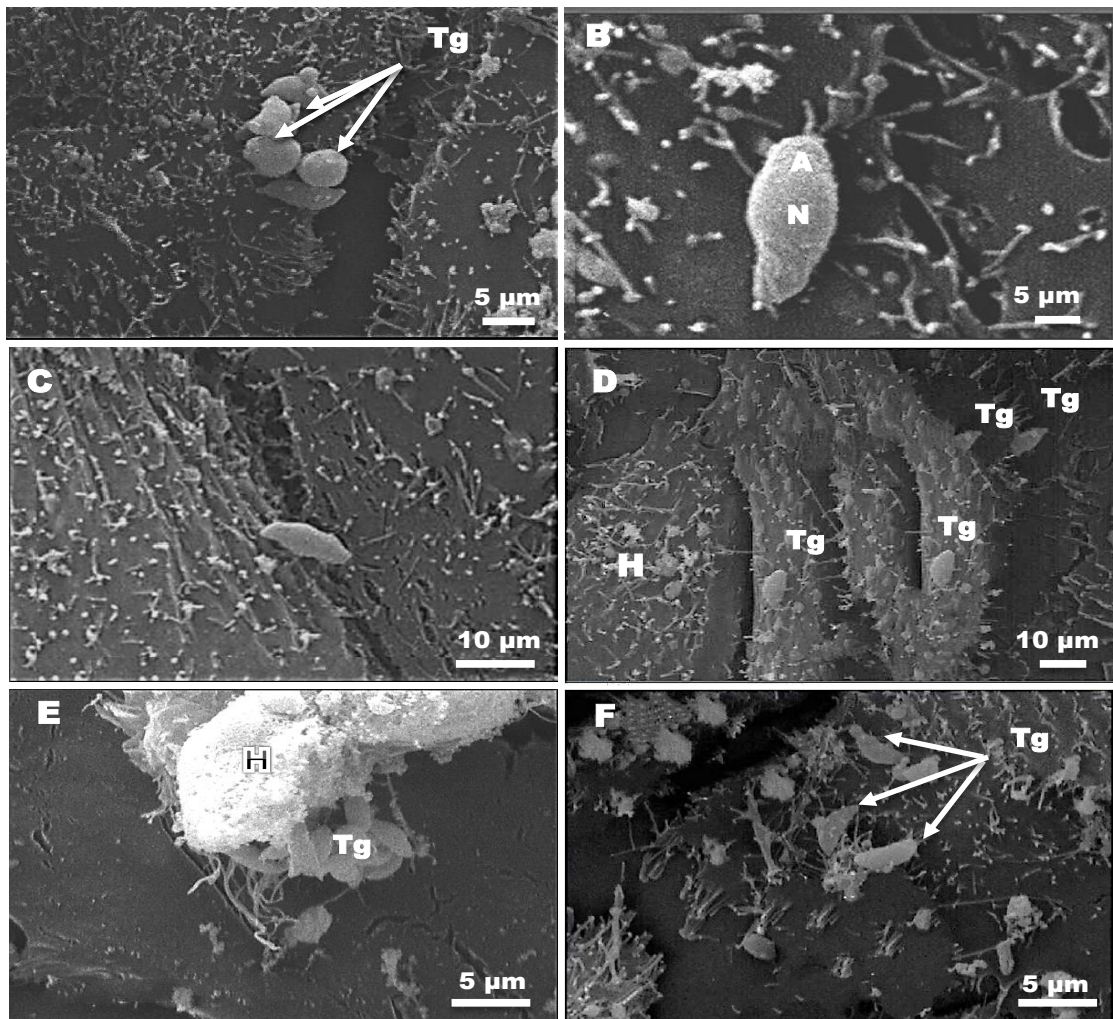


Figure 3.12: SEMs of HBMECs infected with tachyzoites. SEMs show the tachyzoites in close contact with the HBMECs at 6 h PI (A, B, and C). The anterior ends of tachyzoites were embedded in the host cell at 24 h PI (D). The rupture and exit of tachyzoites from the host cell was observed at 24 and 48 h PI (E,F).

3.3.5 TEM imaging of HBMECs infected with *T. gondii*

TEM of infected HBMECs also showed that tachyzoite proliferation disrupted the tight junctions (TJs) in infected cells at 24 and 48 h PI (Figure 3.13C,D) compared to 6 h PI (Figure 3.13B) and in non-infected cells (Figure 3.13A).

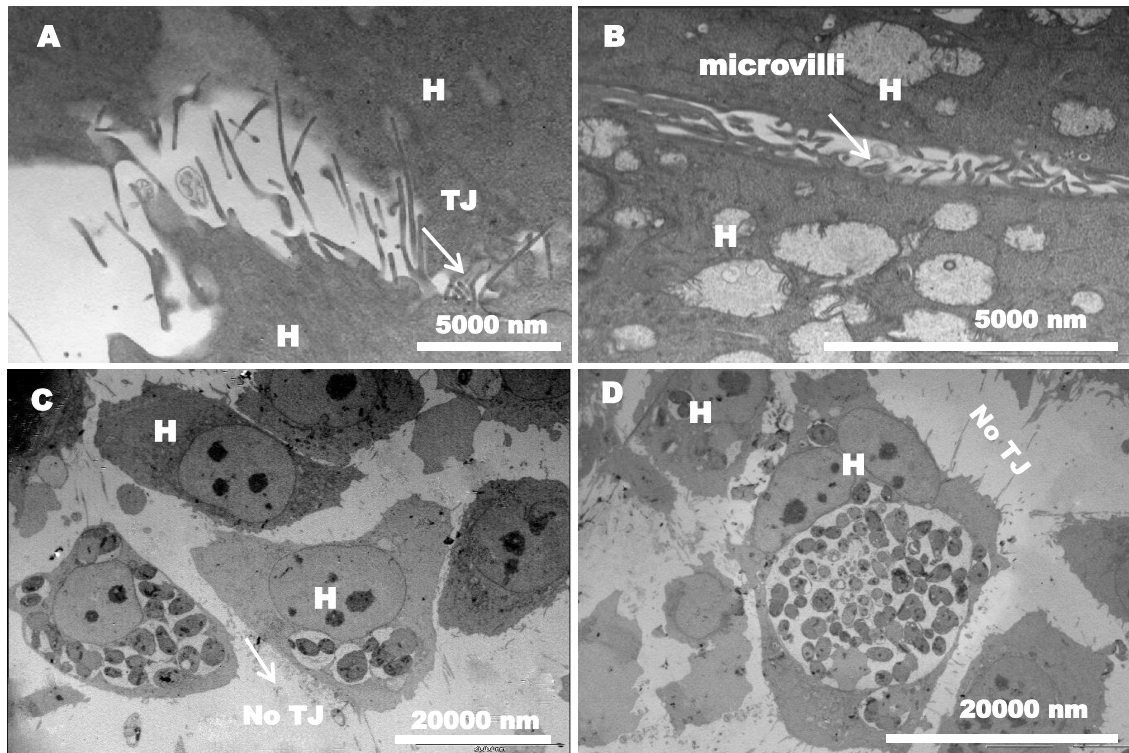


Figure 3.13: Transmission electron micrographs (TEMs) of HBMECs infected and uninfected with tachyzoites. A confluent monolayer of host cells (H) shows microvilli at the cell surface and in the region of intercellular contact (TJ) at 6 h PI (B). A large gap is evident between infected cells at 24 and 48 h PI (C,D) compared to 6 h PI (B) and non-infected ones (A).

TEM of infected HBMECs also showed tachyzoites in the endodyogeny process of cell division (Figure 3.14 A-D).

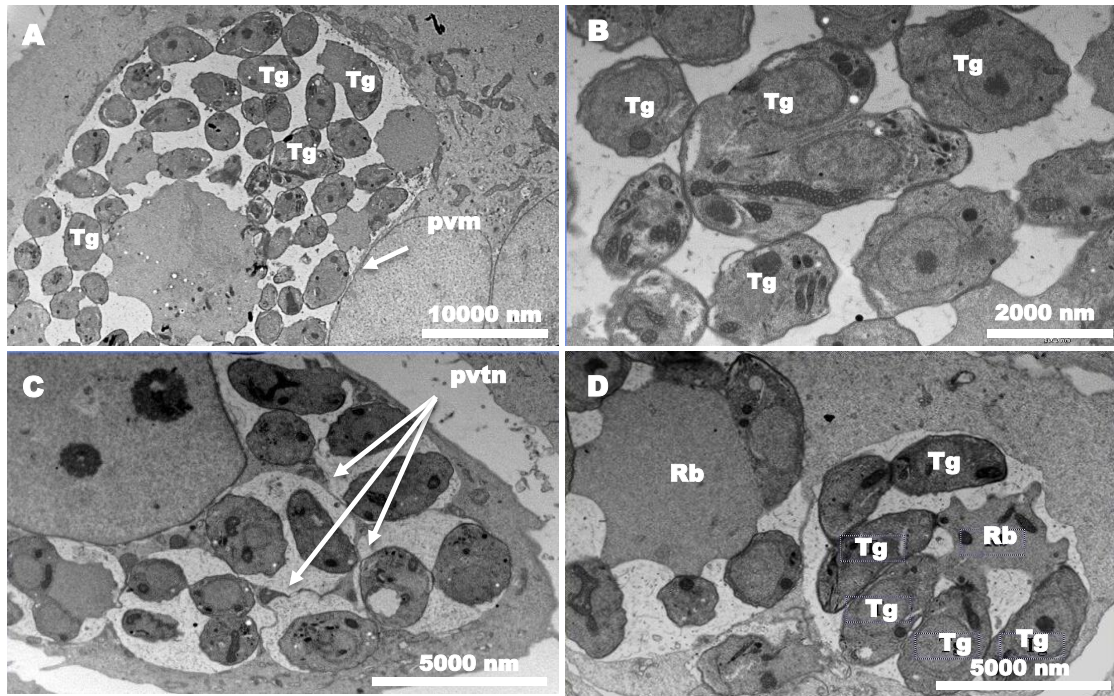


Figure 3.14: TEMs of a tachyzoite undergoing endodyogeny with HBMECs at 24 h PI. TEMs show tachyzoites occupying a parasitophorous vacuole and the parasitophorous vacuole membrane (PVM) is evident (arrow) (A). Two tachyzoites (Tg) in the process of endodyogeny (B). There were more vacuoles containing several tachyzoites within the parasitophorous vacuole tubular network (PVTN) (C). Several tachyzoites in the final stages of endodyogeny, which are still attached by their posterior ends to a common residual body (Rb) (D).

TEM of infected HBMECs showed the presence of host cell mitochondria around the PVM (Figure 3.15 A,B). There were elements of the ER of the host cell around the PVM; the membrane face, which contained ribosomes, was turned toward the cytoplasm and the smooth face was in close contact with the PVM (Figure 3.15 C,D). The presence of the Golgi apparatus around the PV was also noted (Figure 3.15 E,F).

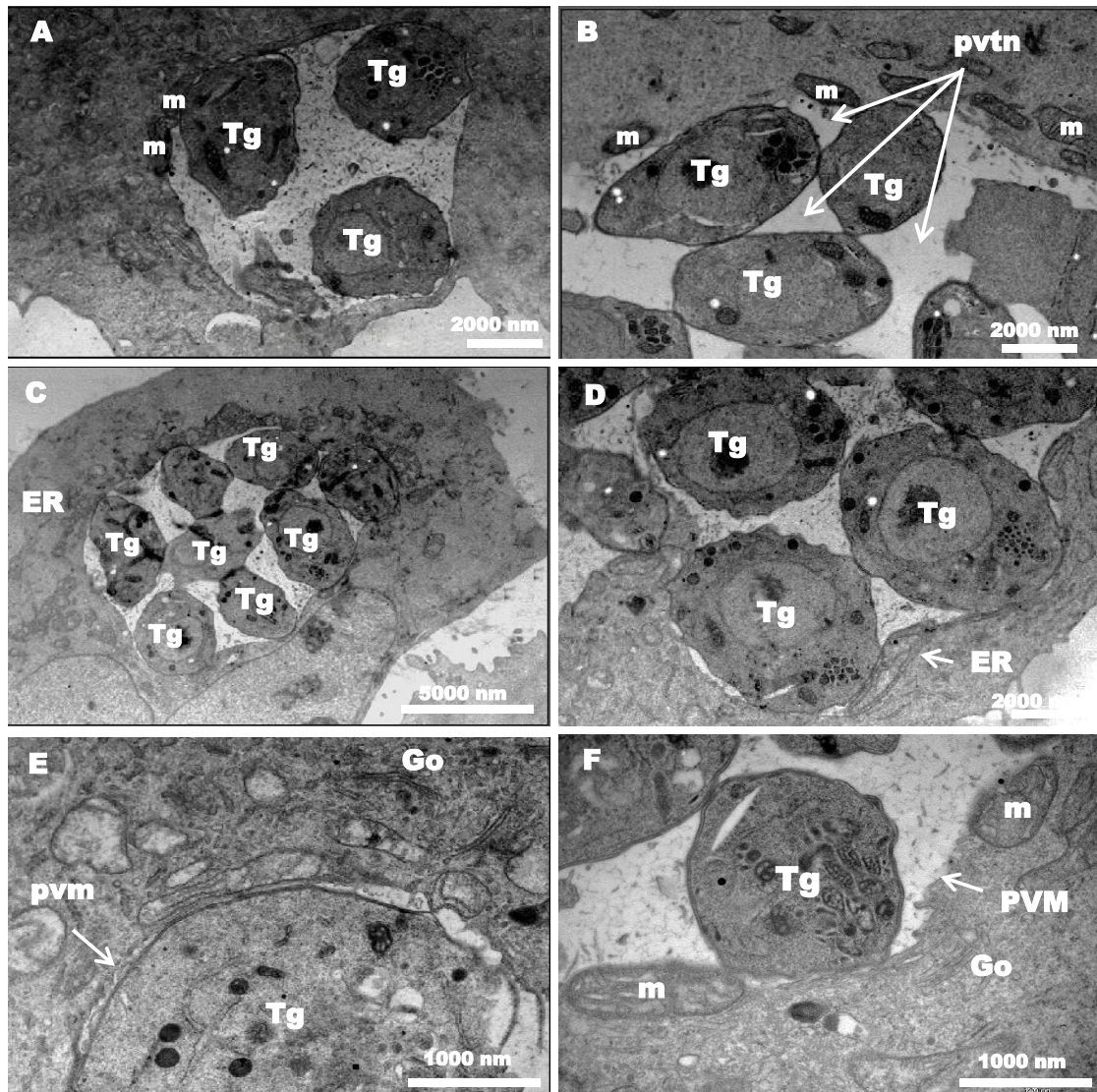


Figure 3.15: TEMs of HBMECs infected with *T. gondii* tachyzoites at 24 h PI. TEMs show mitochondria (m) that are shorter and tend to concentrate around the pvm, which contains tachyzoites embedded within the PVTN (A,B). There were elements of the ER, especially of the smooth reticulum around the PVM (C,D). The presence of the Golgi apparatus (Go) concentrated around the PV, which contains tachyzoites embedded within the PVTN, was also noted (E,F).

TEM of infected HBMECs showed the PV was no longer evident and the tachyzoites were free in the host cell cytoplasm at 48 h PI (Figure 3.16 A,B).

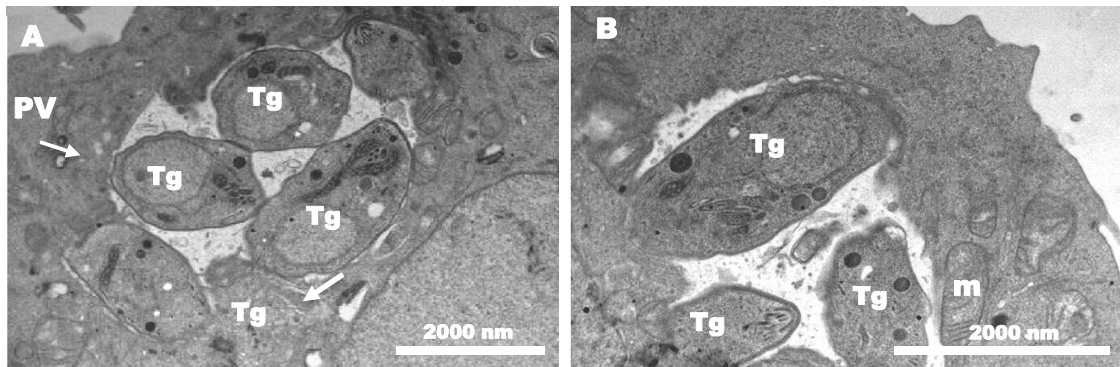


Figure 3.16: TEMs of HBMECs infected with *T. gondii* tachyzoites at 48 h PI. TEMs show several tachyzoites, one of which can be seen escaping from the PV (arrow) (A,B).

3.4 Discussion

T. gondii RH tachyzoites showed the ability to invade and proliferate intracellularly in HBMECs, thereby disrupting cell viability and integrity during the course of infection. AO staining of *T. gondii* tachyzoites infecting HBMECs showed a marked increase in the surface area of tachyzoites during infection. These findings indicate that the HBMECs can support the growth and proliferation of *T. gondii*. The tachyzoites exhibited a common pattern of growth and replication, which has also been observed in macrophages (Sharma et al., 1986), in human monocytic cells (Marin et al. 1996), in human astrocytoma derived cells (Pelloux et al., 1996), and in human retinal cells infected with *T. gondii* (Naemat et al., 2015). As an intracellular parasite, *T. gondii* invades and proliferates within mammalian cells (Tenter et al., 2000) and upon entry, tachyzoites form their own compartments, known as the PV. Within the PV, the parasite proliferates with a generation time of 24 h to form rosettes, eventually leading to cell rupture and exit of the tachyzoites.

A marked decrease was noted in viability of infected cells at 48 h PI, compared to uninfected controls, by alamar blue assay, indicating that growth of the parasites caused a metabolic burden on the host cells.

A decrease was also noted in the electrical resistance of infected cells compared to uninfected controls, indicating that *T. gondii* infection decreased the integrity of the HBMEC monolayer by increasing the permeability of the endothelial monolayer. This finding agrees with previous studies conducted in HBMECs infected with *Trypanosoma brucei* (Sumpio et al., 2014) and in HBMECs infected with *Plasmodium falciparum* (Tripathi et al., 2007), where TEER showed a decrease in infected cells during the course of infection, as compared with controls.

To highlight the morphological changes associated with infection, SEM and TEM were employed to observe the disruption of tight junctions of HBMECs and reorganization of host organelles around the PV. Interestingly, there was a clear

disruption of tight junctions of HBMECs was noted in infected cells at 24 and 48 h PI compared to the 6 h PI and non-infected cells by SEM and TEM. This effect was associated with a reduction in the TEER of the infected cells compared to the uninfected controls during infection. TEER assay showed only a modest change in the tight junctions by *T. gondii* infection as compared with that by SEM and TEM experiment, which showed clearly tight junction damage, because allowed the cells to grow for at least 5 days (in the TEER assay) led to formation of confluent monolayer with reasonably sound tight junctions between the cells. However, allowed the cells to grow for only one day (in the SEM and TEM experiment) might not be enough for the tight junctions to be formed between the cells. This finding agrees with previous study conducted in human retinal pigment epithelial cells infected with *T. gondii* (Nogueira et al., 2015), where EM showed a damage the tight junctions in infected cells during the course of infection, as compared with the controls.

The alteration in the tight junctions permeability may be directly induced by the tachyzoite during the acute phase or indirectly by reactivation of a tissue cyst, as the immune response to infection with *T. gondii* involves the production of cytokines and chemokines, which may contribute to increase paracellular permeability. Disruption of tight junctions in the region of intercellular contact has also been associated with inflammatory mediators and the generation of ROS induced by pathogens (Mayhan 2001; Rao 2008; Hamada et al., 2013). It has been found that a high level of ROS production activates MMPs, such as, MMP-1, -2, and -9 (Haorah et al., 2007), which can alter the tight junction proteins (occludin, claudin and ZO-1, -2 and -3) through phosphorylation of their tyrosine residues (Bauer et al., 2010; Feng et al., 2011).

Our observations using SEM showed the anterior end of tachyzoites embedded in the host cell and tachyzoites exiting from the host cell, suggesting that the host

cells could no longer support the growth of the tachyzoites. This finding agrees with a previous study, where tachyzoites were reported to embed themselves in VERO cells by their anterior end and ruptured the host cell when it could no longer support the growth of tachyzoites (Jones et al., 1972).

TEM revealed that the host mitochondria are shorter and tend to concentrate around the PV throughout the growth of the tachyzoites. This finding is in agreement with previous studies (Tanabe 1985; De Melo et al., 1992), in which the fluorescence microscopy of mouse fibroblast cells and TEM of VERO cells infected by the *T. gondii* showed that the host mitochondria are shorter and tend to concentrate around the PVM, where the parasites grow and multiply. The tendency of the host mitochondria to concentrate around the PVs might provide adenosine triphosphate (ATP) to the parasites, required for sustaining its replication (Gupta et al., 2005). At present, there exists no explanation for this alteration in the dimensions of the mitochondria.

HBMECs infected with *T. gondii* tachyzoites also showed that elements of the host ER concentrated around, and even established physical contact with, the membrane lining the PV. This finding agrees with De Melo et al. (1992), in which the TEM of VERO cells infected by *T. gondii* showed elements of the ER concentrated around the PV, where the parasites grow and multiply. The tendency of portions of the ER to fuse with the membrane lining the vacuole might provide lipid precursors to the PVM, such as choline and serine to ensure appropriate synthesis of phosphatidylcholine and phosphatidylserine required for growth during the development of intracellular parasitism (Gupta et al., 2005). Quantitative measurements of the phospholipid profile of *T. gondii* reveal that phosphatidylcholine and phosphatidylserine accounts for about 75% and 6% of the total phospholipids respectively (Gupta et al., 2005).

Analysis of the organization of host organelles during the interaction of *T. gondii* with HBMECs also showed that the host Golgi apparatus was concentrated around the PV and remained stable throughout the growth of the tachyzoites. This finding is in agreement with a previous study (Romano et al., 2013), in which the TEM of VERO cells infected by *T. gondii* showed the host cell Golgi apparatus concentrated around the PV. The tendency of the host Golgi apparatus to concentrate around the PVs might provide lipid precursors to the parasite, such as ethanolamine to ensure appropriate synthesis of the phosphatidylethanolamine that is required for its replication (Gupta et al., 2005). Quantitative measurement of the phospholipid profile of *T. gondii* reveals that phosphatidylethanolamine accounts for about 10% of the total phospholipids (Gupta et al., 2005). The tight association between the PVM and host organelles was noted immediately after invasion and remained constant throughout the growth of the PV (Magno et al., 2005; Sinai et al., 1997), suggesting the involvement of macromolecules integrated in the PVM for parasite survival, proliferation, membrane biogenesis, and energy requirement. The mechanism used by *T. gondii* to reorganize the host organelles is unknown. However, it is known that the invasion of a cell by tachyzoites is an active and highly organized process mediated by sequential protein secretion from micronemes, rhoptries, and dense granular organelles. The PVM proteins may be involved in nutrient siphoning and recruitment of host organelles around the PV (Laliberté & Carruthers, 2008)

Upon entry, *T. gondii* tachyzoites are surrounded by PVM, which is derived from the host cell membrane during invasion. Tachyzoites gain entry to the host cell by stimulating and hijacking phagocytosis, whereby a portion of the cell membrane envelops the invader and pinch off inside the cell, forming a characteristic lipid-rich structure termed as PV. Shortly after infection, the PVM quickly becomes physically associated with sites of host cell lipid and energy biosynthesis, the mitochondria, ER, and Golgi apparatus (Sinai et al., 1997; Sinai and Joiner, 2001). Microtubules,

the microtubule organizing center, and intermediate filaments are involved in the localization of the PV close to the nucleus and the movement of host organelles around the PV (Halonen and Weidner 1994) (Figure 3.17). The mechanism used by *T. gondii* to siphon nutrient and recruitment of host cell organelles around the PV is unknown. However, it is known that tachyzoites release endosomal/lysosomal vesicles in the PV, and host cell microtubules and the tachyzoite protein, dense granule protein 7 (GRA7) have been implicated in this process (Laliberté & Carruthers, 2008). This pathway could be a source of sterols and other lipids as sources of fatty acids. In addition, rhoptry protein 1 (Rop1), 4 (Rop4), 5 (Rop5), 7 (Rop7), and 18 (Rop18) are involved in the reorganization of the host organelles and remodeling of the cytoskeleton around the PV. Rhoptry protein 2 (Rop2) is involved in the recruitment of host mitochondria to the PVM. Rhoptry protein 14 (Rop14), is involved in the composition of the PVM pore, and Rop16 is involved in the arrest of host cell apoptosis by directing ser15/37 phosphorylation of tumor protein 53 (Chang et al., 2015).

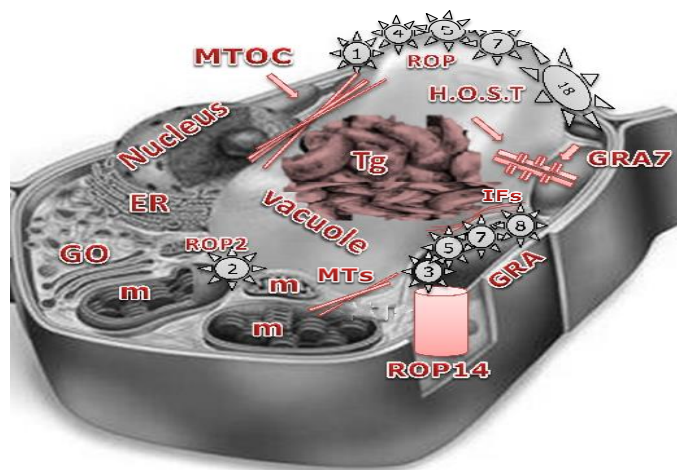


Figure 3.17: Schematic representation of host organelle reorganization and remodeling of the cytoskeleton around the PV. Reorganization of the host mitochondria (m), endoplasmic reticulum (ER), Golgi apparatus (Go), microtubules (MTs), microtubule organizing center (MTOC) and intermediate filaments (IFs) at the (PV). MTs, MTOC and IFs are involved in the localization of the PV close to the nucleus and the movement of host organelles around the PV. MTs and dense granule protein 7 (GRA7) are involved in the composition of the host organelle-sequestering tubulo-structures (H.O.S.T) in the PV. ROP2 is involved in the recruitment of mitochondria at the PVM. ROP14 is involved in the composition of the PVM pore. Dense granule protein 3 (GRA3), 5 (GRA5), 7 (GRA7), and 8 (GRA8) are associated with PV extension.

Our findings indicate that the invasion, growth, and replication of *T. gondii* tachyzoites within HBMECs leads to disruption of cell viability and integrity, involving reorganization of the host cell organelles around the PV to support the parasite growth, replication, and infection. The tight association between the PVM and host organelles may provide lipids and other macromolecules to the intracellular parasite. These results are novel in HBMECs during the course of the parasite intracellular infection cycle. These findings suggest the importance of assessing the molecular metabolic changes in the parasite-infected cells to obtain more insight into the dynamics of host–parasite interactions for the control of this protozoan.

4. Metabolic profiling of HBMECs infected with *T. gondii* in vitro

4.1 Introduction

T. gondii cannot proliferate without a host cell as all of the biomolecules required for its replication are taken from the host cell. As a result, many protozoan parasites have developed complex mechanisms that allow them to exploit the host cellular processes to use them for their own proliferation (Gupta et al., 2005). When a protozoan parasite infects a host cell, it drastically changes the cellular metabolism in an effort to maximise the production of the biomolecules necessary for the production of new infectious protozoans (Gupta et al., 2005). As a result, a protozoan infection can cause many changes in metabolic flux and drastically change the levels of a host cell's biomolecules. If the key aspects of the protozoan parasite's control over host cell metabolism could be identified, therapies that interfere with this control program could be used as antiparasitic agents. Some drug developers have begun to incorporate these biological systems approaches to target selection and have encountered success, as in the case of tryptophan restriction in the HBMECs to prevent parasite growth (Däubener et al., 2001).

Fewer metabolomic studies have aimed to develop biomarkers of protozoan infections. Analysis of the patterns of the brain metabolome in mice infected with *T. gondii*, by applying ^1H NMR, revealed a significant decrease in choline, serine, lipoic acid, cholesterol and fatty acids, which indicates the use of host lipid fractions for increased membrane maintenance or parasite lipid anabolism. Furthermore, the parasite does not produce its own lipids but instead scavenges from the free fatty acid pools of the host cell, which contributes to a decrease in lipid fractions (Gupta et al., 2005; Michael et al., 2006; Isabelle et al., 2000).

Applying high-resolution ^1H NMR spectroscopy and working in mouse models of *Plasmodium berghei* infection revealed a considerable effect on plasma and urine profiles. *Plasmodium berghei* caused a significant decrease in plasma glucose levels

and an increase in lactate and pyruvate levels due to anaerobic glycolysis by the parasite, as well as a decrease in glycerophosphorylcholine, which acts as a choline source for the parasite. Urinalysis from infected mice revealed evidence of changes in the intestinal flora and excretion of pipecolic acid as well as two unknown metabolites missing from the urine of uninfected mice (Li et al., 2008).

Application of high-resolution ^{13}C -or ^{31}P -NMR spectroscopy in a mouse model of infection with the ANKA strain of malaria revealed a notable reduction in brain metabolic activity along with a change in d-glucose-1- ^{13}C utilisation from the mitochondrial respiration cycle to the glutamate/glutamine cycle and the γ -amino butyric acid shunt. The rise in lactate and alanine levels, followed by an increase in the utilisation of the glutamate/glutamine cycle, indicated that elevated glutamatergic metabolism is not the result of brain hypoxia but due to other metabolic disturbances. However, mice infected with the K173 strain of malaria showed an increased (~ 2 -fold) cerebral metabolic rate, with increased glucose utilisation. Energy metabolism as measured by ^{31}P -NMR revealed drastic increases in inorganic phosphate and phosphocreatine content consistent with increased anaerobic metabolism and cerebral hypoxia in mice with cerebral malaria (Rae et al., 2004).

Using magic angle spinning ^1H NMR/ ^{31}P NMR spectroscopy in a mouse model of *Plasmodium berghei* infection again revealed a considerable effect on neurochemical profiles. An estimate of brain metabolite levels revealed increases in brain glutamine and alanine levels concomitant with increased blood ammonia levels, as well as a drop in the levels of brain myo-inositol, which are assumed to compensate for the increased intracellular osmolarity due to glutamine accumulation. Furthermore, this study showed a drop in choline-derived compound levels, significant for membrane and myelin homeostasis (Penet et al., 2007). These metabolic changes occurred along with hepatic encephalopathy and were

accompanied by drastically increased blood bilirubin levels, important shifts in liver enzyme function, and an overall rise in hepatic tissue pathology in infected mice (Penet et al., 2007).

Analysis of the urine metabolome in a mouse model of infection with *African trypanosome* by applying ^1H NMR revealed a significant increase in 3-carboxy-2-methyl -3-oxopropanamine as a unique marker of infection. Plasma analysis from the infected mice revealed drastic increases in lactate, oxaloacetate and glucose levels, as well as reductions in glutamine and lysine levels with infection (Wang et al., 2008).

Thus far, molecular metabolic biomarker studies of protozoan infections have achieved much in the identification of infected animals and exploring how parasites siphon off energy and metabolites and how the host can shift its metabolism to deal with the infection. With the application of metabolomics for measuring complex mixtures of cellular biomolecules, metabolomic studies of host-parasite interactions are critical for an improved understanding and control of protozoan parasites. Thus, defining metabolic changes in an affected target tissue may facilitate delineating the pathogenic mechanisms involved. In the present study, we investigated the hypothesis that pathophysiological effects of *T. gondii* infection on HBMECs involve changes in the metabolic status of target cells during the course of infection. Primary cultures of HBMECs were used as a cellular model to identify the molecular metabolic changes in these cells in response to infection by using nuclear magnetic resonance (^1H NMR).

4.2 Materials and methods

4.2.1 Metabolic profiling

HBMECs were seeded in T-75cm² flasks at 2×10^6 cells/flask and grown in cRPMI medium as described in section 2.2. Once a confluent monolayer of the cells was formed (~24 h), the tachyzoites were added in a host:parasite ratio of 1:2 as described in section 2.4. At 6, 24, and 48 h post-infection (PI), the infected and uninfected cells were harvested by using a cell scraper, followed by washing three times with cold PBS. The cells (infected and uninfected) and tachyzoite pellets were extracted with 2 ml of cold methanol/water (80:20) (Geißler 2011). The cells and tachyzoites were subjected to snap-freezing in liquid nitrogen and defrosted on dry ice in 3 cycles. The cells and tachyzoites were pelleted by centrifugation at 20,400 xg for 5 mins at -9°C. The supernatant was transferred to a clean tube and lyophilized. Prior to NMR analysis, the extract was reconstituted in 550 µl of deuterated water [D₂O, 99.9%; Sigma-Aldrich] containing 0.01% of sodium 3-(trimethylsilyl) propionate-2, 2, 3, 3-d₄ [TSP, Sigma-Aldrich] as internal standards. The total volume (550 µl) of solution was then transferred into 5 mm outer diameter NMR tubes for subsequent analyses on a Bruker Avance 600 NMR spectrometer (Bruker; Rheinstetten, Germany) (Figure 4.1). This method was performed in six replicates, and the data represent from at least three independent experiments to identify any alterations in the molecular metabolism of infected HBMECs in comparison with those in uninfected control cells.

All ¹H NMR spectra from the cells (infected and uninfected) and purified tachyzoites extracts were obtained from Bruker Avance spectrometer (operating at 600 megahertz (MHz)) equipped with 5 mm Broadband/¹H probe (Bruker SMART probe). 32K data points were acquired over 12 ppm using 512 scans.

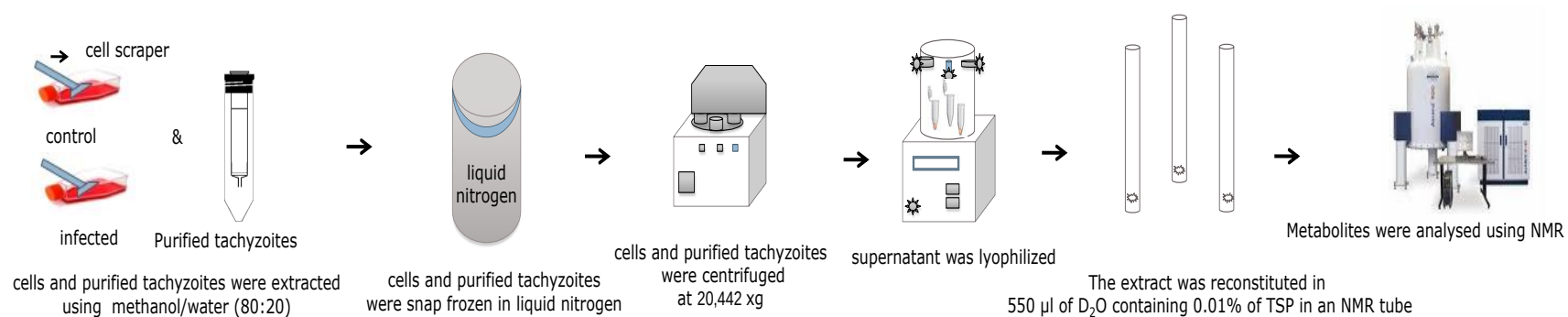


Figure 4.1: Schematic view of sample preparation for metabolic using ^1H NMR. The infected and non-infected cells were harvested with a cell scraper, followed by washing three times with cold PBS. The cell and tachyzoite pellets were extracted with a cold methanol: water solution (80:20). The cells and tachyzoites were then snap frozen in liquid nitrogen, centrifuged, and the supernatant was lyophilized by freeze-dryer and resuspended in 550 µl of D₂O containing 0.01% of TSP. The samples were then put in NMR tubes and analysed using NMR.

4.2.1.1 ^1H NMR analysis

In ^1H NMR, all the nuclei are electrically charged, and any that have a spin create a low magnetic field. As soon as an exterior magnetic field is applied, energy transmission is from a low to a high level of the nuclei. The energy transmission occurs in the radio frequency range. As soon as the spin comes back to its low-level state, energy is emitted at the same frequency. The signal that corresponds to this is calculated in various pathways and processed to provide an ^1H NMR spectrum for the nucleus concerned (Figure 4.2).

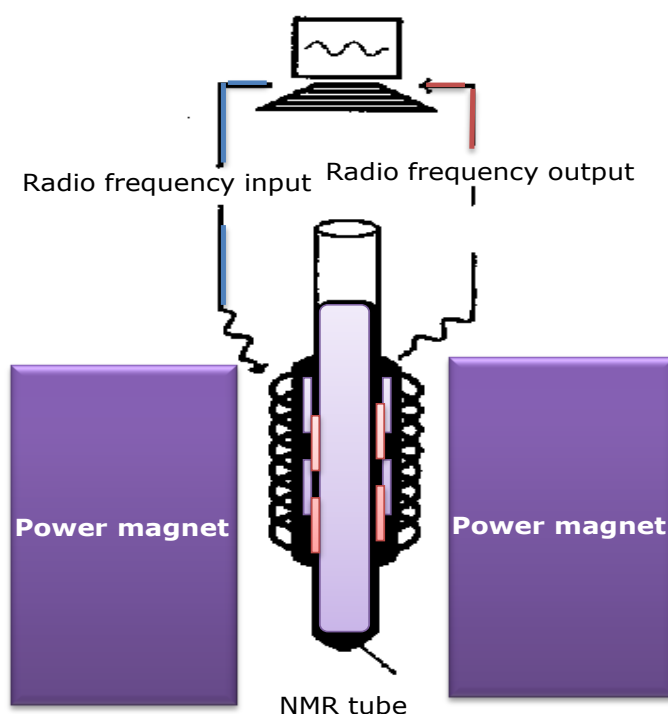


Figure 4.2: Schematic illustration of an ^1H NMR spectrometer. Radiofrequency pulses are transmitted to the NMR tube via transmitter coil (blue). The energy that is absorbed by the sample induces a radiofrequency alternating current in the receiver coil (red) which is feedback to the computer that, in turn, transforms those signals into spectra translate by humans. Modified from Geißler, (2011).

The specific resonance frequency of the energy transmission is based upon the magnetic field at the nucleus, and this field is affected due to shielding by the electrons orbiting the nucleus. As a result, the nuclei in the various chemical environments absorb energy at slightly varying resonance frequencies. This influence is called the chemical shift. This also means that the sample states, such as the pH and ion strength, will influence the spectrum revealed.

The chemical shift for ^1H NMR is recognised as a variation in the fractional units, measured in parts per million (ppm), between the resonance frequency of a specific proton and that of the reference compound. The measured chemical shift of most protons is usually in the range 0-15 ppm. A particular proton usually gives rise to more than one ^1H NMR signal due to the effect of the non-equivalent neighboring protons, an impact known as the spin-spin coupling pattern. The signal density relies on the number of matching nuclei and thus is naturally quantitative (Claridge, 1999).

Other structural information acquired by ^1H NMR spectroscopy involving spin-spin coupling and J-coupling constants (J), which are important parameters in peak assignment of spectra. The characteristic multiplicities of the peaks, following the number of neighbour+1 rule, for instance a proton with no neighbouring protons ($n=0$) will be shown as a single peak in the spectra, a proton with two neighbouring protons will be shown as a triplet peak in the spectra and a proton with five neighbouring protons will be shown as a sixplet peak in the spectra (Figure 4.3). Nuclear Overhauser Enhancement (NOE) is often applied to measure intramolecular distances, and the two features regarding relaxation properties characterising the reversal of the nuclei to equilibrium are longitudinal (T_1) and transversal (T_2) relaxation. For more detailed information of the physics and chemistry that forms the basis of ^1H NMR spectroscopy, several preliminary reference books are available (Friebolin, 1998; Claridge, 1999).

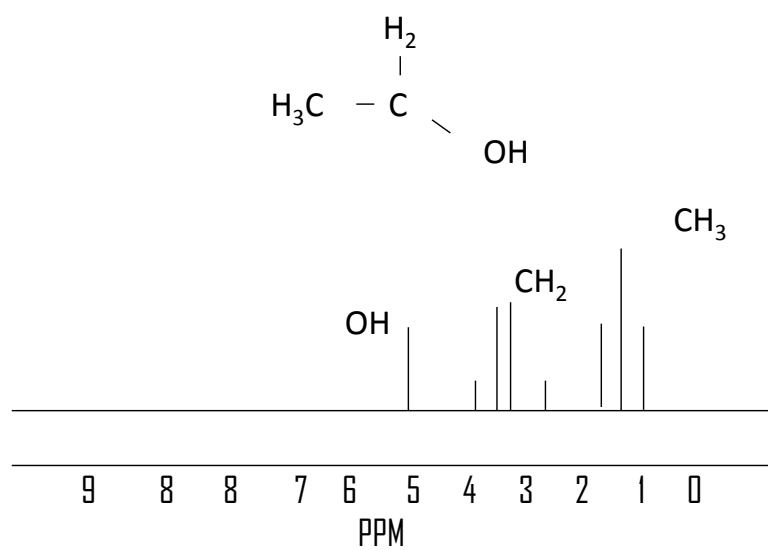


Figure 4.3: Peak multiplicity of ethanol based on the $n+1$ rule for the impact neighbouring protons. Adapted from Jasmina et al. (2010). Abbreviations: PPM = parts per million (ppm).

4.2.1.2 Spectra processing and multivariate analysis

Raw spectra were carefully phase- and baseline-corrected and automatic calibrating to the TSP reference peak at δ 0.00 in Topspin (version 3.1, Bruker). The region containing residual water resonance (4.7 ppm) was removed to avoid any interference with the analysis. The spectra were normalized over the total remaining spectral area using MATLAB (R2008a, Mathworks, Inc., 2008) to improve data quality and remove unwanted biases.

Multivariate statistical analysis, including principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal partial least squares-discriminant analysis (OPLSD-DA) were performed using SIMCA-P 11.0 software (Umetrics, Umea, Sweden). PCA is used to reduce the complexity of the data and display data variation in a lower-dimensionality (Pearson, 1901). The data is created from the original data set is a covariance matrix that is composed of scores matrix and loadings matrix. Scores matrix result in the scores plot that shows the position of each observation, whereas loadings matrix result in the loadings plot that indicates how each spectral variable influenced the position of the scores. The parameter calculated for PCA model is R^2X , since R^2X is only dependent on the X data. The transition of spectral data into PCA is schematically illustrated in Figure 4.4. PLS-DA is a classification tools used to find the difference between two groups. It is applied to find a linear relationship between the X-matrix and the dummy Y-matrix. PLS-DA utilizes prior infomatiom of class membership and hence optimizes separation (Trygg, 2002). The quality of the models was described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, whereas Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability (Figure 4.5). OPLSD-DA is a further developpment of PLS-DA used for optimal recovery of biomarkers and removes systematic variation unrelated to infection status via an orthogonal filter. It was built by X-data containing all spectral information and

dummy Y-data determining class affiliation (Trygg, 2002). To identify the individual metabolites responsible for the differentiation in the score plots between the infected and non-infected cells, color map co-efficient plots were applied. The upward-oriented peaks indicated increased levels of metabolites in the infected cells, while the downward peaks indicated decreased levels of metabolites in the infected cells, with red-colored metabolites contributing more significantly than the blue colored ones (Figure 4.6).

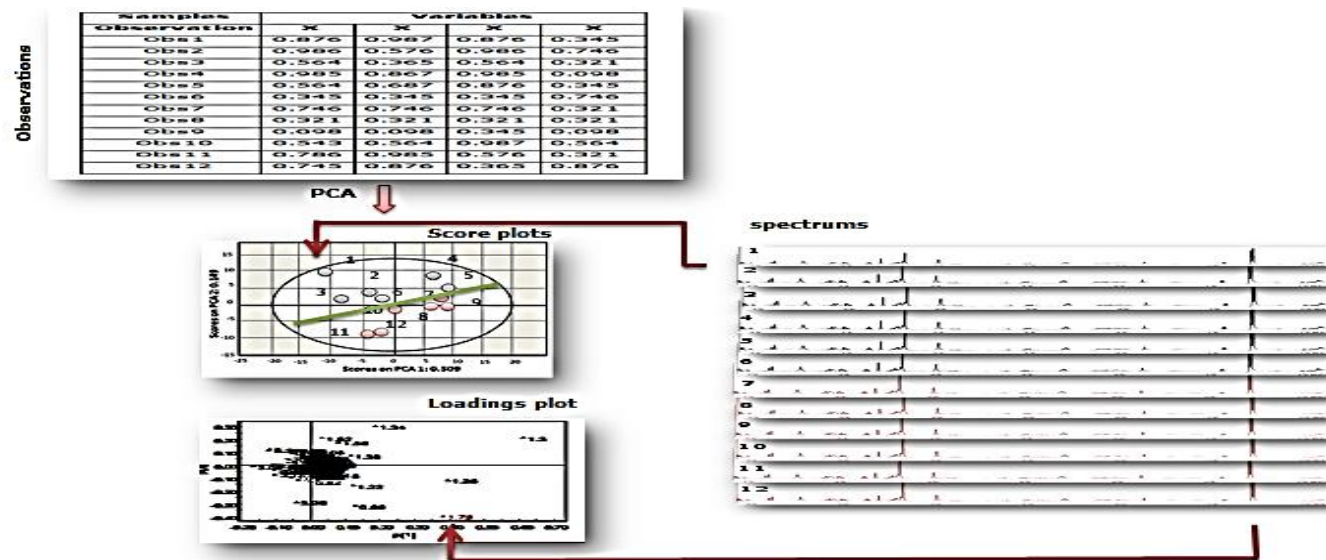


Figure 4.4: Schematic view of PCA. The Original data set X is modeled by PCA: this includes the scores matrix (representing the observations) and the loadings matrix (representing the spectral variables). A number of observations (Obs) listed in rows, whereas variables listed in columns. PCA creates scores and loadings plots visualizing observation (scores) separation and how the variable (loadings) contribute to the score localization.

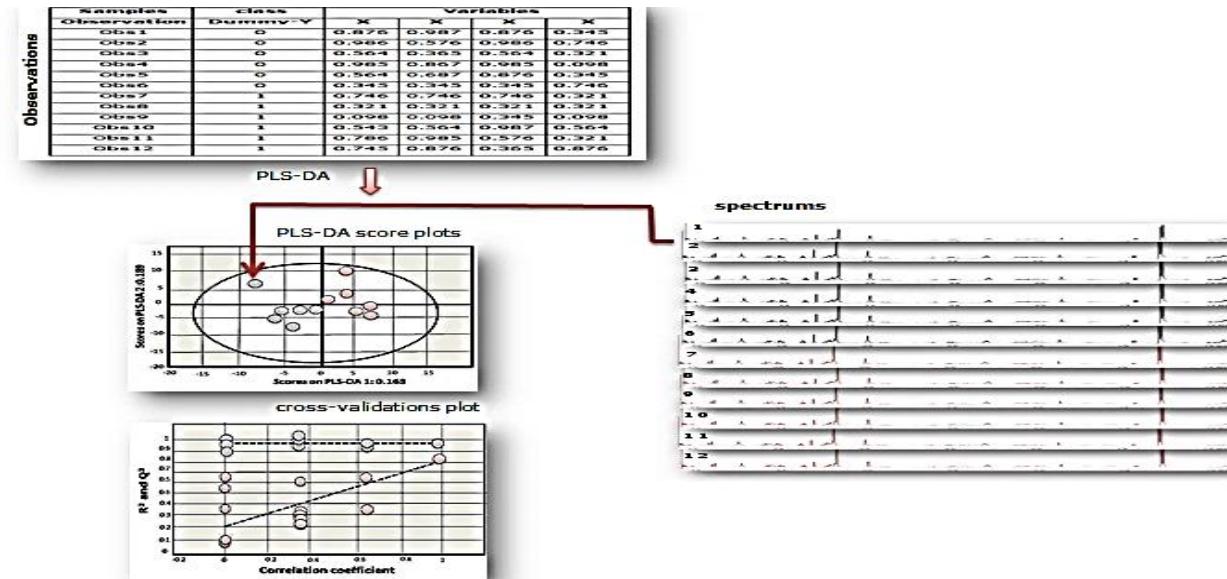


Figure 4.5: Schematic view of PLS-DA. The Original data set X is modeled by PLS-DA {here, Y data [infected and uninfected control (class)] is indicated. It would be used by PLS-DA (class). A number of observations (Obs) listed in rows, whereas variables listed in columns. PLS-DA creates scores plot and cross-validations plot visualizing observation (scores) separation and how the variable (cross-validations) identify the individual metabolites responsible for the differentiation in the score plots between the infected and non-infected cells.

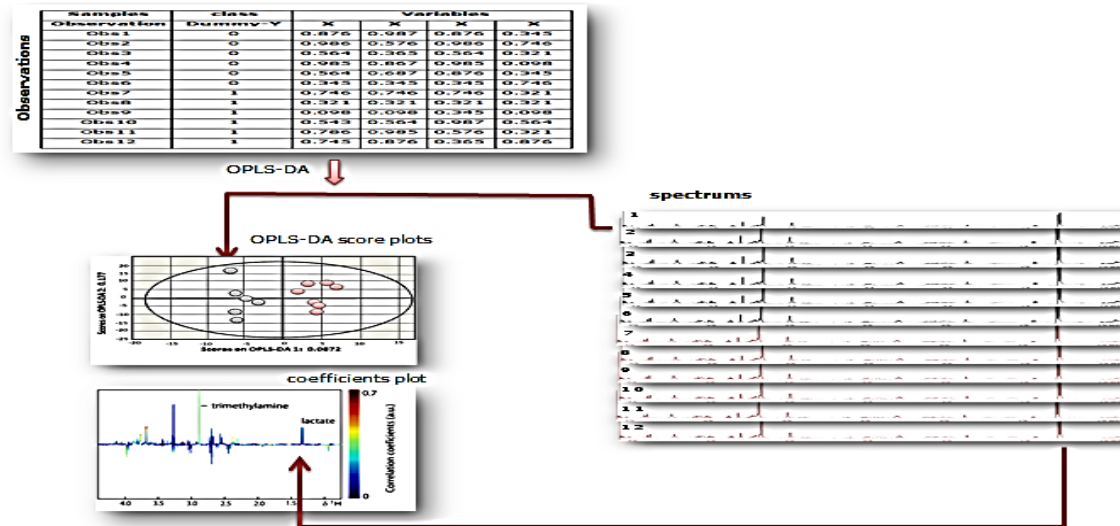


Figure 4.6: Schematic view of OPLS-DA. The Original data set X is modeled by OPLS-DA {here, Y data [infected and uninfected control (class)] is indicated. It would be used by O-PLS-DA (classes). A number of observations (Obs) listed in rows, whereas variables listed in columns. OPLS-DA creates scores and coefficients plots visualizing observation (scores) separation and how the variable (coefficients) identify the individual metabolites responsible for the differentiation in the score plots between the infected and non-infected cells.

4.3 Results

^1H NMR spectra of infected and non-infected HBMECs at different incubation times are shown in Figures 4.7-4.12. More than 25 different metabolites were identified and quantified based on their chemical shifts and signal multiplicity, including isoleucine, leucine, valine, lactate, alanine, acetate, glutamate, glutamine, aspartate, creatine, choline, phosphocholine, glycerophosphocholine, scyllo-inositol, taurine, and myo-inositol (Table 4.1). Visual inspection of the spectra hinted at alterations in the levels of several metabolites as a result of infection, including lactate, choline, and inositol. Lactate seemed elevated at different incubation times (6, 24, and 48 h) in infected compared to non-infected cells. Whereas, reduction of choline was noted in infected compared to non-infected cells. In order to quantify these apparent metabolite changes with infection, we systematically employed PCA, PLS-DA, and OPLS-DA.

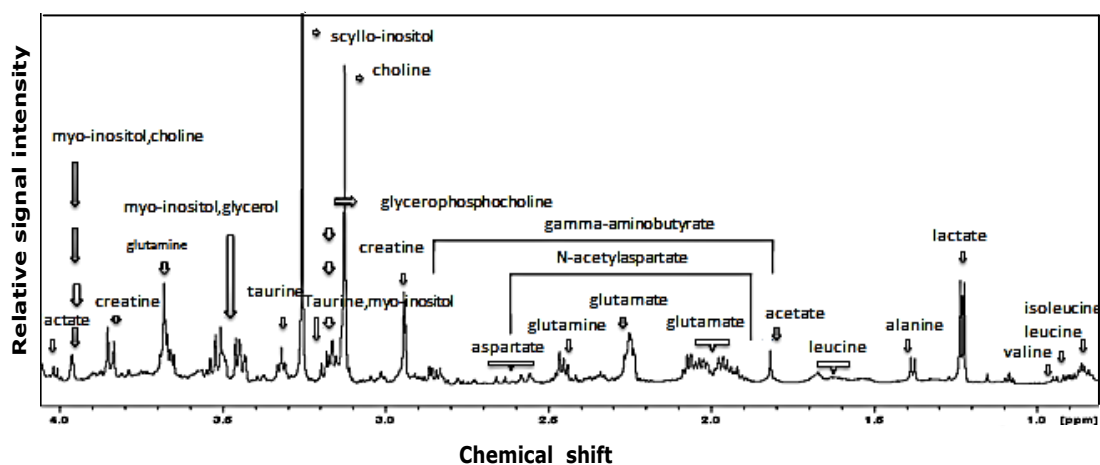


Figure 4.7: ^1H NMR spectra of HBMECs at 6 h incubation time.

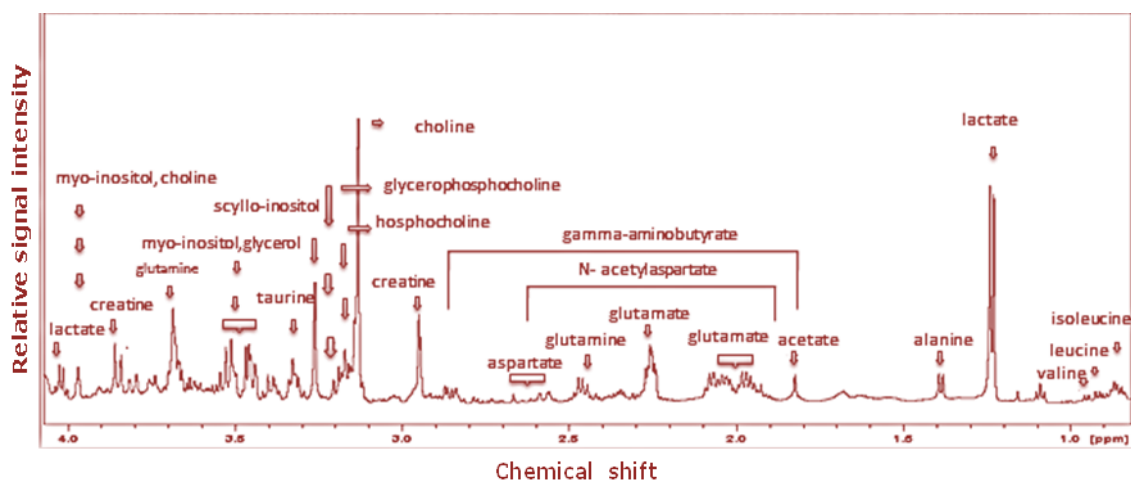


Figure 4.8: ^1H NMR spectra of HBMECs infected with *T. gondii* at 6 h PI.

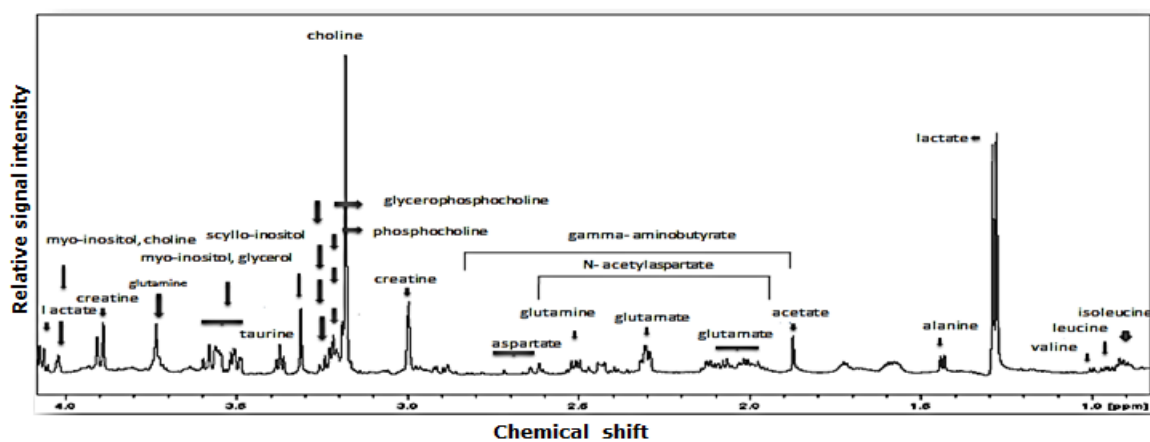


Figure 4.9: ^1H NMR spectra of HBMECs at 24 h incubation time.

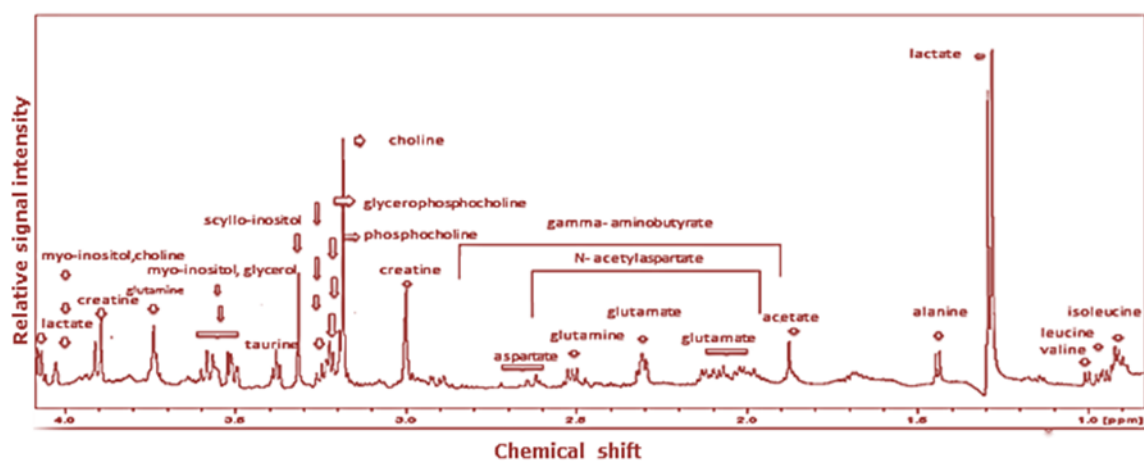


Figure 4.10: ^1H NMR spectra of HBMECs infected with *T. gondii* at 24 h PI.

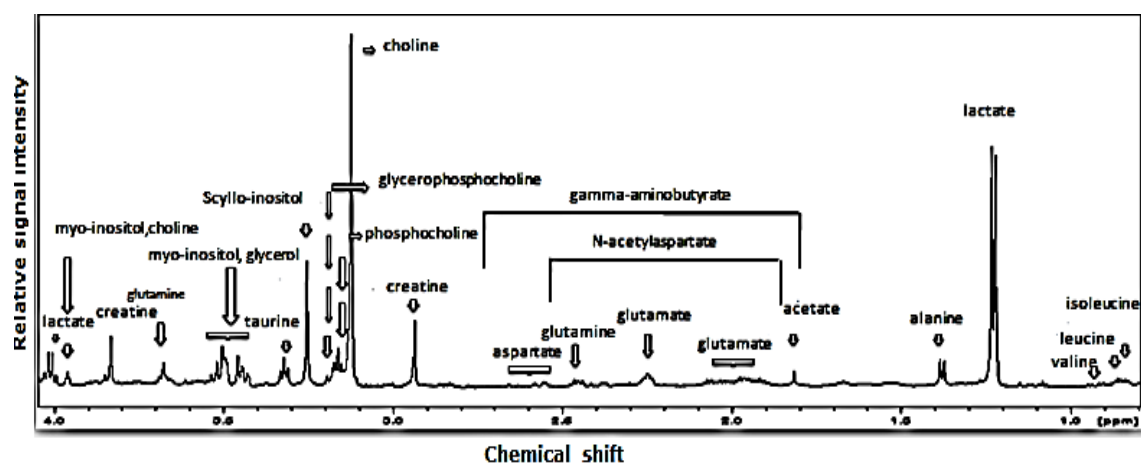


Figure 4.11: ^1H NMR spectra of HBMECs at 48 h incubation time.

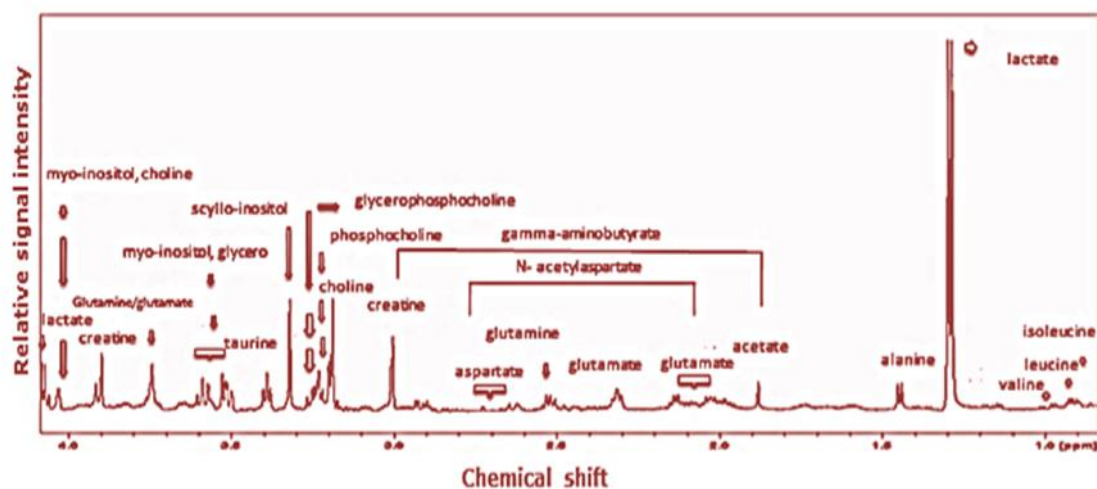


Figure 4.12: ^1H NMR spectra of HBMECs infected with *T. gondii* at 48 h PI.

Table 4.1: Metabolites found in the spectra of infected and non-infected cells

Metabolite	Chemical moiety	Signal multiplicity	Chemical shift in D ₂ O [ppm]	Main functions
acetate	CH ₃	s	1.9250	- fatty acid and steroid biosynthesis - precursor of neurotransmitter acetylcholine
alanine	CH CH ₃	qa d	3.7653 1.4839	amino acid: - protein synthesis - amino acid/ nitrogen transport across organs
aspartate	CH CH ₂ CH ₂	dd dd dd	3.9033 2.6860 2.8165	amino acid: - excitatory neurotransmitter - generation of glutamate - cellular respiration cycle
choline	(CH ₃) ₃ CH ₂ CH ₂ CH ₂ CH ₂ N	s m m m m -	3.2071 4.0627 4.0627 3.5248 3.5248 -199.9	- methylation reactions - precursor of acetylcholine
Creatine	CH ₂ CH ₃	s s	3.9336 3.0396	nitrogenous organic acid: energy metabolism
glutamate	CH ₂ CH ₂ CH CH ₂ CH ₂	m m t m m	2.1361 2.0635 3.7624 2.3494 2.3662	- excitatory neurotransmitter - cellular respiration cycle
glutamine	CH ₂ CH ₂ CH CH ₂ CH ₂	m m t m m	2.1358 2.1554 3.7972 2.4444 2.4714	- cellular respiration cycle - storage form of glutamate - regulating glutamate activity - detoxification - amino acid/nitrogen transport across organs
glycerophosphocholine	CH ₂ CH ₂ (CH ₃) ₃ CH ₂ CH ₂ CH CH ₂ CH ₂ CH ₂ CH ₂	m m s m m m m m m m	3.6792 3.3622 3.2335 3.3444 3.9225 3.6809 4.3327 3.9101 3.6832 3.6096	- cell membrane constituent - osmoregulator
Continued on the next page				

Metabolite	Chemical moiety	Signal multiplicity	Chemical shift in D ₂ O [ppm]	Main functions
isoleucine	CH CH CH ₂ CH ₂ CH ₃ CH ₃	m d m m d t	1.9692 3.6549 1.4589 1.2492 1.0152 0.9430	amino acid: ✚ protein synthesis ✚ propionic fatty acid precursor
lactate	CH CH ₃	m d m m d t	1.9692 3.6549 1.4589 1.2492 1.0152 0.9430	end-product of anaerobic glycolysis: ✚ neuronal energy generation ✚ macrophage activation ✚ nitric oxide production ✚ precursor of glutamate
leucine	CH CH ₂ CH ₂ CH CH ₃ CH ₃	qa d	4.1140 1.3313	amino acid: ✚ protein synthesis
myo -inositol	CH CH CH CH CH CH	m m m t t t	1.6977 1.6740 1.7279 3.7204 0.9591 0.9708	a cyclic sugar alcohol: ✚ Osmoregulator ✚ storage form of glucose ✚ central secondary messenger system ✚ hormonal stimulation ✚ precursor of membrane constituents ✚ glial marker
N-acetylaspartate	CH CH ₂ CH ₂ CH ₃	dd dd dd s	4.3969 2.6991 2.5062 2.0223	amino acid: ✚ Precursor of acetate ✚ Precursor of aspartate ✚ Precursor of N-acetylaspartate ✚ glutamate ion balance ✚ osmosis in neurons ✚ energy metabolism ✚ neuronal marker
phosphocholine	CH ₂ (CH ₃) ₃	m s	3.5743 3.2264	an intermediate in the synthesis of phosphatidylcholine: ✚ cell membrane constituent
scyllo -inositol	CH	s	3.3510	a cyclic sugar alcohol: ✚ modulates protein folding
Continued on the next page				

Metabolite	Chemical moiety	Signal multiplicity	Chemical shift in D ₂ O [ppm]	Main functions
taurine	CH ₂ CH ₂	t t	3.2689 3.4312	an organic acid: ✚ blockage of N-methyl-D-aspartate receptor ✚ free readical scavenger ✚ calcium homoeostasis ✚ astrocytic osmoregulator ✚ immune system activity ✚ inhinitor neuromodulator
valine	CH CH CH ₃ CH ₃	m d d d	2.2620 3.5930 1.0464 0.9952	amio acid: ✚ protein synthesis ✚ propionic fatty acid precursor
gamma-amino butyrate	CH ₂ CH ₂ CH ₂	qi t t	1.9077 2.3025 3.0164	amio acid: ✚ inhibitory neurotrans mitter

Key: s singlet, d doublet, dd double doublet, t triplet, qa quartet, qi quintet, m multiplet.

4.3.1 Multivariate analysis of the ^1H NMR spectra of HBMECs

PCA was initially applied to the extract spectra of infected and non-infected cells at different incubation times. HBMECs were discriminated onto PCA score plots (2 principal components (2 PCs); $R^2 X = 0.422, 0.458, \text{ and } 0.413$ for 6, 24, and 48 h PI, respectively), where spectra of infected cells were separated from those of non-infected cells along PC1 (Figure 4.13). The PCA score plots of infected and non-infected cells showed minor separations at 6 and 24 h and clear visual separation at 48 h, indicating a difference between the metabolic profiles of the infected and non-infected cells being evident at 48 h compared to 6 and 24 h post-infection.

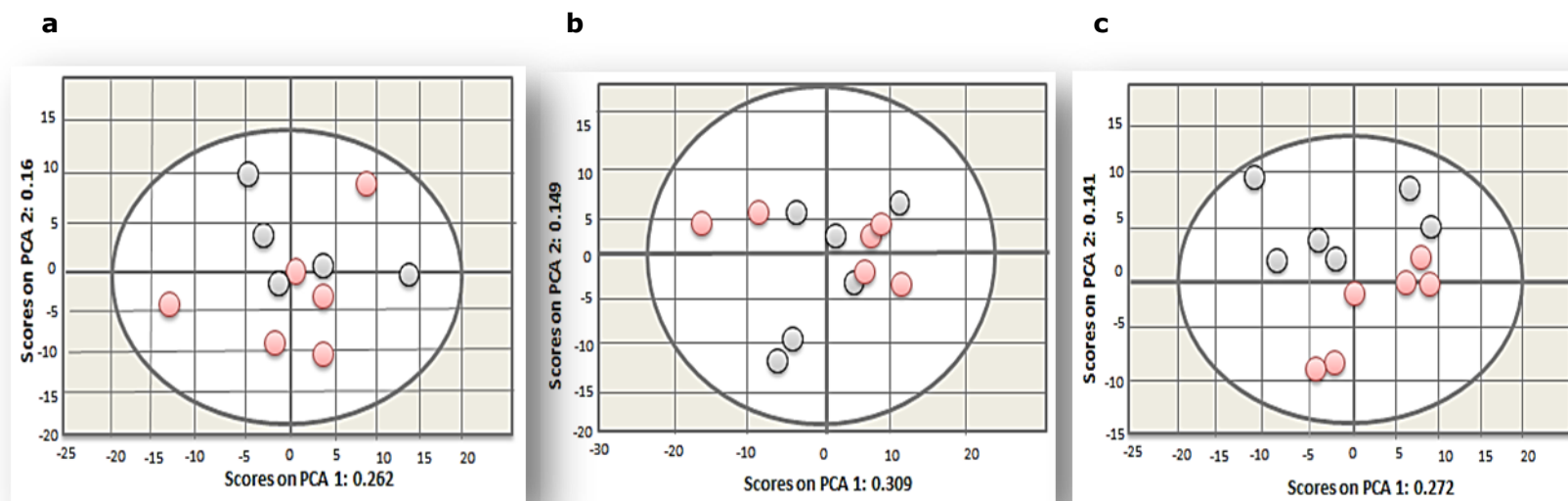


Figure 4.13: PCA score plots of extracts of infected and non-infected HBMECs at 6 h (a), 24 h (b), and 48 h (c) PI. Infected HBMECs were slightly separated from non-infected cells along PC1 ($R^2 X = 0.262, 0.309$, and 0.272 for 6, 24, and 48 h PI, respectively). Each point in the plot represents one replicate ^1H NMR spectra. Gray circle = control, red circle = infected. The center and margin of the ellipse indicate mean and standard deviation, respectively.

PLS-DA was then applied to the extract of the spectra of infected and non-infected cells at different incubation times. HBMECs were discriminated by the PLS-DA score plots (2PCs; $R^2 X = 0.352, 0.244$, and 0.385 for 6, 24, and 48 h PI, respectively), where the spectra of infected cells were separated from those of the non-infected cells along PC1 (Figure 4.14 PLS-DA score plots). Of these, the non-infected cells were clustered in the left section of the PLS-DA model, while the infected cells were clustered in the right section of the PLS-DA model.

On the basis of the PLS-DA model, the *T. gondii* infected and non-infected cells, at different incubation times, were discriminated with $R^2 Y$ and Q^2 (Figure 4.14 cross-validation plot). The PLS-DA validation plot of infected and non-infected cells at 6 ($R^2 Y = 1.662$, $Q^2 = -0.0536$) and 24 h PI ($R^2 Y = 1.782$, $Q^2 = -0.31$) were poor. Therefore, these groups were not explored further. However, the PLS-DA validation plot of infected and non-infected cells at 48 h PI ($R^2 Y = 1.768$, $Q^2 = 0.928$) was strong, indicating a statistically significant biochemical distinction between the metabolic profiles of infected and non-infected cells.

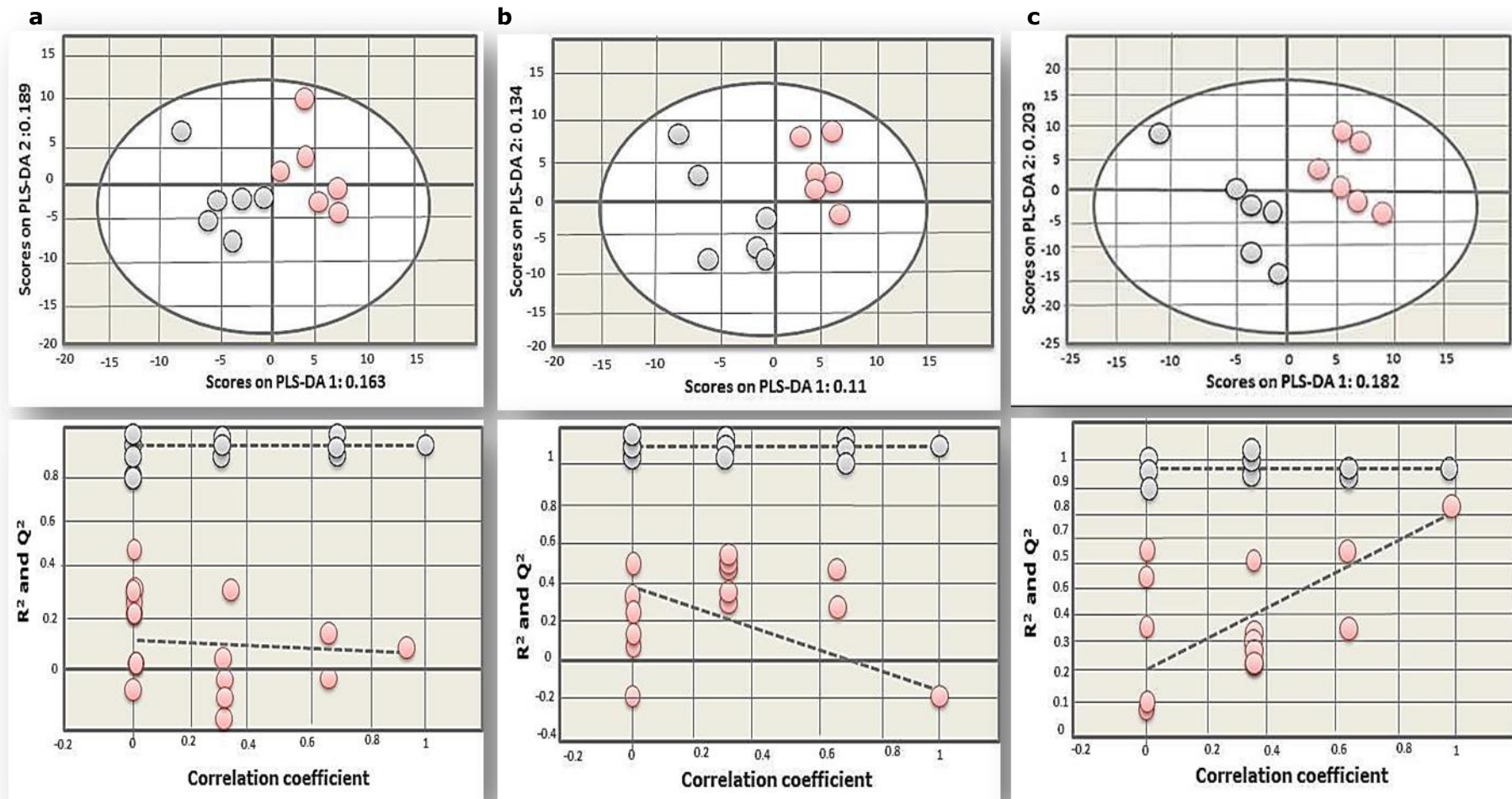


Figure 4.14: PLS-DA score plots (top) and cross-validations plot (bottom) of extracts of infected and non-infected HBMECs at 6h (a), 24 h (b), and 48 h (c) PI. Infected HBMECs were separated from non-infected cells along PLS-DA1 ($R^2X= 0.163, 0.11,$ and 0.182 for 6, 24, and 48 h PI, respectively). The center and margin of the ellipse indicate mean and standard deviation, respectively. R^2 is the explained variance, while Q^2 is the predictive ability of the model. Gray circle = control, red circle = infected.

The metabolic profiles of infected and non-infected HBMECs were overlaid on a OPLS-DA model to focus on discriminatory variation using the ^1H NMR data as the X matrix and group information as the Y matrix. HBMECs were discriminated onto OPLS-DA score plots (2 PCs; $R^2_x = 0.264$ for 48 h PI), where the spectra of infected cells were separated from those of the non-infected cells along PC1 (Figure 4.15a). Of this, the non-infected cells were clustered in the left section of the OPLS-DA model, while the infected cells were clustered in the right section of the OPLS-DA model. The color map coefficient plots were then applied to identify the individual metabolites responsible for the differentiation in the score plots, by using the OPLS-DA model. These plots revealed significant variations between the metabolite levels in the infected and non-infected cells (Figure 4.15b). Lactate, alanine, acetate, glutamine, and creatine seemed elevated at 48 h PI in infected compared with non-infected cells. Whereas, reduction of glutamate, aspartate, choline and myo-inositol, glycerol was noted in the infected compared to the non-infected cells.

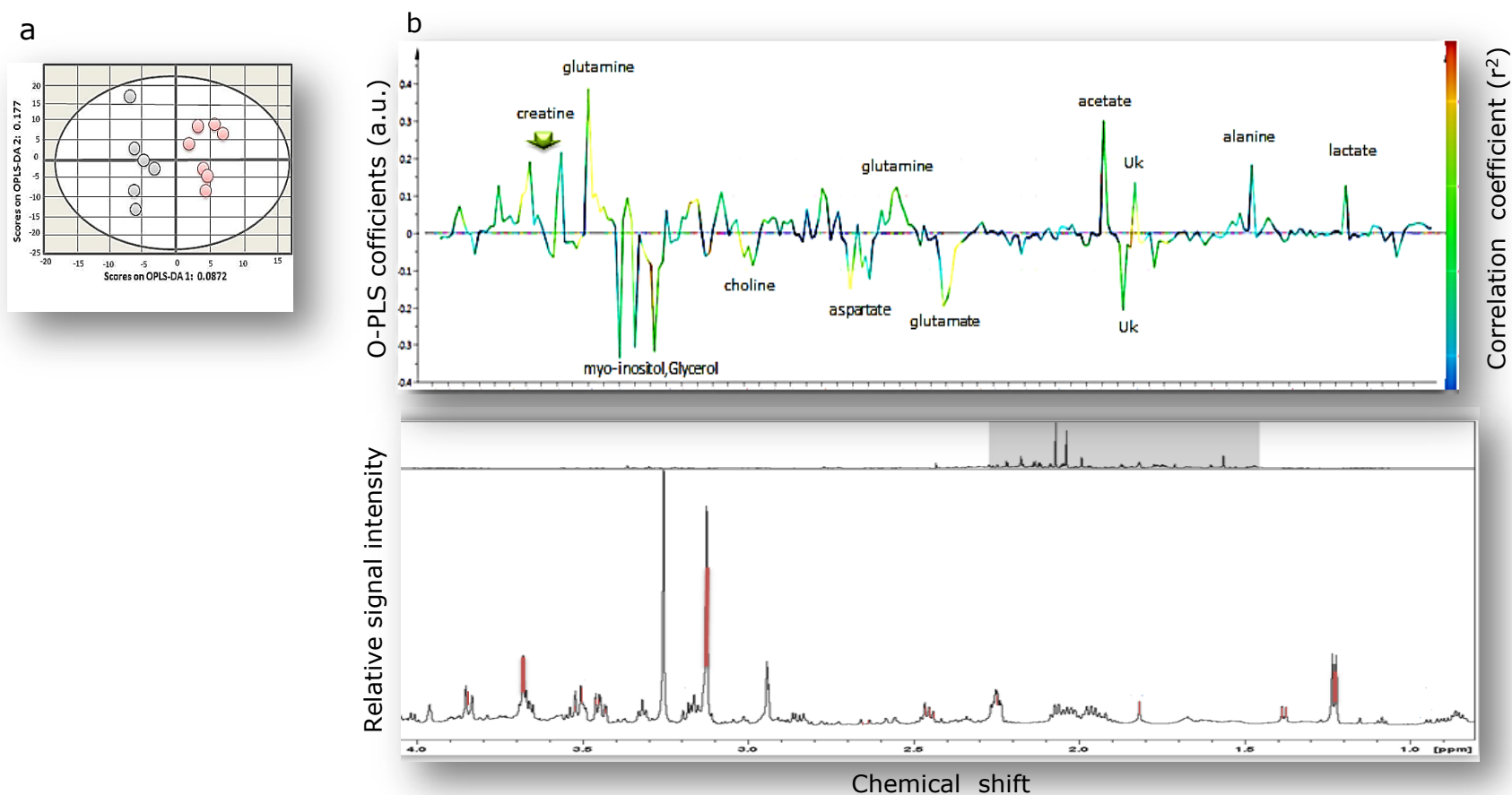


Figure 4.15: OPLS-DA score plots (a) of extracts of infected and non-infected HBMECs at 48 h PI. Infected HBMECs were separated from non-infected ones along OPLS-DA 1 ($R^2X = 0.0872$). The center and margin of the ellipse indicate mean and standard deviation, respectively. Gray circle = control and red circle = infected. Colour map coefficients plot of infected HBMECs and non-infected control cells at 48 h PI (b). Peaks oriented upward indicate increased levels of metabolites in the infected cells, while the downward oriented peaks indicate a decrease in metabolite levels in infected cells, with red-colored metabolites showing greater changes than blue-colored. The color scale indicates the magnitude of contribution to the class difference, and the peak height is expressed in arbitrary units (a.u.) relative to the total peak contribution. Uk: unknown

4.3.2. ^1H NMR spectra of purified parasite preparations

^1H NMR spectrum of purified parasites is shown in Figure 4.16. Seven metabolites were identified and quantified based on their chemical shifts and signal multiplicity, including lactate, acetate, glutamate, glycerophosphocholine, scyllo-inositol, taurine and myo-inositol, glycerol.

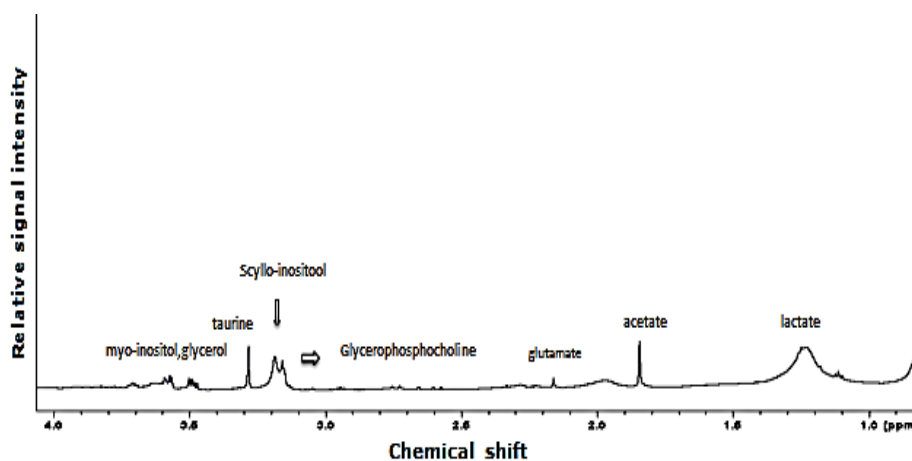


Figure 4.16: ^1H NMR spectrum of purified parasites.

4.4. Discussion

In this study, the metabolic profile of *T. gondii*-infected and non-infected cells was identified using ^1H NMR combined with multivariate analysis. The PCA score plots of infected and non-infected cells showed minor separations at 6 and 24 h PI and clear visual separation at 48 h PI, indicating a difference between the metabolic profiles of the infected and non-infected cells being evident at 48 h PI compared to 6 and 24 h PI. The PLS-DA score plots combined with the validation plots of infected and non-infected cells at 48 h PI was strong, indicating a statistically significant biochemical distinction between the metabolic profiles of infected and non-infected cells. The OPLS-DA score plots combined with the color map coefficient plots revealed significant variations between the metabolite levels in the infected and non-infected cells. Lactate, alanine, acetate, glutamine, and creatine seemed elevated at 48 h PI in infected compared with non-infected cells. Whereas, reduction of glutamate, aspartate, choline and myo-inositol, glycerol was noted in the infected compared to the non-infected cells.

The levels of lactate and alanine were modulated in the HBMECs because of the development of tachyzoites. The elevation of lactate and alanine levels at 48 h PI might indicate a process of the host response to the infection. *T. gondii* was seen to rely on glucose as a carbon source to fuel its energy demands for growth (Blader & Koshy, 2014). An enhanced rate of glycolysis led to increased formation of pyruvate, which could be transaminated to lactate via anaerobic glycolysis and to alanine via aerobic glycolysis. This finding agrees with the results of a previous study conducted on erythrocytes infected with *Plasmodium berghei* (Lang-Unnasch & Murphy 1998; Mehta et al., 2005), where infection with the ANKA strain of malaria was associated with increased lactate and alanine in the infected cells. Along with the lactate and alanine levels, the acetate levels were also increased. This observation agrees with Lamour et al. (2014), in which the proportion of acetate has been higher in erythrocytes infected with the *P. berghei* ANKA strain.

The transporter used by *T. gondii* for glucose uptake is the GLUT1 transporter, where it is converted to lactate, alanine, and acetate via glycolysis (Figure 4.17) (Tielens et al., 2010). In addition, alanine can be synthesized from branched chain amino acids, such as, valine, leucine, and isoleucine. The accumulation of acetate in infected cells may be the result of the biosynthesis activity in *T. gondii* tachyzoites. As can be seen in Figure 4.16, acetate in tachyzoites is the most abundant. In tachyzoites, acetate is produced from acetyl-CoA by mitochondria or apicoplasts, both involving a substrate level of phosphorylation that is catalysed by either acetyl-CoA synthetase (ACS) or succinate-CoA transferase (SCT). The ACS reaction is directly coupled with ATP synthesis, whereas, the SCT reaction yields succinyl-CoA for ATP formation via succinyl-CoA synthetase (Tielens et al., 2010).

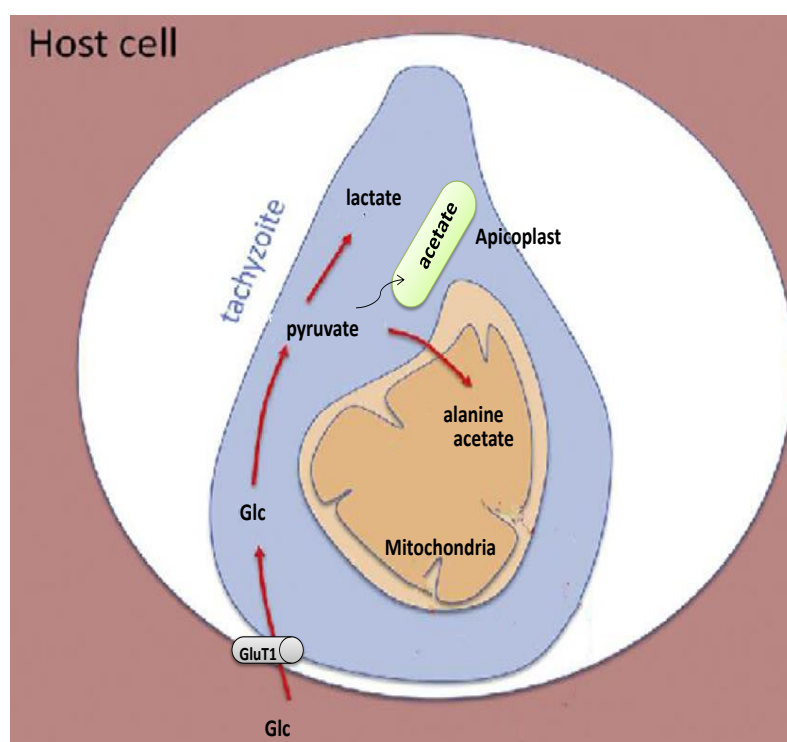


Figure 4.17: Glucose utilization by *T. gondii*. Glucose (Glc) is taken up from the host cell by the GLUT1 glucose transporter, where it is converted to lactate via anaerobic glycolysis and to alanine and acetate via aerobic glycolysis.

The levels of glutamate and glutamine were also modulated in HBMECs because of the development of tachyzoites. Elevation of the glutamine level and reduction of

the glutamate level at 48 h PI could indicate a process of the host response to infection. *T. gondii* was seen to rely on glutamine as a carbon source to fuel its energy demands during glucose starvation (Kim & Weiss 2004; Dubremetz & Lebrun 2012; Hunter & Sibley 2012). The transporter used by *T. gondii* for glutamine uptake is unknown. However, it is known that glutamine is converted to glutamate and utilized by the mitochondria cellular respiration cycle as either α -ketoglutarate (α -KG) or succinate via the γ -aminobutyric acid (GABA) shunt (Figure 4.18). This finding agrees with a previous studies conducted on mice infected with *T. gondii* (Blader & Koshy 2014), in mice infected with *Schistosoma mansoni* (Jasmina et al. 2010), and in primary human foreskin fibroblasts infected with *Vaccinia virus* (Fontaine et al. 2014), where infection with the *T. gondii*, *S. mansoni*, and *Vaccinia virus* are associated with increased glutamine in the infected cells.

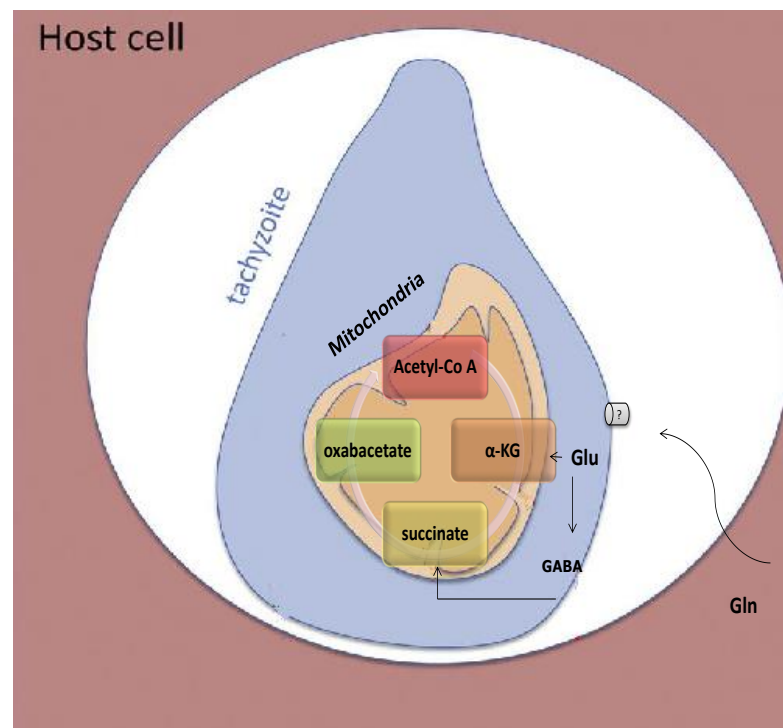


Figure 4.18: Glutamine utilization by *T. gondii*. Glutamine (Gln) is taken up from the host cell by an unknown transporter. It is then converted to glutamate (Glu) that can be utilized by the mitochondrial respiration cycle as either α -ketoglutarate (α -KG) or succinate via the γ -aminobutyric acid (GABA) shunt.

The level of aspartate was also decreased in HBMECs by the development of the tachyzoites. Reduced aspartate level may indicate a process of the host response to infection. The enzyme aspartate transaminase present in the aspartate metabolism pathway can convert glutamate and oxaloacetate to aspartate and 2-oxoglutarate, depending on the availability of amino acids and cellular respiration cycle intermediates.

The level of choline was also decreased in HBMECs by the development of the tachyzoites. The reduced choline level may be because of *T. gondii* importing it from the host (Gupta et al., 2005). Although *T. gondii* can synthesize certain phospholipids, it must obtain choline from the host cell to ensure appropriate synthesis of phosphatidylcholine, which is necessary for replication. Quantification of the phospholipid profile of *T. gondii* reveals that phosphatidylcholine is the most

abundant phospholipid, accounting for about 75% of the total phospholipids (Gupta et al., 2005). The transporter used by *T. gondii* for choline uptake is unknown. However, it is known that choline is converted to phosphocholine by choline kinase (CK). Phosphocholine is then converted to cytidine diphosphate-choline (CDP-choline) by cytidyltransferase (CCT). Following that, CDP-choline is converted to phosphatidylcholine by choline/ethanolamine phosphotransferase (C/EPT) (Figure 4.19) (Vance and Vance, 2004). This finding agrees with a previous studies conducted on human foreskin fibroblasts infected with *T. gondii* (Charron and Sibley 2002; Gupta et al., 2005), in mice infected with *Schistosoma mansoni* (Jasmina et al., 2010), and in rats infected with *Fasciola hepatica* (Jasmina et al., 2010), where infections with the *T. gondii*, *S. mansoni*, and *F. hepatica* are associated with decreased choline in the infected cells.

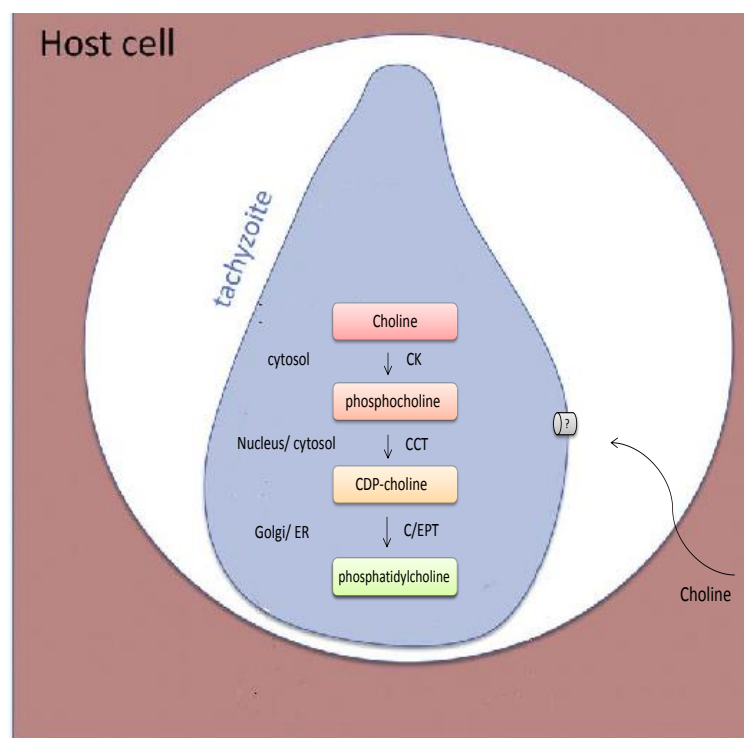


Figure 4.19: Choline utilization by *T. gondii*. Choline is taken up from the host cell by an unknown transporter. It is then converted to Phosphocholine by choline kinase (CK). Phosphocholine is then converted by cytidylyltransferase (CCT) to cytidine diphosphate-choline (CDP-choline) that can be utilized by the endoplasmic reticulum (ER) and Golgi apparatus to form phosphatidylcholine by choline/ethanolamine phosphotransferase (C/EPT).

The level of myo-inositol was also decreased in HBMECs by the development of tachyzoites. A reduced inositol level may be because of *T. gondii* importing it from the host cell. Although *T. gondii* can synthesize certain phospholipids, it must obtain inositol from the host cell to ensure appropriate synthesis of phosphatidylinositol, which is necessary for growth. Quantification of the phospholipid profile of *T. gondii* reveals that phosphatidylinositol accounts for about 7.5% of the total phospholipids (Gupta et al., 2005). Myo-inositol is a building block for all inositol-containing phospholipids in microbes. It can be taken up from the host cells via Na⁺ or H⁺ linked inositol transporters (Reynolds, 2009). Inositol is then converted to phosphatidylinositol in the Golgi apparatus by phosphatidylinositol synthase (Reynolds, 2009). This observation agrees with Wu et al. (2010),

in which the proportion of inositol was lower in mice infected with *Schistosoma japonicum* compared to the non-infected controls.

The level of creatine was modulated in HBMECs because of the development of tachyzoites. An elevated creatine level at 48 h PI may indicate a process of the host response to the infection. Creatine is a nitrogenous organic acid, which is converted to phosphocreatine by mitochondrial creatine phosphokinase (CPK) (Govindaraju et al., 2000; Block et al., 2002). CPK is assayed in the serum as a marker of toxoplasmosis (Gherardi et al., 1992) and malarial symptoms (Davis et al., 1999), where infection with *T. gondii* and *P. berghei* are associated with increased CPK in the serum.

Our findings indicate that invasion, growth, and replication of *T. gondii* tachyzoites within HBMECs lead to molecular metabolic alterations for supporting parasite growth, replication, and infection. Any alterations in the molecular metabolic composition of cells may involve alterations in the fatty acid composition of total phospholipids in the *T. gondii* infected cells. These findings suggest the importance of assessing the fatty acid composition of total phospholipids in parasite-infected cells, to obtain a better insight into the dynamics of host–parasite interactions, for the control of this protozoan.

5. Fatty acid profiling of HBMECs infected with *T. gondii* in vitro

5.1 Introduction

Most of the common diseases in the developing world occur due to infection with intracellular pathogens such as malaria (*Plasmodium falciparum*), toxoplasmosis (*T. gondii*), tuberculosis (*Mycobacterium tuberculosis*), and legionnaire's disease (*Legionella pneumophila*). These intracellular vandals have evolved the ability to manipulate host cell processes to invade, adapt, grow, and proliferate within infected cells. These pathogens gain entry to the host cell by stimulating and hijacking phagocytosis, whereby a portion of the cell membrane envelops the invader and pinches off inside the cell, forming a characteristic lipid-rich structure termed as PV. Inside this protected compartment, the infection is shielded from the host immune response and is ideally positioned for the uptake of nutrients from the host's metabolism as a fuel for its replication and continued dissemination.

In addition to taking advantage of cellular lipids that are usually located inside host cells, *T. gondii* induces metabolic changes in infected cells, leading to the gain of their usual lipid metabolism to facilitate their proliferation (Gupta et al., 2005). These alterations can produce manipulation of intracellular membranes of the host cell, highlighting the intimate connections between *T. gondii* and host lipid metabolism. During *T. gondii* invasion, tachyzoites modify the PVM derived from the host cell membrane. These modifications include a marked increase in the membrane fluidity by manipulation of the host cell fatty acid composition (e.g. arachidonic acid) (Li et al., 2008), manipulation of the phospholipid transbilayer distribution (e.g. phosphatidylcholine) (Gupta et al., 2005), and increased permeability through PVM pores, rendering the PVM permeable to small molecules (Laliberté & Carruthers, 2008).

Recently, interest in understanding the role of lipid metabolism in the apicomplexan parasites adaptation to the host cells, host-parasite interactions at the cellular level, and the identification of different pathogen-specific targets for anti-parasitic drug therapy has increased. Considerable progress has been made in the study of lipid metabolism of parasite and interactions between parasite and its host cell. Van der Meer-Janssen et al. (2010) and D'Avila et al. (2011) have proposed a dynamic role for lipid in the host response to parasitic infection. Increased lipid formation has been described in skeletal muscle cells infected with *T. gondii* (Gomes et al., 2014), in HBMECs infected with *N. caninum* (Elsheikha et al., 2014), in erythrocytes infected with *Plasmodium falciparum* (Jackson et al., 2004), and in macrophage cells infected with *Trypanosoma cruzi* (Melo et al., 2003; D'Avila et al., 2011) where a role for lipid in the survival and replication of parasites has been suggested. However, lipid formation in *T. gondii*-infected cells and the transfer of host cell lipids to the parasite across the PVM for the maintenance of the intracellular parasites remains uncertain. During *T. gondii* infection, tachyzoites modify the membrane of host cells. These modifications include an increase in arachidonic acid C20:4n6 levels. Li et al. (2008) have been shown that *T. gondii* is involved in the release of arachidonic acid from phospholipids of the murine monocyte-macrophage-like cells and murine fibroblast cells membrane, whereby they alter the host membrane permeability. Although some progress has been made in this area through the identification of specific phospholipids (e.g. phosphatidylcholine) as key factors for *T. gondii* multiplication, their specific functions remain unknown. Understanding the interactions between *T. gondii* and the host cell's lipid pools is necessary to identify the prospective lipid targets and the druggable metabolic pathways suitable for anti-*T. gondii* therapies.

To date, no information is available on the alteration of the fatty acid profile of HBMECs during the progression of *T. gondii* infection. In the present study, we investigated the hypothesis that *T. gondii* infection can alter fatty acid compositions of total phospholipids and modify cellular membrane fluidity. Primary cultures of HBMECs were used as a cellular model to study changes in fatty acid compositions of the total phospholipids in HBMECs during the development of *T. gondii* infection by using gas chromatography (GC). Quantification of the lipid surface area of the HBMECs and tachyzoites by Lipid^{Tox} stain was also performed.

5.2 Materials and methods

5.2.1 Lipid^{Tox} staining

Lipid^{Tox} is a green neutral lipid stain that is used to identify intracellular lipid droplets by using confocal laser scanning microscopy. This assay is based on the permeation of Lipid^{Tox} into cell and tachyzoite membranes, emitting green fluorescence when bound to lipid bodies. Lipid^{Tox} was performed as per the manufacturer's instructions [InvitrogenTM detection technologies]. Briefly, the HBMECs were cultured in 6-well cell plate on coverslips (Corning Incorporated, USA) at a concentration of 1×10^5 /ml in cRPMI medium. Once a confluent monolayer of cells was formed (~24 h), tachyzoites were added in a host:parasite ratio of 1:2 as described in section 2.4. The coverslips were removed from the wells at 6, 24, and 48 h post-infection (PI), washed twice in PBS, fixed in cold acetone:methanol (1:1 v/v) for 10 min, stained with Lipid^{Tox} in 1:400 dilution for 30 min, and then mounted with ProLong Gold antifade reagent with 4',6-diamino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Inc., Eugene, OR) on to a microscope slide. Slides were observed under a Zeiss LSM880 confocal laser scanning microscope equipped with a monochrome digital camera (Hamamatsu C4742-95) (Figure 5.1). This assay was performed in triplicate, and the data represent the mean and standard error of the mean (S.E.M).

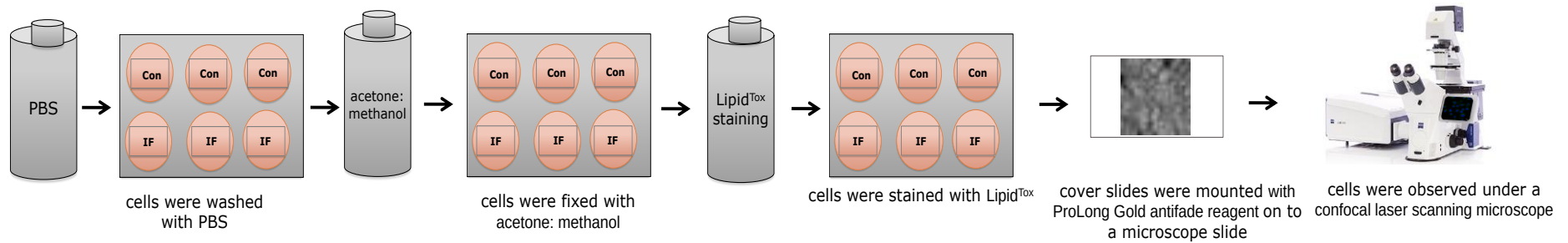


Figure 5.1: Schematic illustration of the Lipid^{Tox} staining assay. HBMECs were seeded in 6-well plates cover slides. When the cells became confluent (~24 h), the tachyzoites were added in a host: parasite ratio of 1:2. Then, the culture medium was removed from the wells. The cells were then washed twice in PBS, fixed in cold acetone: methanol, stained with Lipid^{Tox}, and mounted with ProLong Gold antifade reagent on to a microscope slide. Intracellular lipid droplets were observed under a Zeiss confocal laser scanning microscope imaging system. Con: control cells; IF: infected cells.

5.2.2 Fatty acid profiling

HBMECs were seeded in T-75cm² flasks at 2×10^6 cells/flask and grown in cRPMI medium as described in section 2.2. Once a confluent monolayer of the cells was formed (~24 h), the tachyzoites were added in a host:parasite ratio of 1:2 as described in section 2.4. At 6, 24, and 48 h PI, the infected and uninfected cells were harvested by using a cell scraper, followed by washing three times with PBS. The cells (infected and uninfected) and purified tachyzoites were centrifuged for 5 min at 193 xg, and the cell and tachyzoite pellets were lyophilized by a Modulyo freeze-dryer (Thermo Savant, USA). The cell and tachyzoite pellets were measured using a Mettler Toledo AT251 analytical balance. The cell and tachyzoite pellets were then extracted by using a cold chloroform:methanol solution (2:1, v/v), followed by shaking in an orbital shaker for 15–20 min at room temperature. The samples were filtered through folded filter paper and 3 ml of 0.9% NaCl solution was added to the filtrate; the mixture was then vortexed for a few seconds and centrifuged for 10 min at 2400 xg. The upper phase and the gel-like interphase were removed by siphoning via pipette, and the lower phase (chloroform phase) was collected into labeled eppendorf vials. The chloroform phase was evaporated by applying a nitrogen stream for 30 min, and 2 ml of hexane was added to redissolve each sample. The samples were transesterified as described previously by Christie (1982) and the modified method of Chouinard et al. (1999). Methyl acetate (40 µl) was added to the phospholipid samples. Next, 40 µl of the methylation reagent (0.9 ml of 30% sodium methoxide in 4.1 ml methanol) was added to each reaction mixture and then 60 µl of the termination reagent (0.2 g oxalic acid in 6 ml diethyl ether) was added. Calcium chloride (200 mg) was added to this mixture. The samples were then centrifuged for 5 min at 2400 xg, and the supernatant was transferred to a GC vial and used directly for GC analysis (Figure 5.2). This assay was performed in triplicate, and the data represent the mean and standard error of the mean (S.E.M) from at least three independent experiments.

Separation of fatty acids was performed by GC using the Varian CP-Sil 88 (Crawford ScientificTM Ltd., Strathaven, UK) capillary column with hydrogen as a carrier gas. The temperature of the oven was programmed from 59°C to 100°C at 8 °C/min, then to 170°C at 6°C/min for 10 min, and then to 240°C at 3°C/min for 10 min. Injection and detection temperatures were set at 255°C and 250°C, respectively. The fatty acid compositions of phospholipids in *T. gondii* tachyzoites and HBMECs (infected and uninfected) were identified by comparing their respective retention times with those of a standard mixture (SigmaAldrich Co., LLC, Gillingham, UK). A total of 37 fatty acids were analyzed in this study, including saturated (no double bonds between carbons in the chain) fatty acids ((butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), undecanoic acid (C11:0), lauric acid (C12:0), tridecanoic acid (C13:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), stearic acid (C18:0), arachidic acid (C20:0), heneicosanoic acid (C21:0), behenic acid (C22:0), tricosanoic acid (C23:0), lignoceric acid (C24:0)), monounsaturated (one double bond between carbons in the chain) fatty acids ((myristoleic acid (C14:1), pentadecenoic acid (C15:1), palmitoleic acid (C16:1n7c), heptadecenoic acid (C17:1), and nervonic acid (C24:1n9c)), and polyunsaturated (more than one double bond in the chain) fatty acids ((linolenic acid (C18:3n3), eicosatrienoic acid (C20:3n3), eicosapentaenoic acid (C20:5n3), docosahexaenoic acid (C22:6n3), linolelaidic acid (C18:2n6t), linoleic acid (C18:2n6c), γ-linolenic acid (C18:3n6), eicosadienoic acid (C20:2n6), eicosatrienoic acid (C20:3n6), arachidonic acid (C20:4n6), docosadienoic acid (C22:2n6), elaidic acid (C18:1n9t), oleic acid (C18:1n9c), eicosenoic acid (C20:1n9), and erucic acid (C22:1n9)).

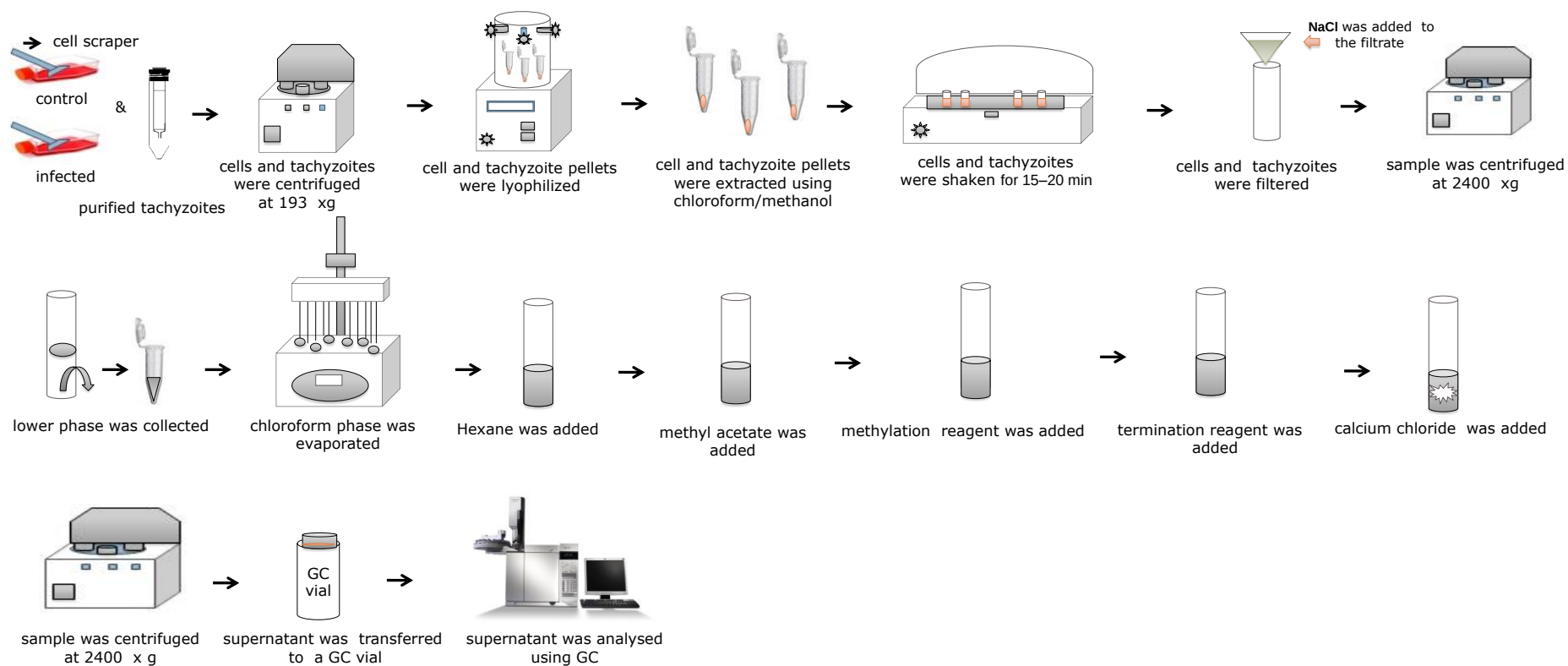


Figure 5.2: Schematic view of sample preparation for fatty acid analysis. The infected and non-infected cells were harvested with a cell scraper, followed by washing three times with PBS. The cells and purified tachyzoites were then centrifuged, and the cell and tachyzoite pellets were lyophilized by freeze-dryer and extracted using a cold chloroform:methanol solution. The chloroform phase was then evaporated by using sample concentrator, to which hexane, methyl acetate, methylation reagent, termination reagent, and calcium chloride were added. Then, supernatant was transferred to a GC vial and used directly for GC analysis.

5.2.3 Statistical analysis

Quantification of the surface area of the lipids by Lipid^{Tox} stain was measured by using Volocity[®] software. The surface area of the lipids and fatty acid composition of total phospholipids between control and infected cells at different time points were assessed by two-way ANOVA, followed by Tukey's multiple comparison test using Graphpad Prism version 5.0 (Graphpad Software, Inc., San Diego, CA). As the data was not normally distributed, statistical analysis was performed on the transformed data (log10).

5.3. Results

5.3.1. Lipid^{Tox} staining

Analysis of the Lipid^{Tox} staining data revealed a significant main effect of group ($F_{(1, 12)} = 6.000$, $P = 0.0306$), but no significant main effect of time ($F_{(2, 12)} = 2.009$, $P = 0.1768$) or group $\times$ time interaction ($F_{(2, 12)} = 0.8105$, $P = 0.4676$). Post-hoc analysis indicated that the surface area of lipids was significantly increased in infected, compared to control, cells ($n = 9$ cells/group) (Figure 5.3-5.4).

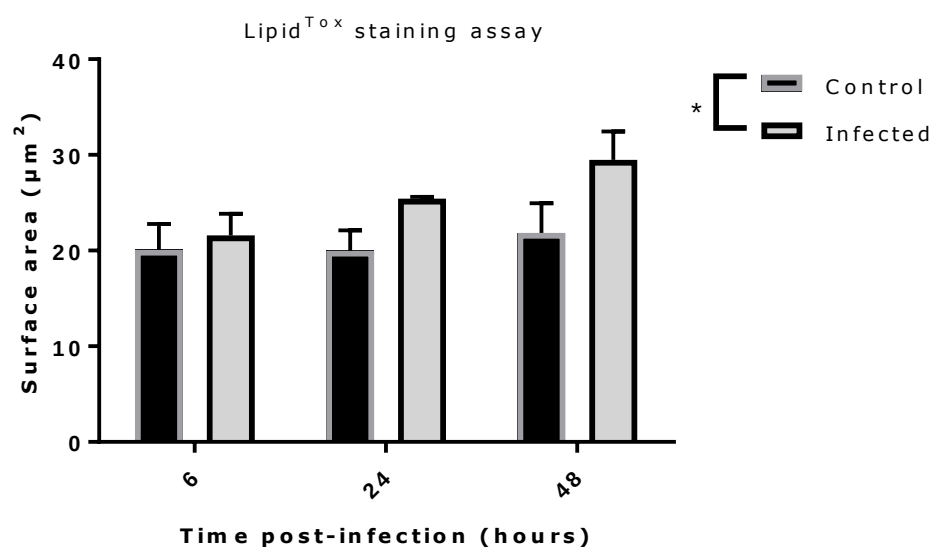


Figure 5.3: Lipid^{Tox} staining in HBMECs infected and uninfected with *T. gondii* over 48 h PI. Significant increases were noted in the surface area of lipids in infected, compared to control, cells across all times (* $P = 0.0306$).

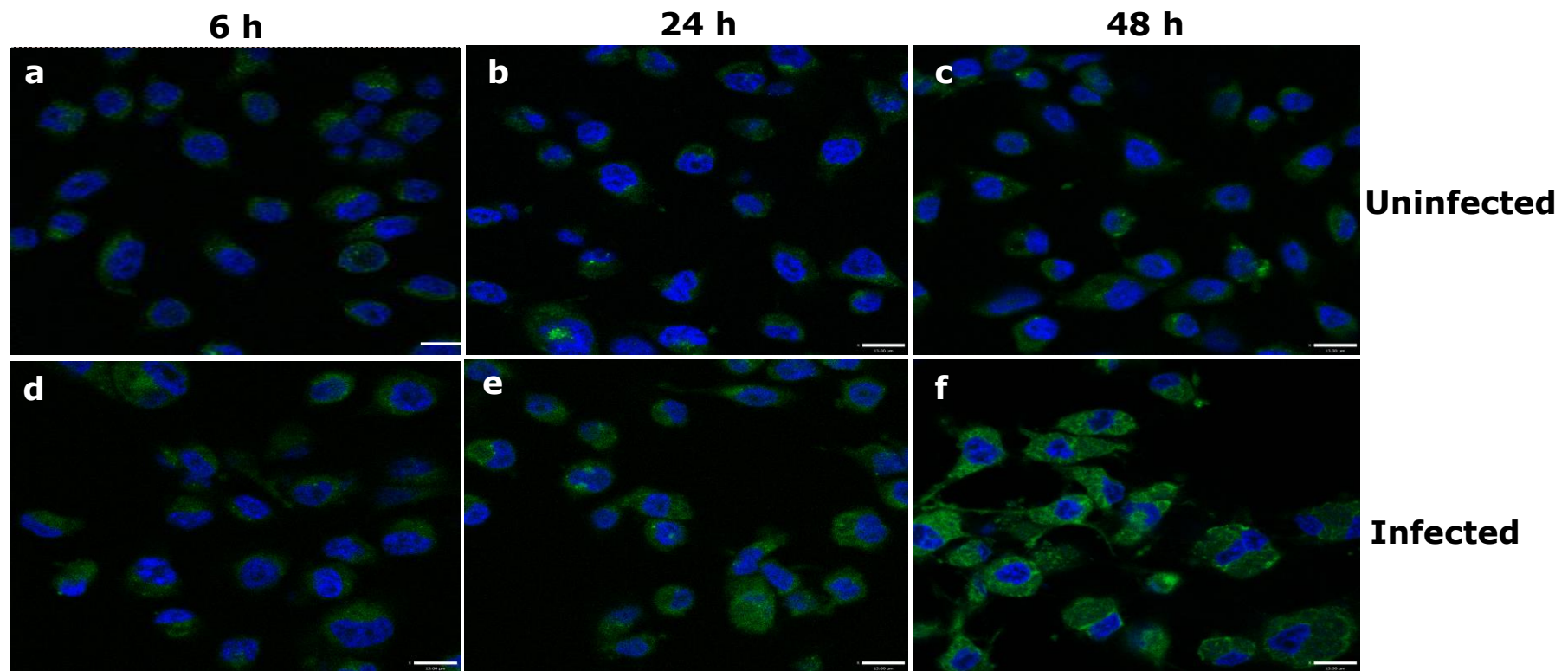


Figure 5.4: Lipid^{Tox} staining of HBMECs showing a progressive increase in lipids over time as indicated at the top. Cell and tachyzoite nuclei are shown in blue and lipid in green. Scale bar= 13 μ m

5.3.2 Fatty acid levels in HBMECs

Fatty acids found in HBMECs infected with *T. gondii* and non-infected controls are shown in Table 5.1. The following eight fatty acids did not show any significant differences between control and infected cells at different time points PI: C14:0, C16:1n7, C18:2n6c, C18:3n3, C20:2n6, C20:3n6, C20:4n6, C22:6n3 (all $P > 0.05$). However, significant differences in C16:0, C18:0 and C18:1n9c levels were observed at certain time points PI.

Table 5.1: Fatty acid composition of total phospholipid found in HBMECs infected with *T. gondii* and non-infected controls at different incubation times.

Fatty acids	Mean±S.E.M								
	6 h		24 h		48 h		*P value		
	Control	Infected	Control	Infected	Control	Infected	a	b	c
C14:0	0.84±0.190	0.97±0.075	0.98±0.055	1.21±0.320	1.11±0.165	1.01±0.025	0.5571	0.5355	0.6298
C16:0	0.63±0.06	0.88±0.19	0.85±0.066	1.08±0.081	1.04±0.18	1.2±0.123	0.0655	0.0419	0.9435
C16:1n7	1.32±0.160	1.36±0.045	1.39±0.09	1.55±0.240	1.22±0.140	1.19±0.045	0.6537	0.2341	0.7831
C18:0	0.45±0.026	0.55±0.057	0.41±0.0473	0.70±0.038	0.55±0.146	0.77±0.055	0.0051	0.1392	0.4354
C18:1n9c	0.55±0.081	0.81±0.0353	0.54±0.032	1.00±0.038	0.83±0.22	1.03±0.088	0.0037	0.0951	0.4328
C18:2n6c	0.99±0.185	1.06±0.125	0.95±0.235	1.29±0.115	1.52±0.235	1.51±0.035	0.3756	0.0631	0.5666
C18:3n3	0.68±0.090	0.66±0.135	0.68±0.090	0.80±0.01	0.61±0.160	0.55±0.065	0.9097	0.3553	0.6623
C20:2n6	1.33±0.155	1.36±0.105	1.35±0.135	1.17±0.290	0.85±0.115	1.02±0.035	0.9509	0.0891	0.5812
C20:3n6	0.84±0.225	0.59±0.530	1.00±0.06	1.08±0.040	0.93±0.270	0.18±0.345	0.2527	0.3166	0.4202
C20:4n6	1.39±0.195	1.43±0.280	1.75±0.160	1.71±0.000	1.48±0.085	1.30±0.060	0.6614	0.1340	0.8070
C22:6n3	1.18±0.200	1.33±0.005	1.14±0.240	1.47±0.135	0.87±0.225	0.77±0.120	0.4118	0.0587	0.5176

Results are expressed as Mean ± Standard Error of the mean of the three replicates. * a: P value for the main effect of group (control cells vs infected cells), b: P value for the main effect of time (6 vs 24 vs 48 hs), and c: P value for the group x time interaction.

Analysis of the C16:0 data revealed a significant main effect of time ($F_{(2, 12)} = 4.182$, $P = 0.0419$), but no significant main effect of group ($F_{(1, 12)} = 4.107$, $P = 0.0655$) or group $\times$ time interaction ($F_{(2, 12)} = 0.05849$, $P = 0.9435$) (Figure 5.5a). Post-hoc analysis indicated that C16:0 was significantly increased at 48 h compared to 6 h PI, regardless of group ($P = 0.0419$).

Analysis of the C18:0 data revealed a significant main effect of group ($F_{(1, 12)} = 11.67$, $P = 0.0051$), but no significant main effect of time ($F_{(2, 12)} = 2.334$, $P = 0.1392$) or group $\times$ time interaction ($F_{(2, 12)} = 0.8919$, $P = 0.4354$) (Figure 5.5b). Post-hoc analysis indicated that C18:0 was significantly increased in infected, compared to control, cells ($P = 0.0051$).

Analysis of the C18:1n9c data revealed a significant main effect of group ($F_{(1, 12)} = 12.85$, $P = 0.0037$), but no significant main effect of time ($F_{(2, 12)} = 2.880$, $P = 0.0951$) or group $\times$ time interaction ($F_{(2, 12)} = 0.8989$, $P = 0.4328$) (Figure 5.5c). Post-hoc analysis indicated that C18:1n9c was significantly increased in infected, compared to control, cells ($P = 0.0037$).

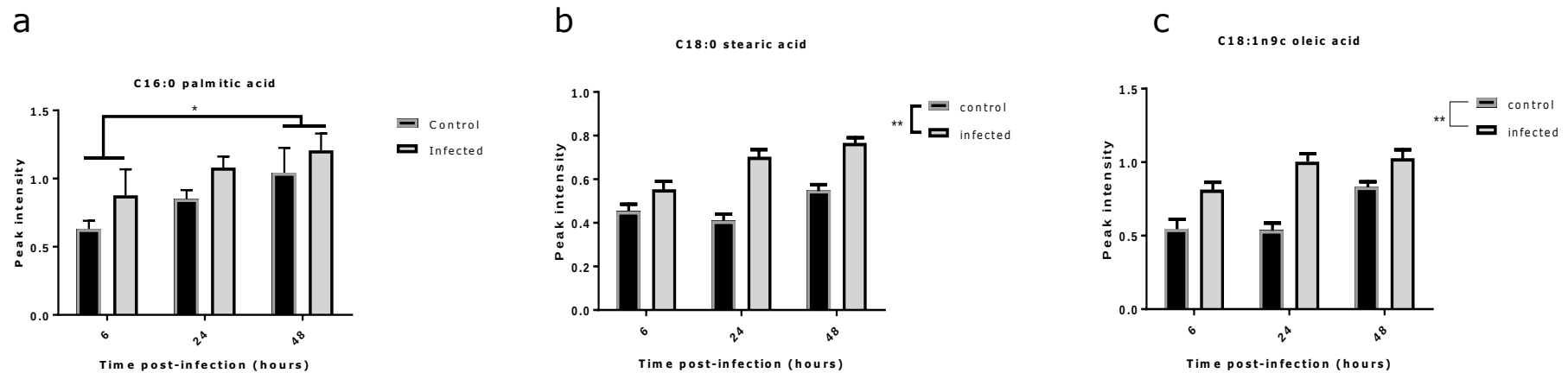


Figure 5.5: Fatty acid composition of total phospholipid in HBMECs infected and uninfected with *T. gondii* over 48 h PI. a) A significant increase was noted in the level of C16:0 at 48 h, compared to 6 h, across both groups (* $P = 0.0419$). Significant increases were noted in the levels of b) C18:0 (** $P = 0.0051$) and c) C18:1n9c (** $P = 0.0037$) in infected, compared to control, cells across all times.

5.3.3 Fatty acid levels in purified parasite preparations

The following pie chart shows the proportions of fatty acid in *T. gondii* tachyzoites. C6:0 and C16:0 were the most abundant fatty acid as they represented 24% and 21% of the total parasite phospholipid, respectively. C24:1n9c, C18:0, and C22:6n3 were also considerably abundant, constituting 16%, 14%, and 14% respectively, whereas C14:0 accounted for ~11% of the total (Figure 5.6).

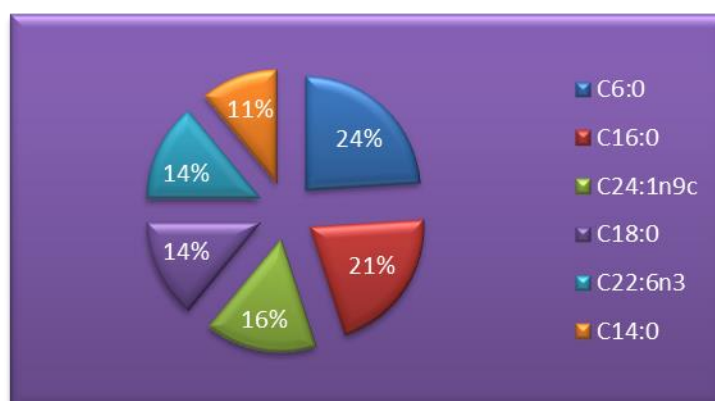


Figure 5.6: Fatty acid composition of total phospholipid in *T. gondii* tachyzoites. Caproic acid (C6:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), nervonic acid (C24:1n9c), and docosahexaenoic acid (C22:6n3).

5.4. Discussion

The aim of this work was to assess alterations in free fatty acids in HBMECs after infection with *T. gondii*. Lipid^{Tox} staining of *T. gondii*-infecting HBMECs showed a marked increase on the surface area of lipids during infection. Structurally, lipid droplets are neutral-lipid cores consisting mainly of triacylglycerols and cholesterylesters surrounded by a monolayer of phospholipid containing cholesterol and proteins (Bozza et al., 2009). Recent studies have proposed a dynamic role for lipid droplets in the host response to parasitic infection (Van der Meer-Janssen et al., 2010; D'Avila et al., 2011). Increased lipid droplet formation has been described in *T. gondii* (Charron and Sibley, 2002; Gomes et al., 2014), *N. caninum* (Elsheikha et al., 2014), *Plasmodium falciparum* (Jackson et al., 2004), and *Trypanosoma cruzi* infection (Melo et al., 2003; D'Avila et al., 2011) where a role for lipid droplet in the survival and replication of parasites has been suggested. However, lipid droplet formation in *T. gondii*-infected cells and the transfer of host cell lipids to the parasite across the PVM for the maintenance of the intracellular parasites remains uncertain.

In the present study, no significant differences in the levels of C14:0, C16:1n7, C18:2n6c, C18:3n3, C20:2n6, C20:3n6, C20:4n6, and C22:6n3 were observed. However, a marked increase in the levels of C18:0 and C18:1n9c was noted in infected cells compared to non-infected controls, indicating that C18:0 and C18:1n9c are involved in host-*T. gondii* interactions (Vial et al., 2003). In addition, the finding that monounsaturated fatty acid is elevated in infected cells suggests that the key role of C18:1n9c in the pathogenesis of toxoplasmosis might be to modulate the host cell's membrane fluidity. This finding is in agreement with previous studies conducted in C8166 lymphoid cells infected with lentivirus (Apostolov et al., 1989) and in HeLa cells infected with poliovirus (Guinea and Carrasco, 1991), where the incorporation of C18:1n9c into host cell membranes led to increased membrane fluidity. It is possible that the parasite uses lipid

synthetic/metabolic abilities to produce some of modifications to the host cell membrane. Genomic and experimental analyses have identified pathways for the synthesis of fatty acids in *T. gondii* (Waller et al., 1998). Tachyzoite uses three mechanisms of fatty acid synthesis. The tachyzoite harbors both type I (FASI) and II (FASII) pathways of fatty acid synthesis. The main roles of the FAS-I and -II systems, which are found in the mitochondria and apicoplast of most eukaryotes, are to supply the demand for lipoic acid, C14:0 and C16:0. Lipoic acid is an organosulfur compound, which is used as a cofactor for pyruvate dehydrogenase in the Krebs's cycle. Although *T. gondii* can synthesize lipoic acid inside the apicoplast, it must obtain lipoic acid from the host cell to utilize this lipid in its own mitochondrion (Thomsen-Zieger et al., 2003; Crawford et al., 2006). The glycolysis process in the cytoplasm of *T. gondii* produces phosphoenolpyruvate (PEP), which is imported into the apicoplast by triose phosphate transporters (TPTs) and converted to acetyl-CoA (Ac-CoA) by the subsequent action of pyruvate kinase and pyruvate dehydrogenase. Ac-CoA is then carboxylated to malonyl-coenzyme A (Mal-CoA) by acetyl-CoA carboxylase (ACCase). Mal-CoA is then reduced, dehydrated, and re-reduced by the sequential actions of FabD, -H, -G, -Z, -I, and -B/F, resulting in the formation of lipoic acid, C14:0, and C16:0.

In addition, the tachyzoite seems to possess fatty acid elongation machinery (Ramakrishnan et al., 2013). The enzymatic steps involved in the elongase process are similar to those in the FASI and II systems, albeit the growing chain is held by acetyl-CoA, instead of by acyl carrier protein (ACP). Genomic analysis has identified three elongase genes in *T. gondii* and the activity of these genes could be a source of synthesis of longer chain fatty acids (Bisanz et al., 2006; Mazumdar et al., 2006). Ramakrishnan et al. (2013) suggested that C14:0 and C16:0 synthesized by FASII are further elongated by the ER elongases (ELO) to produce long-chain monounsaturated fatty acids including C18:0, C18:1n9c, C22:1, and C26:1 (Figure 5.7).

Furthermore, intracellular parasites synthesize various enzymes that may target host lipids or exploit the host cell's lipid metabolizing enzymes. During *T. gondii* invasion, both tachyzoites and host-derived phospholipase are involved in the release of arachidonic acid C20:4n6 from phospholipids of the host cell membrane, whereby they alter the host membrane fluidity and facilitate the invasion by tachyzoites (Li et al., 2008). The host membrane's fluidity is largely determined by its composition, including the type of phospholipid, fatty acid, cholesterol and protein (Hsiao et al., 1991). Any change in the fatty acid composition of total phospholipid, such as the degree of unsaturation of fatty acyl chains and their length, results in the alterations of the biophysical characteristics of the membrane. Interaction of monounsaturated and polyunsaturated fatty acids (e.g. oleic acid [C18:1n9c], linoleic acid [C18:2n6c], arachidonic acid [C20:4n6]) with animal cells (e.g. embryonated egg, mouse brain, buffalo green monkey, Vero cells, monkey epithelial cells) induces a change in the fluidity of their membranes (Kohn et al., 1980). It is also possible that the membrane structure gets altered by the numerous *T. gondii* proteins exported to the host cell membrane during invasion. These include microneme proteins, which are secreted from the microneme organelle to allow the parasite to glide and adhere to its host cell. A second wave of secretion originates from the rhoptry organelle to allow the parasite to enter its host cell and form the PV. A third secretion originates from dense granules to modify PVMs (Bonhomme et al., 1992; Carruthers and Sibley, 1997). During *T. gondii* invasion, tachyzoites modify the PVM to maintain a suitable intracellular environment. These modifications include a marked increase in the membrane fluidity by manipulation of the host cell fatty acid composition (e.g. arachidonic acid) (Li et al., 2008), manipulation of the phospholipid transbilayer distribution (e.g. phosphatidylcholine) (Gupta et al., 2005), and increased permeability through PVM pores (Laliberté & Carruthers, 2008), rendering the PVM permeable to the entry of small molecules. Coppens et al. (2000) have shown that a variety of host lipids are exchanged across the PVM, including fatty acids, phospholipids and

cholesterol, some of which are further metabolized by the parasite to ensure its survival and proliferation. Intracellular *T. gondii* can uptake selected exogenous fatty acids (Tomavo et al., 1989; Quittnat et al., 2004). Inside parasites, exogenous fatty acids are rapidly manufactured into triacylglycerols (Quittnat et al., 2004) and cholesteryl esters (Nishikawa et al., 2005) with a two-fold higher incorporation of palmitic acid (C16:0), as compared to other fatty acids, including oleic acid (C18:1n9c), stearic acid (C18:0), linoleic acid (C18:2n6c) and arachidonic acid (C20:4n6). In addition, triacylglycerol is synthesized *de novo* through the acyl-CoA:diacylglycerolacyl transferase-mediated pathway in the *T. gondii* ER (Quittnat et al., 2004). These glycerolipids are stored in *T. gondii* lipid bodies. *T. gondii* apicoplasts contain the complete pathway to synthesize galactosyl glycerolipids from fatty acids and glycerol-phosphate (Marechal et al., 2002). Quittnat et al. (2004) used fluorescent lipid 1-stearoyl-2-[1-14C]arachidonoyl-sn-glycerol suggested that lipid is internalized by extracellular parasites and then converted into triacylglycerol.

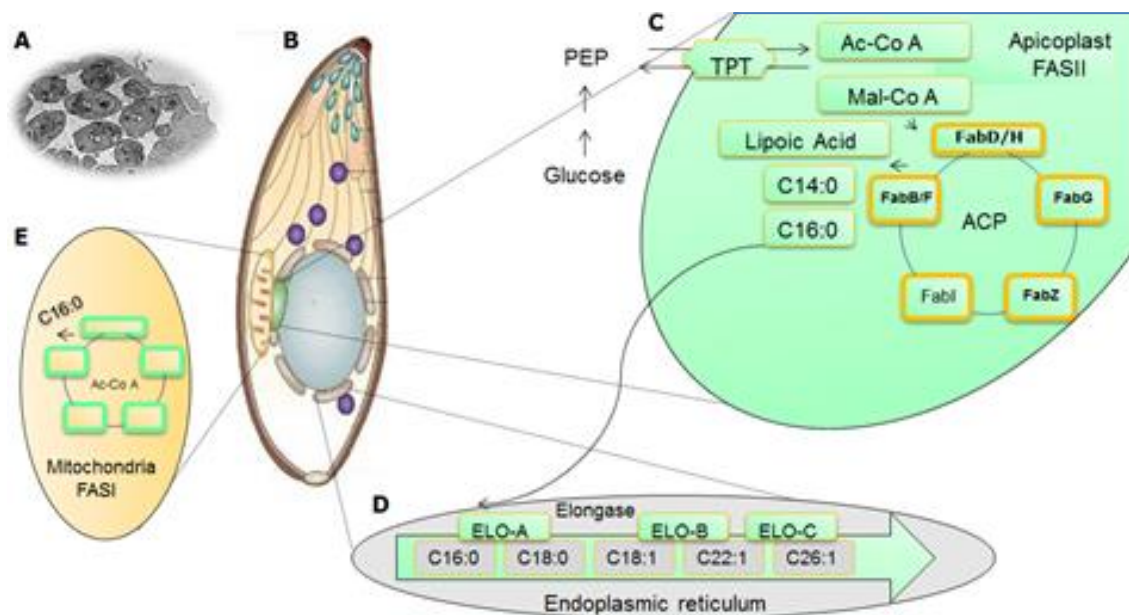


Figure 5.7: *T. gondii* acquires fatty acids through a complex network of synthesis and scavenging. *T. gondii* can salvage lipid precursors from the host cell (A). In addition, tachyzoites harbor three fatty acid synthesis pathways in the apicoplast, mitochondria, and ER (B). The apicoplast localized FASII pathway (green) produces significant amounts of myristic acid (C14:0), palmitic acid (C16:0), and lipoic acid, relying on cytoplasmic glycolysis for precursors (C). *T. gondii* also maintains an ER-associated elongase (ELO) system that synthesizes very long chain monounsaturated fatty acids using the activities of ELO-B and ELO-C (D). The mitochondria-localized FAS I pathway produces significant amounts of palmitic acid (C16:0) (E). PEP: Phospho enolpyruvate; TPTs: triose phosphate transporters; Ac-CoA: acetyl-CoA; Mal-CoA: malonyl-coenzyme A; ACP: acyl carrier protein.

In the present study, a significant increase was noted in C16:0 level at 48 h compared to 6 h PI, indicating that C16:0 accumulation in cells may be the result of biosynthesis activity in HBMECs (Meng et al., 2004). Saturated fatty acids are synthesized from Ac-CoA and Mal-CoA to form C16:0. Then, C16:0 is further elongated to produce the long-chain fatty acids C18:0, C20:0, C22:0, and C24:0. These long-chain fatty acids are integrated into biological membranes reducing their fluidity.

Taken together, these findings suggest that the invasion, growth, and proliferation of *T. gondii* tachyzoites within HBMECs alter the degree of unsaturation of fatty acyl chains and their length, potentially resulting in modulation of the host cell's membrane fluidity. Since the physicochemical structure or fluidity of a membrane is largely determined by its composition, including phospholipid type, fatty acid composition, cholesterol content, and protein content. This, in turn, may involve changes in content or distribution of trace elements in *T. gondii* infected cells. Several lines of evidence suggest that the acquisition and homeostasis of trace elements within cells are tightly regulated, and any alteration in their levels can have adverse impacts on the host cell and its ability to respond to parasitic infection. These findings suggest the importance of assessing the essential and non-essential trace elements in parasite-infected cells to obtain better insight into the dynamics of host-parasite interactions for the control of this protozoan.

6. Trace element profiling of HBMECs infected with *T. gondii* *in vitro*

6.1 Introduction

The interaction of host cells with intracellular parasites is responsible for numerous events including activation of immune cells, increased synthesis and release of cytokines, production of antibodies, activation of complement, and the movement of trace elements between the blood and tissues, which are crucial for regulating immunity, with the aim of countering the invading parasites and maintaining the tissue homeostasis (Beisel 2004; Ilback and Friman 2007).

All of these interactions may also cause an imbalance of trace elements, especially with those directly involved with immune function (e.g. Zn, Fe, Cu, Mg, and Se) (Beck et al. 1994; Beck et al., 2001; Ilback and Friman 2007). These elements are required for a multitude of cellular housekeeping functions, including replication, transcription, and signal transduction. In addition, these elements are essential for maintenance of membrane barrier integrity, protection against pathogens, and regulation of immune responses (Chaturvedi et al. 2004; Modlin 1990). Zn status affects the normal functioning of the cells involved in innate immunity, such as macrophages, DCs and NK cells. The macrophage, an essential cell in many immunologic functions, is affected by the levels of Zn, which can dysregulate intracellular killing, cytokine production, and phagocytosis. Zn levels also affect the development of acquired immunity by preventing both the growth and certain functions of T-lymphocytes such as cytokine production. Likewise, B-lymphocyte development and antibody production is compromised by altered Zn levels (Kimberly, 2010). Hence, both reduced and excess levels of Zn may have marked effects on virtually all components of the immune system (Ibs & Rink, 2003).

The role of trace elements in the health and disease of the brain has been well identified. Alterations in the levels of Zn, Fe, and Cu have been reported in relation to pathological conditions such as brain tumor (Alam and Kelleher, 2012), Parkinson's disease (Dexter et al., 1989), Alzheimer's disease (Chazot and

Broussolle, 1993; Waggoner et al., 1999; Gallagher et al., 2012), and toxoplasmosis (Geng et al., 2000; Seyerk et al., 2004; Al-Khshab and Al-Bakry, 2009). Seyerk et al. (2004) have shown that the concentration of Zn in serum is lower in *T. gondii* sero-positive sheep, while Fe, Mg, and Cu concentrations are higher. Al-Khshab and Al-Bakry (2009) have shown that Zn, Fe, and Mg concentrations in the serum of women sero-positive for *T. gondii* are decreased, while Cu concentrations are increased. Decreased levels of Zn were also noted in the plasma of A/J mice during the pre-inflammatory stage of Coxsackie B virus infection (Funseth et al., 2000) and in serum of children during *Giardia lamblia* infection (Yones et al., 2015). However, increased levels of Zn were noted in lung tissues of DBA/2 mice during chronic infection of *Pseudomonas aeruginosa* (Pompilio et al., 2014). In a study carried out on rats experimentally infected with toxoplasmosis, the level of Fe, Mg, and Cu in the brain tissue in the experimental group was significantly reduced (Geng et al., 2000).

Although levels of Zn, Fe, Mg, and Cu in serum and brain tissue during toxoplasmosis have been reported (Seyerk et al., 2004; Al-Khshab and Al-Bakry, 2009; Geng et al., 2000), changes in trace element balance in HBMECs during *T. gondii* infection *in vitro* remain unknown. In the present study, we investigated the hypothesis that trace element abundance in HBMECs changes in response to infection with *T. gondii*. Primary cultures of HBMECs were used as a cellular model to study changes in the levels of trace elements in HBMECs during the development of *T. gondii* within the host cell by using inductively coupled plasma mass spectrometry (ICP-MS).

6.2 Materials and methods

6.2.1 Trace element profiling

HBMECs were seeded in T-175cm² flasks at 1×10^{10} cells/flask and grown in cRPMI medium as described in section 2.2. Once a confluent monolayer of the cells was formed (~24 h), the tachyzoites were added in a host:parasite ratio of 1:2 as described in section 2.4. At 6, 24, and 48 h PI, the growth medium was collected from the infected and non-infected cultures and analysis of extracellular trace elements using ICP-MS was performed. The infected and uninfected cells were harvested by using a cell scraper, followed by washing three times with deionized water. The cells (infected or uninfected) and purified tachyzoites were centrifuged for 5 min at 193 xg. The cell and tachyzoite pellets were lyophilized by a Modulyo freeze-dryer (Thermo Savant, USA). The cell and tachyzoite pellets were then digested by using a mixture containing 3 ml HNO₃, 3 ml H₂O, and 2 ml H₂O₂ and allowed to stand for 1 h at room temperature (James et al., 2014). The samples were then heated in a microwave for 90 min. The concentration of trace elements was then measured by using ICP-MS (Varian Ultramass, Melbourne, VIC, Australia) fitted with a direct injection nebulizer (CETAC, Omaha, NE, USA) using Rh (10 ng/l) as an internal standard (Figure 6.1). This assay was performed in triplicate, and the data represent the mean and standard error of the mean (S.E.M) from at least three independent experiments.

A total of 23 trace elements were analysed in this study: boron (B), sodium (Na), magnesium (Mg), phosphorous (P), sulfur (S), potassium (K), calcium (Ca), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), rubidium (Rb), strontium (Sr), molybdenum (Mo), cadmium (Cd), cesium (Cs), and barium (Ba).

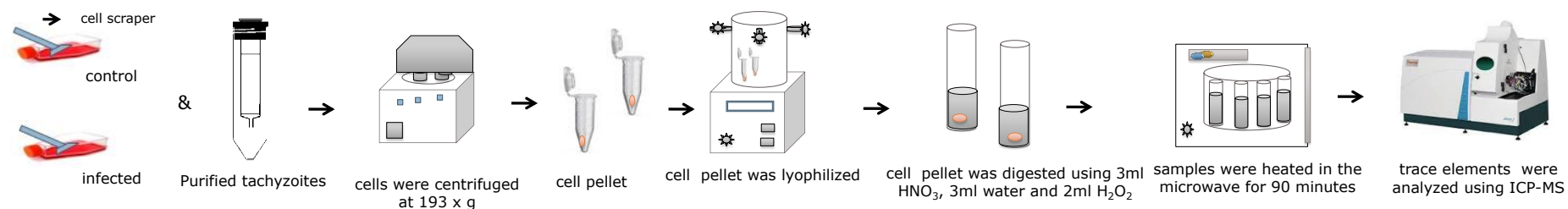


Figure 6.1: Schematic view of sample preparation for trace element analysis. The infected and non-infected cells were harvested by using a cell scraper, followed by washing three times with deionized water. The cells and purified tachyzoites were then centrifuged, and cell and tachyzoite pellets were lyophilized by freeze-dryer. The cell and tachyzoite pellets were then digested in a mixture containing 3 ml HNO₃, 3 ml H₂O, and 2 ml H₂O₂, and allowed to stand at room temperature for 1 h. The samples were then heated in the microwave for 90 min and the concentrations of trace elements were analysed using ICP-MS.

6.2.2 Statistical analysis

Trace element concentrations between control and infected cells at different time points were assessed by two-way ANOVA, followed by Tukey's multiple comparison test using Graphpad Prism version 5.0 (Graphpad Software, Inc., San Diego, CA). As the data was not normally distributed, statistical analysis was performed on the transformed data (log10).

6.3 Results

6.3.1 Trace element levels in HBMECs

Trace element levels in the HBMECs infected with *T. gondii* and in non-infected controls are shown in Table 6.1. The following 12 trace elements did not show any significant differences between control and infected cells at different time points: Na, Ca, V, Cr, Co, Ni, As, Sr, Mo, Cs, Ba, and B (all $P > 0.05$). However, significant differences were noted in the levels of Zn, Fe, Mg, and Cd between the control and infected cells across time and between different time points across both groups ($P < 0.05$). Significant differences were also noted in the levels of P, K, Mn, Cu, Se, Rb, and S at different time points across both groups ($P < 0.05$) (Table 6.1).

Mean±S.E.M									
Elements	6 h		24 h		48 h		*P value		
	Control	Infected	Control	Infected	Control	Infected	a	b	c
B	0.023±0.003	30.01±0.003	0.016±0.003	30.02±0.003	0.020±0.000	30.03±0.006	0.73	0.2374	0.1352
Na	0.37±0.07	0.45±0.09	0.32±0.08	0.70±0.16	0.45±0.10	0.47±0.03	0.14	0.5289	0.1076
Mg	1.863±0.05	2.36±0.14	2.10±2.99	2.66±0.30	3.34±0.44	4.06±0.50	0.04	0.0009	0.9421
P	26.36±2.89	35.04±3.25	48.54±3.86	44.97±2.46	60.34±4.44	59.59±2.99	0.06	<0.0001	0.2071
S	17.66±1.252	21.04±2.28	24.65±2.166	24.58±2.21	28.75±1.859	29.27±0.86	0.41	0.0008	0.6195
K	4.575±0.60	5.59±0.73	7.504±1.72	6.87±0.80	13.18±1.09	13.22±1.17	0.87	< 0.0001	0.7502
Ca	1.371± 0.549	1.51±0.32	2.099± 0.785	3.01±1.48	3.078 ±0.835	2.24±0.40	0.90	0.2921	0.5658
V	0.06±0.01	0.04±0.01	0.06±0.01	0.08±0.01	0.07±0.003	0.27±0.7	0.27	0.2293	0.2944
Cr	0.98±0.10	0.90±0.03	0.99±0.03	1.04±0.04	1.07±0.03	1.71±0.59	0.32	0.1912	0.3240
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Mean±S.E.M									
Elements	6 h		24 h		48 h		*P value		
	Control	Infected	Control	Infected	Control	Infected	a	b	c
Mn	1.03±0.02	1.02±0.02	1.06±0.03	1.15±0.02	1.18±0.03	1.45±0.21	0.11	0.0155	0.3082
Fe	125.0±19.82	157±12.73	225.3±19.18	295.8±39.29	283.7±33.17	386.8±45.66	0.01	0.0001	0.5285
Co	0.043±0.01	0.04±0.003	0.04±0.006	0.06±0.006	0.06±0.010	0.093±0.028	0.29	0.3478	0.3867
Ni	0.65±0.13	0.57±0.03	0.60±0.03	0.64±0.03	0.66±0.04	1.32±0.58	0.33	0.2512	0.3129
Cu	1.33±0.04	1.44±0.16	1.27±0.09	1.27±0.01	1.47±0.06	1.79±0.10	0.09	0.007	0.2803
Zn	202.1±20.05	282.9±16.29	251.02± 32.03	314.03±29.16	466.7±20.22	582.17±15.14	0.0006	< 0.0001	0.5294
As	0.07±0.00	0.08±0.01	0.04±0.01	0.06±0.01	0.05±0.01	0.01±0.04	0.09	0.3048	0.4095
Se	0.46±0.02	0.85±0.1	1.321±0.21	1.12±0.14	1.47±0.16	1.59±0.17	0.40	0.0002	0.1731
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Mean±S.E.M									
Elements	6 h		24 h		48 h		*P value		
	Control	Infected	Control	Infected	Control	Infected	a	b	c
Rb	7.47±0.74	9.29±0.78	12.98±2.496	11.72±1.02	21.40±1.57	22.42±1.66	0.67	< 0.0001	0.5851
Sr	0.67±0.19	0.59±0.10	0.83±0.20	0.93±0.27	0.95±0.19	0.79±0.14	0.73	0.3672	0.7791
Mo	0.40±0.06	0.33±0.03	0.38±0.03	0.41±0.02	0.43±0.02	0.49±0.09	0.36	0.1801	0.2933
Cd	0.032±0.01	0.07±0.01	0.06±0.02	0.10±0.01	0.07±0.02	0.13±0.03	0.0037	0.0194	0.7852
Cs	0.01±0.003	0.06±0.04	0.02±0.0	0.02±0.00	0.03±0.00	0.03±0.00	0.26	0.6050	0.2955
Ba	1.09±0.25	0.72±0.04	1.22±0.04	0.97±0.06	0.95±0.11	1.17±0.09	0.19	0.2793	0.0671

Results are expressed as Mean ± Standard Error of the mean of the three replicates. * a: P value for the main effect of group (control cells vs infected cells), b: P value for the main effect of time (6 vs 24 vs 48 h), and c: P value for the group x time interaction.

Analysis of the Zn data revealed a significant main effect of group ($F_{(1, 12)} = 21.13$, $p = 0.0006$) and time ($F_{(2, 12)} = 87.76$, $P < 0.0001$), but no significant group $\times$ time interaction ($F_{(2, 12)} = 0.6709$, $P = 0.5294$) (Figure 6.2a). Post-hoc analysis indicated that a significant increase in Zn levels was observed in infected compared to control cells ($p = 0.0006$). It also indicated that Zn was significantly increased at 48 h compared to 6 and 24 h PI, regardless of group ($P < 0.0001$).

Analysis of the Fe data revealed a significant main effect of group ($F_{(1, 12)} = 7.483$, $P = 0.0181$) and time ($F_{(2, 12)} = 20.38$, $P = 0.0001$), but no significant group $\times$ time interaction ($F_{(2, 12)} = 0.6728$, $P = 0.5285$) (Figure 6.2b). Post-hoc analysis indicated that a significant increase in Fe levels was observed in infected compared to control cells ($P = 0.0181$). It also indicated that Fe was significantly increased at 48 h compared to 6 and 24 h PI, regardless of group ($P = 0.0001$).

Analysis of the Mg data revealed a significant main effect of group ($F_{(1, 12)} = 4.806$, $p = 0.0488$) and time ($F_{(2, 12)} = 13.42$, $P = 0.0009$), but no significant group $\times$ time interaction ($F_{(2, 12)} = 0.05989$, $P = 0.9421$) (Figure 6.2c). Post-hoc analysis indicated that a significant increase in Mg levels was observed in infected compared to control cells ($P = 0.0488$). It also indicated that Mg was significantly increased at 48 h compared to 6 and 24 h PI, regardless of group ($P = 0.0009$).

Analysis of the Cd data revealed a significant main effect of group ($F_{(1, 12)} = 12.92$, $p = 0.0037$) and time ($F_{(2, 12)} = 5.574$, $P = 0.0194$), but no significant group $\times$ time interaction ($F_{(2, 12)} = 0.2467$, $P = 0.7852$) (Figure 6.2d). Post-hoc analysis indicated that a significant increase in Cd levels was observed in infected compared to control cells ($p = 0.0037$). It also indicated that Cd was significantly increased at 48 h compared to 6 and 24 h PI, regardless of group ($P = 0.0194$).

Analysis of the P data revealed a significant main effect of time ($F_{(2, 12)} = 37.59$, $P < 0.0001$), but no significant main effect of group ($F_{(1, 12)} = 0.2781$, $P = 0.0676$) or group $\times$ time interaction ($F_{(2, 12)} = 1.801$, $P = 0.2071$) (Figure 6.2e). Post-hoc analysis indicated that P was significantly increased at 48 h compared to 6 and 24 h PI, regardless of group ($P < 0.0001$).

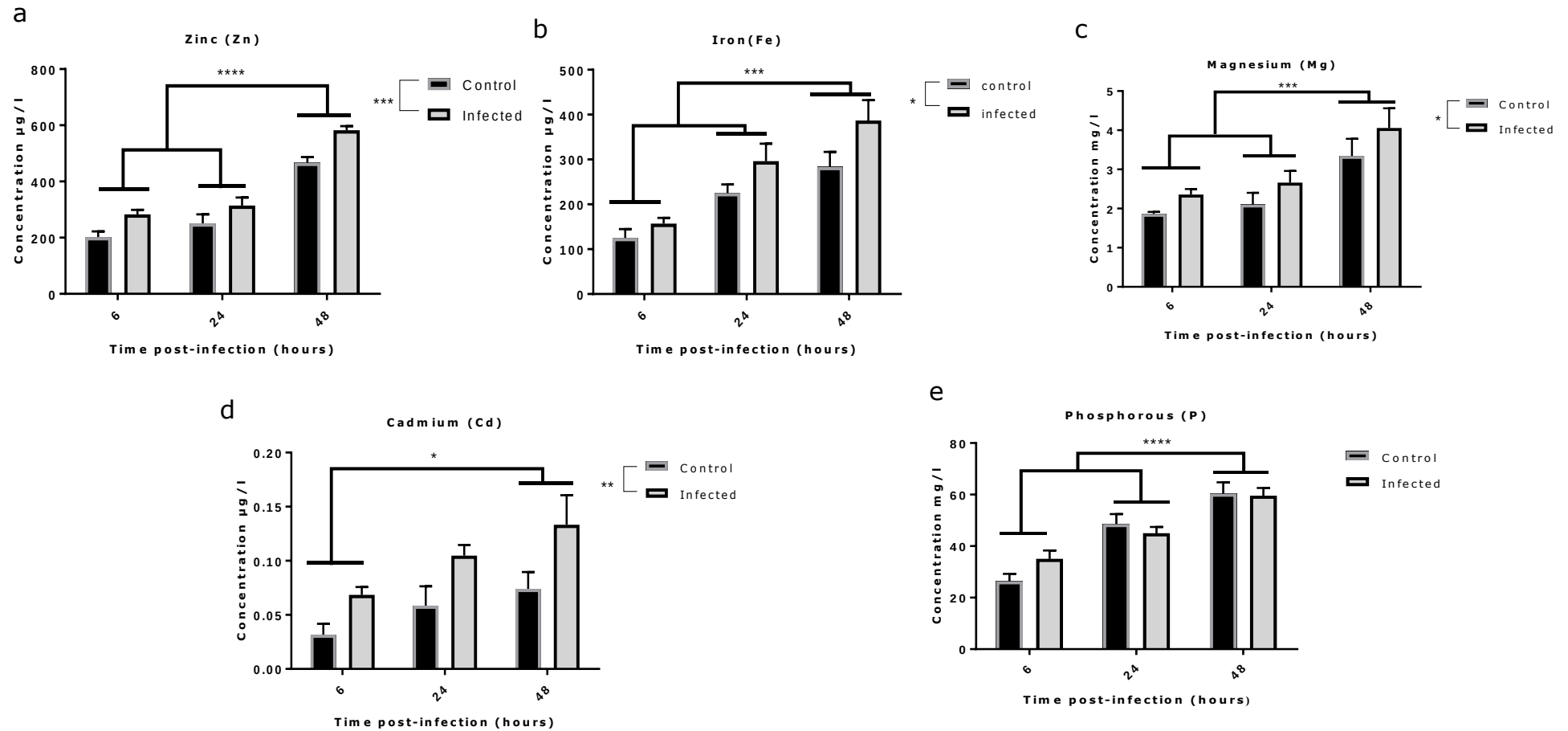


Figure 6.2: Concentrations of Zn, Fe, Mg, Cd, and P in HBMECs infected and non-infected with *T. gondii* over 48 h PI. Significant increases were noted in the level of Zn (** $P = 0.0006$) (Figure 6.2a), Fe (* $P = 0.0181$) (Figure 6.2b), Mg (* $P = 0.0488$) (Figure 6.2c), and Cd (* $P = 0.0037$) (Figure 6.2d) between the control and infected cells across all times. Significant increases in these and in P levels (**** $P < 0.0001$) (Figure 6.2e) were also noted at 48 h compared to 6 and/or 24 h across both groups .

Analysis of the K data revealed a significant main effect of time ($F_{(2, 12)} = 30.49$, $P < 0.0001$), but no significant main effect of group ($F_{(1, 12)} = 0.02485$, $P = 0.8774$) or group $\times$ time interaction ($F_{(2, 12)} = 0.2945$, $P = 0.8774$) (Figure 6.3a). Post-hoc analysis indicated that K was significantly increased at 48 h compared to 6 and 24 h PI, regardless of group ($P < 0.0001$).

Analysis of the Mn data revealed a significant main effect of time ($F_{(2, 12)} = 6.019$, $P = 0.0155$), but no significant main effect of group ($F_{(1, 12)} = 2.810$, $P = 0.1195$) or group $\times$ time interaction ($F_{(2, 12)} = 1.300$, $P = 0.3082$) (Figure 6.3b). Post-hoc analysis indicated that Mn was significantly increased at 48 h compared to 6 h PI, regardless of group ($P = 0.0155$).

Analysis of the Cu data revealed a significant main effect of time ($F_{(2, 12)} = 7.463$, $P = 0.0078$), but no significant main effect of group ($F_{(1, 12)} = 3.304$, $P = 0.0942$) or group $\times$ time interaction ($F_{(2, 12)} = 1.417$, $P = 0.2803$) (Figure 6.3c). Post-hoc analysis indicated that Cu was significantly increased at 48 h compared to 24 h PI, regardless of group ($P = 0.0078$).

Analysis of the Se data revealed a significant main effect of time ($F_{(2, 12)} = 17.96$, $P = 0.0002$), but no significant main effect of group ($F_{(1, 12)} = 0.7480$, $P = 0.4041$) or group $\times$ time interaction ($F_{(2, 12)} = 2.037$, $P = 0.1731$) (Figure 6.3d). Post-hoc analysis indicated that Se was significantly increased at 48 h compared to 6 and 24 h PI, regardless of group ($P = 0.0002$).

Analysis of the Rb data revealed a significant main effect of time ($F_{(2, 12)} = 42.52$, $P < 0.0001$), but no significant main effect of group ($F_{(1, 12)} = 0.1869$, $P = 0.6732$) or group $\times$ time interaction ($F_{(2, 12)} = 0.5607$, $P = 0.5851$) (Figure 6.3e). Post-hoc analysis indicated that Rb was significantly increased at 48 h compared to 6 and 24 h PI, regardless of group ($P < 0.0001$).

Analysis of the S data revealed a significant main effect of time ($F_{(2, 12)} = 13.66$, $P = 0.0008$), but no significant main effect of group ($F_{(1, 12)} = 0.7131$, $P = 0.4149$)

or group $\times$ time interaction ($F_{(2, 12)} = 0.4984$, $P = 0.6195$) (Figure 6.3f). Post-hoc analysis indicated that S was significantly increased at 48 h compared to 6 and 24 h PI, regardless of group ($P = 0.0008$).

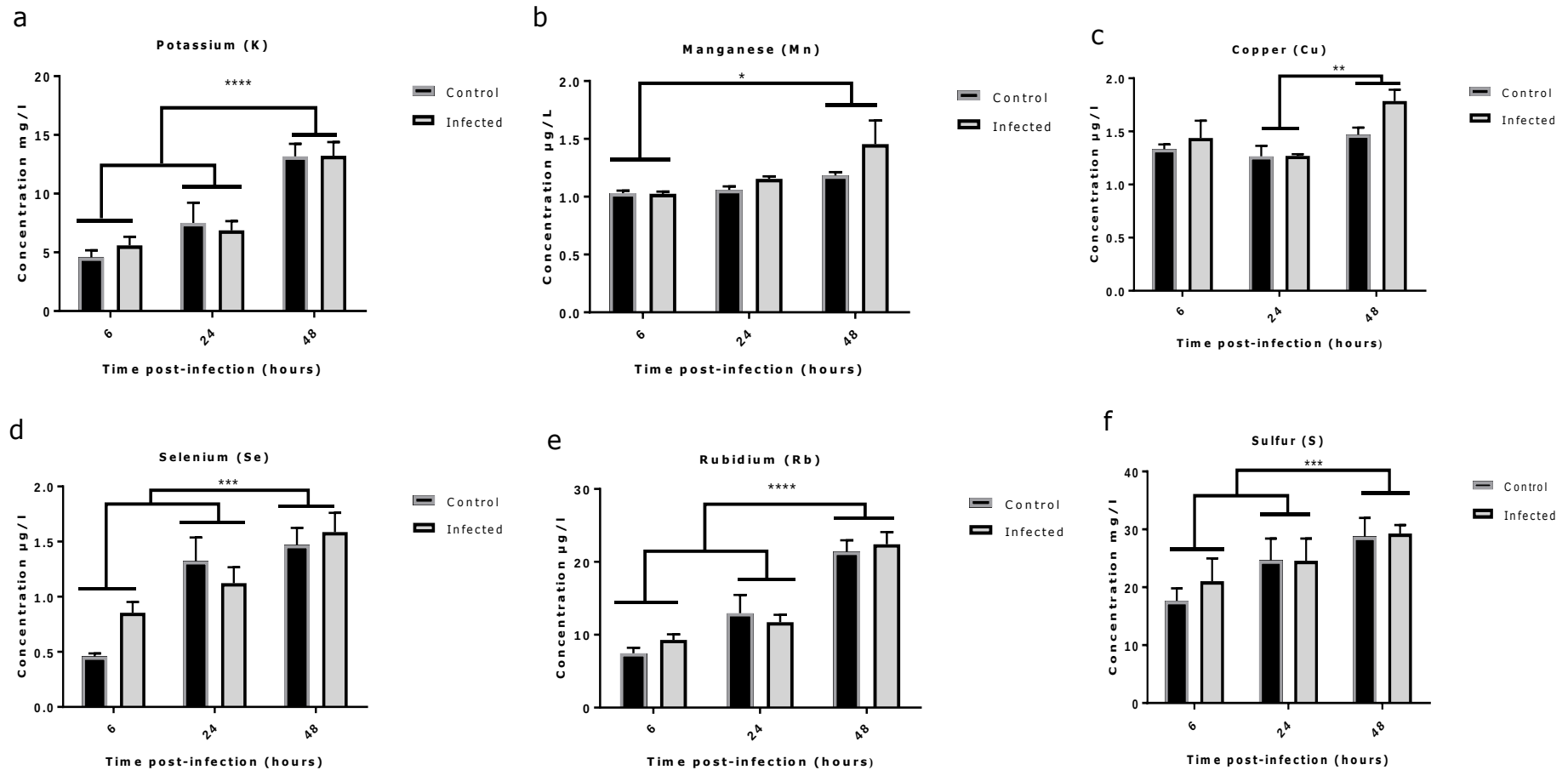


Figure 6.3: Concentrations of K, Mn, Cu, Se, Rb, and S in HBMECs infected and non-infected with *T. gondii* over 48 h PI. Significant increases were noted in the level of K (**** $P < 0.0001$) (Figure 6.3a), Mn (* $P = 0.0155$) (Figure 6.3b), Cu (** $P = 0.0078$) (Figure 6.3c), Se (*** $P = 0.0002$) (Figure 6.3d), Rb (**** $P < 0.0001$) (Figure 6.3e), and S (*** $P = 0.0008$) (Figure 6.3f) at 48 h compared to 6 and/or 24 h across both groups.

6.3.2 Trace element levels in growth media of HBMECs

Trace elements in the growth medium obtained from the infected and non-infected cell cultures at different time points PI are shown in Table 6.2. The following 22 trace elements did not show any significant differences between media from control and infected cells at different time points: B, S, Ca, Cr, Fe, Ni, Cu, As, Se, Mo, Cd, Cs, Ba, Na, Mg, P, K, V, Mn, Zn, Rb, and Sr (all $P > 0.05$). However, significant differences were noted in the levels of Co between media from the control and infected cells at 24 h PI ($P < 0.05$) (Table 6.2).

Mean±S.E.M									
Elements	6 h		24 h		48 h		*P value		
	Control	Infected	Control	Infected	Control	Infected	a	b	c
B	0.728± 0.002	0.72±0.001	0.729±0.001	0.73±0.0003	0.729± 0	0.73±0.001	0.06	0.2621	0.2621
Na	3079±32.34	3106±47.56	3212±33.33	3160±37.34	3123±100.24	2976±86.50	0.29	0.12	0.40
Mg	11.310±0.12	11.47±0.33	11.92±0.15	11.84±0.15	11.65±0.37	11.08±0.32	0.47	0.122	0.39
P	163.90±2.44	166.90±5.98	174±5.29	174.18±2.76	168.24±4.69	154.6±7.74	0.43	0.08	0.27
S	274.19±2.721	287.347±1.196	289.89± 3.450	290.89±4.37	286.73±10.03	272.09±7.78	0.97	0.16	0.094
K	198.14±2.29	199.76±2.73	206.75±1.735	202.89±2.34	199.11±6.21	189.54±5.75	0.24	0.06	0.39
Ca	1.57±0.003	1.59±0.031	1.59±0.003	1.58±0.003	1.59±0.012	1.56±0.01	0.93	0.79	0.31
V	0.92±0.03	0.95±0.03	0.82±0.23	0.74±0.01	0.76±0.04	0.74±0.004	0.30	0.82	0.82
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Mean±S.E.M									
Elements	6 h		24 h		48 h		*P value		
	Control	Infected	Control	Infected	Control	Infected	a	b	c
Cr	0.979±0.05	1.02±0.07	0.936±0.0357	0.83±0.06	0.995±0.08	0.84±0.06	0.15	0.1691	0.2570
Mn	0.53±0.01	0.55±0.04	0.51±0.01	0.50±0.01	0.55±0.08	0.51±0.04	0.79	0.71	0.78
Fe	173815.86	1793±5.10	1786±19.99	1782±18.38	1785±52.01	1726±88.46	0.93	0.81	0.45
Co	1.78±0.01	1.82±0.04	1.84±0.3	1.65±0.05	1.82±0.03	1.78±0.02	0.03	0.2007	0.0154
Ni	0.91±0.07	1.04±0.04	0.100±0.06	0.87±0.07	0.96±0.08	0.99±0.05	0.78	0.7333	0.1728
Cu	216.2±7.20	215.9±1.14	213.3±3.33	199.5±4.08	213.0±3.41	209.8±2.34	0.11	0.0989	0.2519
Zn	2.69±0.02	2.69±0.01	2.71±0.01	2.72±0.01	2.70±0.01	2.68±0.01	0.61	0.05	0.32
As	0.19±0.05	0.18±0.04	0.35±0.07	0.54±0.24	0.48±0.17	0.50±0.19	0.59	0.1195	0.7695
Continued on the next page									

Mean±S.E.M									
Elements	6 h		24 h		48 h		P value		
	Control	Infected	Control	Infected	Control	Infected	a	b	c
Se	1.170±0.00	1.183±0.003	1.190±0.012	1.187±0.003	1.19±0.015	1.163±0.015	0.5893	0.3654	0.2153
Rb	80.92±0.79	81.09±1.26	81.84±1.48	81.25±0.81	80.58±2.50	76.35±2.39	0.28	0.19	0.41
Sr	32.66±0.43	33.350±1.80	34.04±0.46	34.34±0.52	34.60±1.14	33.15±1.02	0.86	0.51	0.55
Mo	1.94±0.037	1.89±0.06	1.92±0.046	1.74±0.03	1.81±0.05	1.81±0.05	0.07	0.0959	0.1890
Cd	0.31±0.003	0.31±0.000	0.31±0.000	0.30±0.003	0.31±0.003	0.31±0.009	0.0821	0.516	0.7462
Cs	0.13±0.009	0.13±0.006	0.12±0.00569	0.12±0.003	0.126±0.006	0.13±0.015	0.76	0.2421	0.9056
Ba	1.06±0.08	1.04±0.01	0.98±0.003	0.96±0.01	0.99±0.01	0.99±0.02	0.58	0.0780	0.9749

Results are expressed as Mean ± Standard Error of the mean of the three replicates. * a: P value for the main effect of group (media from control vs infected cells), b: P value for the main effect of time (6 vs 24 vs 48 h), and c: P value for the group x time interaction.

Analysis of the Co data revealed a significant main effect of group ($F_{(1, 12)} = 5.921$, $p = 0.0315$) and group $\times$ time interaction ($F_{(2, 12)} = 6.110$, $P = 0.0148$), but no significant main effect of time ($F_{(2, 12)} = 1.757$, $P = 2141$) (Figure 6.4). Post-hoc analysis indicated that a significant increase in Co levels was observed in infected compared to control cells ($P = 0.0315$). It also indicated that Co was significantly decreased at 24 h in infected cells compared to controls ($P = 0.0148$).

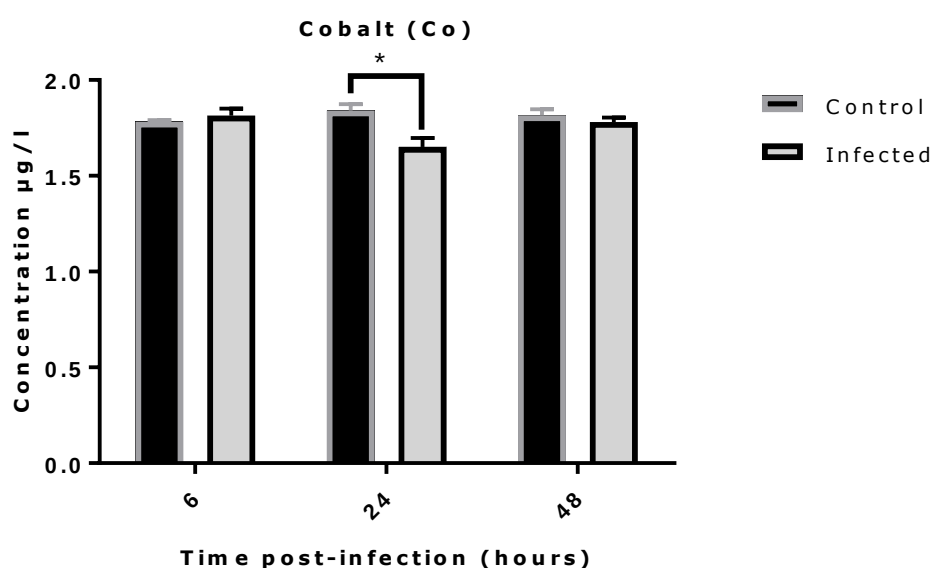


Figure 6.4: Concentrations of Co in the growth media obtained from HBMECs infected and non-infected with *T. gondii* over 48 h PI. A significant decrease was noted in the levels of Co (* $P = 0.0148$) between the control and the infected cells at 24 h PI.

6.3.3 Trace element levels in purified parasite preparations

The following pie chart shows the proportions of trace elements in *T. gondii* tachyzoites. Zn and Cu were the most abundant trace element as they represented 74% and 13% of the total of trace elements, respectively. Fe, Mn, P were also considerably abundant, constituting 5%, 3%, 2% respectively, whereas Na, S, and Rb accounted for ~1 % each (Figure 6.5).

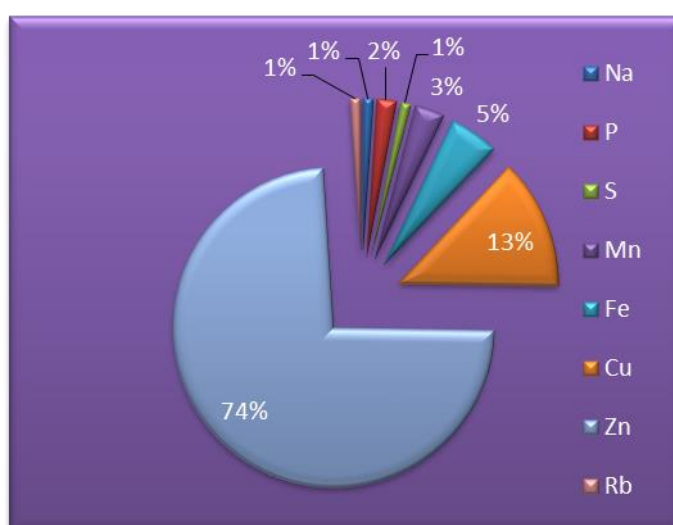


Figure 6.5: Proportions of trace elements in *T. gondii* tachyzoites. Sodium (Na), phosphorous (P), sulfur (S), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), and rubidium (Rb).

6.4 Discussion

The present work investigated alterations in the levels of trace elements in HBMECs over the course of *T. gondii* infection. Dynamic changes in the concentrations of 23 trace elements were determined in infected and non-infected cells at 6, 24, and 48 h PI. Significant increases were noted in the levels of Zn, Fe, Mg, and Cd in infected compared to control cells at all time points. The levels of these trace elements were also significantly increased at 48 h compared to 6 and/or 24 h across both groups. Significant increases were also noted in the levels of P, K, Mn, Cu, Se, Rb, and S at 48 h compared to 6 and/or 24 h across both groups. However, no significant differences in B, Na, Ca, V, Cr, Co, Ni, As, Sr, Mo, Cs, and Ba levels were observed between groups or over time.

Dynamic changes in the concentrations of 23 elements were also determined in the growth medium obtained from the infected and non-infected cell cultures at the same time points PI. A significant decrease was noted in the level of Co from the media of infected compared to control cells at 24 h PI. However, no significant differences in B, S, Ca, Cr, Fe, Ni, Cu, As, Se, Mo, Cd, Cs, Ba, Na, Mg, P, K, V, Mn, Zn, Rb, and Sr levels were observed between groups or over time.

A marked increase in the level of Zn was noted in infected cells compared to non-infected controls. It is possible that increased Zn levels, whether a parasite-induced or host-cell response, is important for protection against cellular oxidative stress and DNA damage during infection (Oteiza et al., 2000; Ho et al., 2003), and for suppressing cell apoptosis (Beyersmann and Haase, 2001). Zn is a major intracellular regulator of apoptosis because a decrease in the level of Zn can induce apoptosis, whereas increased Zn levels inhibit the induction of apoptosis via the modulation of caspase-3 activity (Sunderman, 1995; Truong-Tran et al., 2001). It is known that the exposure of culture cells to membrane-permeable Zn chelators can induce apoptosis in virally transformed cells, while normal cells remain unaffected under these conditions (Fernandez-Pol et al., 2001). The effects of Zn

have been linked to several potential mechanisms, including on the pathogen invasion and replication (Prasad, 2000; Prasad et al., 2007; Overbeck et al., 2008). An *in vitro* study revealed that Zn inhibits the invasion and replication of influenza virus (Uchide et al., 2002), respiratory syncytial virus (Suara and Crowe, 2004), SARS-coronavirus (Te Velthuis et al., 2010), equine arteritis virus (Te Velthuis et al., 2010), and transmissible gastroenteritis virus (Wei et al., 2012) by preventing viruses binding to ICAM-1 receptor.

The present results also show that *T. gondii*-infected cells exhibit elevated levels of Fe. Alteration in the level of Fe raises the possibility that this trace element is essential for *T. gondii* intracellular growth. Fe is a cofactor of many enzymes involved in normal functioning of the nervous system, mitochondria electron transport, and metabolism (Soetan et al., 2010). Decreased or increased levels of Fe may cause mitochondrial DNA damage (Gao et al., 2009). Any damage to mitochondrial DNA can cause an imbalance in oxidative stress, which may result in an increased ability of microorganisms to replicate in the host cell. Schaible & Kaufmann (2004) have shown that Fe is crucial for both intracellular pathogens and their hosts. Intracellular protozoan parasites such as *T. gondii* possess mechanisms to obtain Fe from their host to sustain their replication. This pathogen hijacks Fe in the host cells by binding to transferrin receptor (TfR) during cell entry. The host expression of TfR is up-regulated in *T. gondii*-infected cells, suggesting that the parasite uses host endocytic pathway and H.O.S.T. structure for Fe acquisition. In the H.O.S.T. structures, it can be envisioned that host Fe transporters of the Nramp family pumps Fe across the endo-lysosomal membrane, where *T. gondii* Fe transporters in the PVM could transport Fe into the PV for uptake by *T. gondii* (Gail et al. 2004). Drakesmith & Prentice (2008) have also shown that viruses, such as human immunodeficiency virus (HIV) and hepatitis C, hijack Fe in the host cells to sustain their replication. These viruses hijack Fe either by binding to TfR during cell

entry or alter the expression of proteins involved in Fe homeostasis, such as human hemochromatosis and hepcidin proteins.

We also showed a marked increase in the levels of Mg in *T. gondii*-infected cells compared to non-infected controls. The altered levels of Mg raises the possibility that this trace element is essential for the intracellular development of *T. gondii*. Mg is a component of metalloenzymes, which play a significant role in the normal functioning of the immune system (Galland, 1988). Decreased levels of Mg may reduce some aspects of immune function and increase susceptibility to a wide range of infectious agents. However, excess amounts of Mg can also impair immune function (Galland, 1988). Seyerk et al. (2004) have shown that the concentration of Mg in serum is higher in sero-positive toxoplasmosis sheep. However, Mg concentrations in the serum of sero-positive women was decreased. In a study carried out on rats experimentally infected with *Toxoplasma*, the levels of Mg in the brain of the infected group was significantly reduced (Geng et al., 2000).

A marked increase in the levels of Cd was noted in infected cells compared to non-infected controls, indicating that Cd might be involved in the growth of the parasite. Cd inhibits antioxidant enzymes (Gupta et al., 1991; Bagchi et al., 1996; Asagba, 2007; Chater et al., 2009), which can lead to increased oxidative stress and may result in an increased ability of pathogens to replicate in host cells. Increased replication of influenza virus was noted in MDCK cells after exposure to Cd (Checconi et al., 2013). A study carried out on Cd-exposed mice found that animals exposed to Cd were more susceptible to *Listeria monocytogenes* and to other opportunistic infections than control mice (Simonyte et al., 2003).

A marked decrease in the level of Co was noted in the growth medium obtained from infected cells compared to non-infected controls at 24 h PI, indicating that Co might be involved in the building of newly generated parasites with a generation time of 24 h. Co is involved in the synthesis of vitamin B12, which is required for

the methylation of choline and thiamine. The latter is involved in the synthesis of DNA, regulating proliferation and growth of the parasite (Zock and Katan, 1998). Furthermore, Co is required as a cofactor for many enzymes involved in DNA biosynthesis and amino acid metabolism (Arinola et al., 2008). Bakhshi et al. (2013) have shown that the serum concentration of Co was higher in cattle infected with *Theileria*. Increased levels of Co were also noted in the plasma of A/J mice during the pre-inflammatory stage of Coxsackie B virus infection (Funseth et al., 2000). However, the levels of Co in the heart of infected animals were reduced (Funseth et al., 2000).

In the present study, a significant increase was noted in the levels of P, K, Mn, Se, Rb, and S at 48 h compared to 6 and/or 24 h across both groups, indicating that these trace elements might act as a cofactor of many enzymes involved in DNA replication and protein synthesis (Chaturvedi et al. 2004; Modlin 1990). Cu, like P, K, Mn, Se, Rb, and S, is an essential trace element. It is necessary for the normal functioning of the nervous system and is required for maintaining the balance of useful metals in the body, such as Zn and Mo.

Taken together, these findings indicate that infection of HBMECs with *T. gondii* is associated with alterations in the levels of Zn, Fe, Mg, Cd, and Co. Further studies are needed to confirm these results by using the available tools in cell biology, molecular genetics and array technology. Moreover, these findings warrant further investigations to plan the strategies for trace element supplementation or reduction in addition to evaluating their potential as diagnostic parameters for monitoring the progression of infection or responses to anti-*T. gondii* therapy. Further studies are also required to establish the role of these trace elements in host defence against *T. gondii* by profiling the immunological changes in response to infection and correlate findings with the trace element profile.

7. General discussion

T. gondii is a major food-borne parasite (Tenter et al., 2000). About one-third of the world's human population is latently infected by this parasite (Ajzenberg, 2011; Montoya and Liesenfeld, 2004). In humans, *T. gondii* causes severe morbidity and mortality not only in congenital infections but also in immune-deficient individuals. In addition to its public health aspects, *T. gondii* causes major economic losses in the sheep and goat farming industries as it is one of the most important abortifacient agents in these ruminants (Dubey et al., 1981). Unlike other infections, *T. gondii* infection does not always lead to the development of clinical signs in infected animals. As a result, the farmers remain unaware of the presence of the parasite on their farms. Infection of livestock, in turn, creates a source of infection in humans, and consumption of raw or undercooked meat is considered to be the major source of *T. gondii* infection (Boyer et al., 2005; Cook et al., 2000).

T. gondii infection is critical not only in pregnant women but also in individuals with compromised immune systems such as patients with HIV/AIDS and on those undergoing cancer therapies. Moreover, *T. gondii* can be passed along from mother to the fetus, putting the baby at risk of severe neurological disease. It has long been known that *T. gondii* can affect the brain. However, the manner in which the parasite breaches the BBB, the biological barrier intended to keep pathogens out of the brain, remains debatable.

Over the years, several theories have been considered to explain how *T. gondii* enters the brain. It has been proposed that *T. gondii* can breach the BBB paracellular route by disrupting the tight junctions or by infecting leukocytes (i.e. 'Trojan horse' mechanism), in which the parasite squeezes between the barrier cells while hiding inside infected monocytes and macrophage (Kim, 2008). Recently, Konradt et al. (2016) identified the mechanism of entrance of parasite into the brain. By using multi-photon microscopy, researchers found that *T. gondii* infects the brain's endothelial cells lining the BBB and then proliferates inside them to

move forward to invade the brain. The ability of intracellular pathogens to infect the brain's microvascular endothelial cells is the key to their ability to cause brain damage (Däubener et al., 2001).

Hence, in the present study, HBMECs were used as an *in vitro* BBB model, as these cells closely resemble the native BBB cells in terms of barrier tightness and paracellular permeability (Eddy et al., 1997). The aim of using these cells was to elucidate the morphological and metabolic changes in HBMECs following infection with *T. gondii*. It is well known that *T. gondii* can infect a wide range of mammalian cells including HBMECs (Däubener et al., 2001). However, little is known about the role of *T. gondii* infection in morphological and metabolic changes in HBMECs.

In the first part of this thesis, the intracellular development of *T. gondii* was studied by using AO staining and EM at different time-points (6, 24 and 48 h) PI. The integrity of the HBMEC monolayer and tight junction permeability during *T. gondii* infection and the morphological changes associated with infection were assessed by TEER and EM at the same time-points. These time-points were selected to cover the spectrum of changes that happened in infected HBMECs during the course of the parasite intracellular infection cycle.

The major part of this study was to (i) identify cell viability and metabolic changes associated with infection using alamar blue and $^1\text{H-NMR}$, (ii) evaluate the changes in lipid content and fatty acid composition of the total phospholipids using lipid^{Tox} staining and GC and (iii) assess the changes in the content of trace elements in response to *T. gondii* infection using ICP-MS (Figure 7.1).

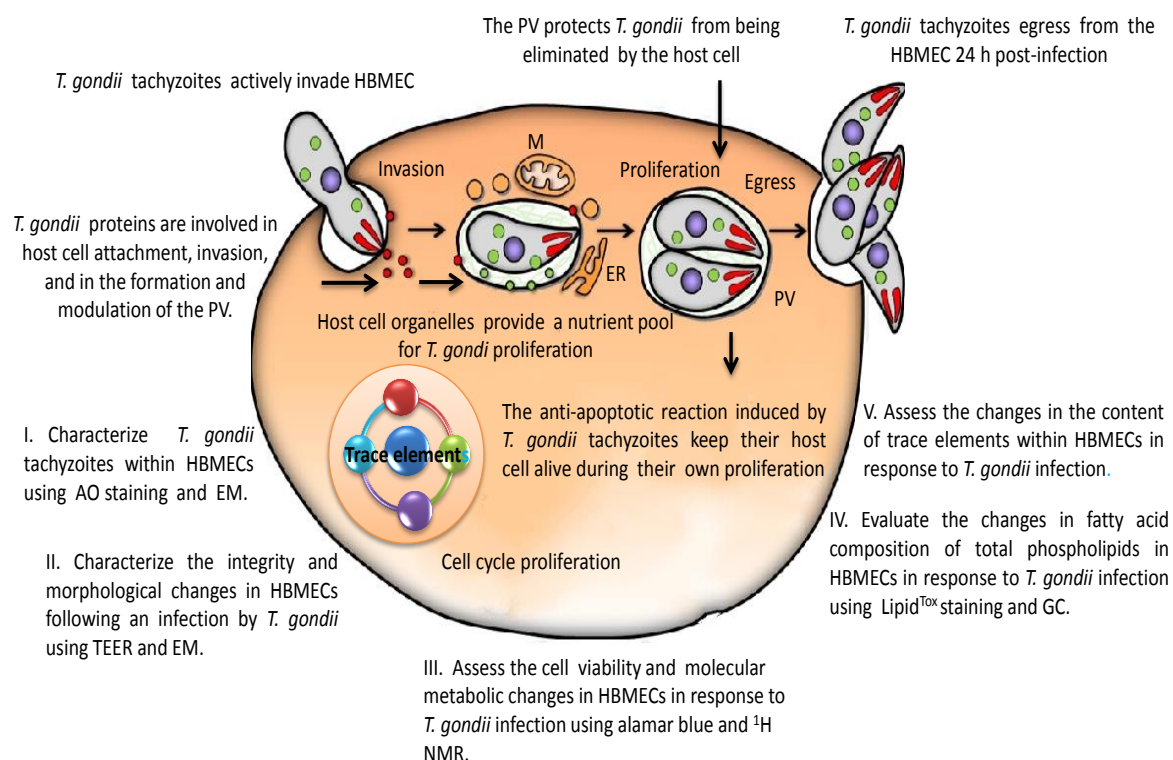


Figure 7.1: Schematic representation of interaction of *T. gondii* with HBMECs *in vitro*. The tachyzoites actively invade their host cell and form their own compartments (PV) in which tachyzoites proliferate with a generation time of 24 h, eventually leading to cell rupture and exit of the parasites. During the course of the infection, the tachyzoite organelles fuse with the parasite membrane and discharge their (protein) contents in the PV. Host cell organelles are recruited around the PV, potentially to serve as a nutrient source. *T. gondii* inhibits host cell apoptosis by altering the trace element levels. I–V represent the range of studies referred to in this thesis. M: mitochondria; ER: endoplasmic reticulum; PV: parasitophorous vacuole. Modified from Sébastien, (2015).

T. gondii RH tachyzoites can invade and proliferate intracellularly in HBMECs, thereby disrupting the integrity of the HBMEC monolayer during the course of an infection. Tachyzoites exhibited a pattern of growth and replication which has also been observed in *T. gondii* infected macrophage, human monocyte, human astrocytoma, human retinal, Hela and VERO cells (Sharma et al., 1986; De Melo et al., 1992; Marin et al. 1996; Pelloux et al., 1996; Toulah et al., 2011; Romano et al., 2013; Naemat et al., 2015). It has been shown that tachyzoites actively invade their host cell and form their own compartments (PV) in which tachyzoites proliferate with a generation time of 24 h, eventually leading to cell rupture and exit of the parasites.

Intracellular development of *T. gondii* revealed a decrease in the resistance of infected cells by TEER assay as compared to that in uninfected controls, indicating that the invasion of the HBMECs by *T. gondii* has detectable effects on the integrity of the HBMEC monolayer. A decrease in the TEER has been observed in HBMECs infected with arenavirus (Lander et al., 2014) and in porcine brain capillary endothelial cells infected with *Plasmodium falciparum* (Treeratanapiboon et al., 2005).

To highlight the morphological changes associated with infection, EM was employed to observe the disruption of tight junctions of HBMECs and reorganization of host organelles around the PV. Interestingly, there was a clear disruption of tight junctions between HBMECs during the intracellular development of tachyzoites within these cells by EM experiment as compared with that by TEER assay. EM experiment showed clearly tight junction damage by *T. gondii* infection as compared with that by TEER assay, which showed only a modest change in the tight junctions, because allowed the cells to grow for only one day (in the EM experiment) might not be enough for the tight junctions to be formed between the cells. However, allowed the cells to grow for at least 5 days (in the TEER assay) led to formation of confluent monolayer with reasonably sound tight junctions between the cells. Allowed the cells to grow for 5 days (as in the TEER assay) and then

conduct the EM experiment Or allowed the cells to grow for only one day (as in the EM experiment) and then conduct the TEER experiment is required to assess disruption of tight junctions between HBMECs during the intracellular development of tachyzoites within these cells at 1 versus 5 days. It is well known that several neuropathic pathogens such as arenavirus, *P. falciparum* and *T. gondii* (Lander et al., 2014; Treeratanapiboon et al., 2005; Nogueira et al, 2015) can disrupt the endothelium junction complexes by different factors such as direct toxic effects, interfering with the junctional formation and the induction of an inflammatory response, which in turns may compromise the endothelial barrier integrity (Zougbedé et al., 2011; Kang & McGavern 2010; Prato et al., 2011; Lacerda-Queiroz et al., 2011).

Furthermore, some HBMEC organelles (e.g. the mitochondria, ER and Golgi apparatus) reorganized around the PVM, and this reorganization remained stable throughout the growth of the tachyzoites. This pattern of reorganization of the host cell organelles has also been observed in Hela and VERO cells infected with *T. gondii* (De Melo et al., 1992; Toulah et al., 2011; Romano et al., 2013). It has been shown that the mitochondria in uninfected cells are elongated structures that extend from the nuclear region toward the cell membrane. After parasite invasion, mitochondria become shorter and tend to concentrate around the PVM. At present, there exists no explanation for this alteration in the dimensions of the mitochondria. It is believed that increased ATP production as source for energy to the parasites, primarily via glycolysis, and modulation of host cell death via the mitochondrial pathway results in changes in the dimensions of the mitochondria. The host ER and Golgi apparatus also concentrate around, and even establish physical contact with, the PVM (De Melo et al., 1992; Romano et al., 2013). The tendency of the HBMEC organelles to concentrate around the PVM may provide energy and essential nutrients to the parasites (e.g. choline, serine and ethanolamine) to ensure appropriate synthesis of phosphatidylcholine,

phosphatidylserine, and phosphatidylethanolamine required for parasite replication (Gupta et al., 2005). It is well known that *T. gondii* can take up their lipids precursors (e.g. choline, serine, and ethanolamine) from host cells for incorporation into complex phospholipids (Charron and Sibley, 2002; Gupta et al., 2005). Quantitative measurements of the phospholipid profile of *T. gondii* revealed that phosphatidylcholine is the most abundant phospholipid, accounting for about 75% of all phospholipids (Gupta et al., 2005). The next most abundant phospholipids are phosphatidylethanolamine (10%) and phosphatidylserine (6%). A study by Charron and Sibley (2002) using fluorescent lipid [oleic-1-14C] L- α -phosphatidic acid has shown that only *T. gondii* tachyzoites inside host cells further metabolize the scavenged lipid into phosphatidylcholine.

The invasion of a cell by tachyzoites is an active and highly organized process mediated by the sequential protein secretion from MICs, ROPs and GRA organelles. *T. gondii* presents three organelles involved in host cell attachment, invasion and the formation and modulation of the PV. It is known that MICs, ROPs and GRAs fuse with the parasite membrane and discharge their (protein) contents in the PV (Dubremetz et al., 1993; Carruthers and Sibley 1977). MIC proteins play a central role in the attachment to host cells via the binding of specific receptors (e.g. ICAM 1) (Carruthers 1999; Carruthers et al., 2000). ROPs, which are discharged immediately following MIC secretion, accumulate on the parasite surface to form the moving junction (Alexander et al., 2005). GRAs are released into the PV after invasion, forming a tubular network inside the PV that supports the characteristic rosette arrangement of parasites (Mercier et al., 2002; Magno et al., 2005). Whereas, the PVM helps protect the parasite from host cell elimination as well as isolates it from the abundant source of nutrients in the host cytosol. To avoid this issue, the parasite elaborates pores in the PVM that permits bidirectional diffusion of small molecules (Schwab et al., 1994). PVM proteins ensure nutrient acquisition and the recruitment of host cell organelles, including mitochondria, ER and Golgi

apparatus. In addition, the tight association of the host mitochondria and ER to the PVM suggests a possible transfer of metabolites to the parasite (Sinai et al., 1997).

To survive and ensure division, *T. gondii* must acquire nutrients from its host. It is well known that *T. gondii* uptakes tryptophan (Pfefferkorn 1984; Pfefferkorn et al., 1986; Dai et al., 1994), arginine (Fox et al., 2004; Gail et al., 2004), polyamines (Seabra et al., 2004), choline (Gupta et al., 2005), purines (Schwartzman and Pfefferkorn 1982; Chaudhary et al., 2004), cholesterol (Coppens & Joiner 2003; Coppens et al., 2004), iron (Gail et al., 2004; Dimier and Bout 1998), and long-chain fatty acids (Charron & Sibley 2002) through the PVM pores. Although *T. gondii* surrounded by the PVM are protected against acidification from the fusion of endocytic vesicles, the non-fusion state deprives *T. gondii* of an abundant source of nutrients from the host cell cytosol. *T. gondii* instead uptakes small molecules that cross the PVM via pores in a passive manner that is independent of the temperature and energy, thereby forming bidirectional molecular exchange with the host cells. These pores allow the acquisition of small soluble metabolites (<1300–1900 Da) such as glucose, amino acids, nucleotides, and ions for parasite survival, proliferation, membrane biogenesis and energy requirement (Schwab et al., 1994). Catabolic waste products generated by *T. gondii* are possibly discarded into the host cell cytosol in the same manner.

Although passive diffusion of some compounds is sufficient to supply intracellular parasites, some metabolites are found in low concentrations in the host cell cytosol bound to large proteins or are insoluble, thereby limiting acquisition by diffusion. Therefore, active transport is necessary at the PVM and at the plasma membrane of *T. gondii*. Proteins associated with the PVM are issued from a ROP during *T. gondii* invasion and also secretion from GRAs after entry. Rhoptry protein 4 (ROP4), 5 (ROP5), 7 (ROP7), 8 (ROP8), 16 (ROP16), and 18 (ROP18) get associated with the PVM following invasion, suggesting their likely role in host interaction. Dense

granule protein 3 (GRA3), 5 (GRA5), 7 (GRA7), 8 (GRA8), and 10 (GRA10) have been shown to associate with the PVM after entry (Ossorio et al., 1994; Ahn et al., 2005; Lecordier et al., 1999; Mercier et al., 2005). These PVM proteins are likely involved in several processes such as nutrient acquisition, remodeling of the IFs and MTs around the PV, reorganization of intracellular organelles, and the modulation of certain processes of the host cell (Martin et al., 2007). *T. gondii* infection causes the remodeling of host IFs and MTs around the PV (Halonen and Weidner 1994; Coppens et al., 2006). IF association with the PVM plays a role in positioning the PV close to the nucleus, whereas MTs are responsible for the movement and localization of intracellular organelles around the PVM (Halonen and Weidner 1994). Remodelling of MTs around the PV is dependent on tubulin polymerisation and depolymerisation. In addition, the MTOC is recruited from the nuclear membrane to the PVM, but how this benefits the parasite remains unclear (Coppens et al., 2006; Romano et al., 2007). Interestingly, endosomal/lysosomal vesicles are also detected close to the PV and have been implicated in the transfer of endolysosomal components, including cholesterol, from the host cell cytosol to parasites (Coppens 2006). Host cell cholesterol is crucial for *T. gondii* invasion and replication (Coppens et al., 2000). *T. gondii* does not synthesize its own cholesterol and instead uptakes it from the host. Coppens et al. (2000) reported that the growth of the parasite is enhanced by the addition of free cholesterol or cholesterol bound to LDL particles to the extracellular medium. Both extracellular parasites and parasites inside the PV can incorporate free cholesterol, suggesting the existence of cholesterol-acquisition machinery at the plasma membrane of the parasite and PVM (Sehgal et al., 2005).

The association between the PV and host cell organelles (e.g. mitochondria, ER and Golgi apparatus) was observed shortly after invasion, and the association remained stable throughout the growth of the PV, suggesting the involvement of molecules initially incorporated in the PVM. The tight association between host cell organelles

has been observed with the mitochondria, ER and Golgi apparatus of *T. gondii*-infected VERO and Hela cells, as well as has been implicated in the direct transfer of phospholipids in a manner independent of vesicular transport (Achleitner et al., 1999; Voelker 2005). Similarly, the tight association between the PVM and host cell organelles may provide a key source of new phospholipids (Sinai & Joiner 2001), which could be incorporated into the PVM to ensure PV expansion during the endodyogeny process of cell division. Although *T. gondii* can synthesize certain phospholipids, it must acquire lipid precursors (e.g. choline, serine, and ethanolamine) from the host cells for incorporation into complex phospholipids that are necessary for its replication. As a likely indicator of parasite siphoning, the production of choline and myo-inositol by the HBMECs is decreased in response to *T. gondii* invasion and replication. In the present study, intracellular development of *T. gondii* showed a decrease in cell viability of the infected cells by alamar blue staining, as compared to uninfected controls, indicating that the growth of the parasites incurred a metabolic burden on the host cells. Lactate and glutamine are other essential nutrients that are increased in response to *T. gondii* infection. Lactate and glutamine are used as sources for energy, primarily via glycolysis and glutaminolysis (Blader & Koshy, 2014; Kim & Weiss 2004; Dubremetz & Lebrun 2012; Hunter & Sibley 2012). It is believed that increased lactate and glutamine levels result in increased paracellular permeability. Supplementing glutamine to the jejunal mucosa led to an increase in the ion pump activity and increased paracellular permeability (Yang et al., 1999).

Folates and fatty acids can be acquired from the host cells or synthesized *de novo* by *T. gondii* (Charron & Sibley 2002; Massimine et al., 2005; Quittnat et al., 2004). Both the synthesis and scavenging actions indicate that the genomic presence of biosynthetic genes for essential nutrients does not necessarily mean that the parasite is a prototroph for such metabolites. Intracellular *T. gondii* can uptake selected fatty acids (Tomavo et al., 1989; Quittnat et al., 2004). Inside parasites,

fatty acids are rapidly manufactured into triacylglycerols (Quitnat et al., 2004) and cholesteryl esters (Nishikawa et al., 2005), with a two-fold higher incorporation of palmitic acid (C16:0) than other fatty acids, including oleic acid (C18:1n9c), stearic acid (C18:0), linoleic acid (C18:2n6c) and arachidonic acid (C20:4n6). After the uptake of butyric acid (C4:0) by *T. gondii*, the lipid is anabolized into phosphatidylcholine (Charron and Sibley, 2002). It is believed that increased phosphatidylcholine levels result in altered monolayer permeability. Sawai et al. (2001) have shown that phosphatidylcholine can be hydrolyzed into lysophosphatidylcholine by the enzyme phospholipase, resulting in significantly decreased TEER.

Genomic searches have identified three elongase genes (i.e. ELO-A, ELO-B, ELO-C) in *T. gondii*, and their activity could be a source of synthesis of longer chain fatty acids (Bisanz et al., 2006; Mazumdar et al., 2006). It is well known that myristic acid (C14:0) and palmitic acid (C16:0) synthesised by FASII are further elongated by ER elongases to produce long-chain monounsaturated fatty acids. Furthermore, intracellular parasites synthesise various enzymes, such as phospholipase, that may target host lipids or take advantage of the host's lipid metabolizing enzymes. It has been shown that phospholipids can be hydrolyzed into lysophospholipids and free fatty acids by phospholipase, resulting in altered monolayer permeability (Sawai et al., 2001). Moreover, both tachyzoite and host-derived phospholipase are involved in the release of arachidonic acid (C20:4n6) from phospholipids of the host cell membrane, whereby they alter the host membrane fluidity and facilitate the invasion by tachyzoites (Li et al., 2008). Kohn et al. (1980) have shown that interaction of monounsaturated and polyunsaturated fatty acids (e.g. oleic acid [C18:1n9c], linoleic acid [C18:2n6c], arachidonic acid [C20:4n6]) with animal cells (e.g. embryonated egg, mouse brain, buffalo green monkey, Vero cells, monkey epithelial cells) induces a change in the fluidity of their membranes. It is also believed that increased monounsaturated and polyunsaturated fatty acids result in

increased tight junction permeability via modulated occludin and ZO-1 localization. Moreover, the fatty acid levels affect occludin protein expression in the umbilical vein endothelial cell line and in the brain capillary endothelial cells (Yamagata et al., 2003). Immunofluorescent staining of Caco-2 cell epithelial monolayers grown in media supplemented with linoleic acid (C18:2n6c) revealed that this polyunsaturated fatty acid modulated occludin and ZO-1 localization, which, in turn, involved significantly increased tight junction permeability (Roche et al. 2001). Similarly, thin-layer chromatography of Caco-2 cell epithelial monolayers grown in media supplemented with palmitoleic acid (C16:1n7c) and oleic acid (C18:1n9c) increased the tight junction permeability (Sawai et al., 2001).

Trace elements are required for a multitude of cellular housekeeping functions, including replication, transcription, and signal transduction. In addition, trace elements are essential for the maintenance of membrane barrier integrity, protection against pathogens and regulation of immune responses (Chaturvedi et al. 2004; Modlin 1990). Notably, Zn levels increase in HBMECs infected with *T. gondii*. It is possible that increased Zn levels, either parasite-induced or as a host-cell response, is important for protection against cellular oxidative stress, DNA damage during infection (Oteiza et al., 2000; Ho et al., 2003), and suppression of cell apoptosis (Beyersmann and Haase, 2001). The latter helps *T. gondii* avoid rapid clearance by macrophages, which are activated by signals emitted from apoptotic cells. It is well known that *T. gondii* actively blocks host apoptosis to their advantage to keep the host cell alive during their own proliferation. In a previous study, *T. gondii*-infected cells inhibited the apoptotic death receptor pathway by targeting caspase 8 via parasite-dependent proteolytic cleavage and degradation (Vutova et al., 2007). In addition, *T. gondii*-infected cells showed only low levels of cytochrome c release after induction of apoptosis via the mitochondrial pathway, thus reducing the activation of caspase 9 in the apoptosome, thereby preventing the activation of caspase 3 (Carmen et al., 2006; Keller et al., 2006). Furthermore,

it appears that *T. gondii* also independently targets caspase 3 by altering the Zn levels as this enzyme remains inactive.

Zn is a major intracellular regulator of apoptosis, because a decrease in the level of Zn can induce apoptosis, whereas increased Zn levels inhibit the induction of apoptosis via the modulation of caspase 3 activity (Sunderman, 1995; Truong-Tran et al., 2001). It is known that the exposure of culture cells to membrane-permeable Zn chelators can induce apoptosis in virally transformed cells, while normal cells remain unaffected under these conditions (Fernandez-Pol et al., 2001). The concentrations of trace elements in the target tissues of a specific infection also change during microbial infections (Beisel 1998; Edvinsson et al. 2008; Ilback et al., 2003; Ilback and Friman 2007; Ilback et al., 2004). These changes in trace element balance may be identified by increased synthesis of metalloproteins such as Zn carboxypeptidase A that occur in the serum and tissues during infections (Bremner and Beattie 1990; Del Razo et al. 2001; Ilback et al. 2004). It is believed that these events may occur to provide trace elements for the synthesis of proteins in the tissues as well as to change the serum balance of trace elements in order to suppress the growth and replication of microbes (Beisel 2004; Pekarek and Engelhardt 1981). The increase in metalloproteins may also cause an increased tissue uptake of non-essential trace elements, resulting in potentially toxic concentrations of these elements in the tissues (Ilback et al. 2004). In addition, the host inflammatory response to protozoal infection can generate ROS and NO species, which in turn stimulate inflammatory cytokines and MMP (e.g. Zn or co-dependent enzymes) secretion by endothelial cells of the BBB. Thus, protozoal infections of the brain have the potential to cause the BBB disruption with the induction of oxidative damage that alters the MMP activity (Mayhan 2001; Rao 2008; Hamada et al., 2013). Changes in the balance of essential or non-essential trace elements may affect replication/virulence of the invading pathogen as well as the outcome of an infection (Molin, 2010). Based on the reports of previous studies,

it is reasonable to assume that restoration of the disturbed balance, i.e. supplementation or reduction of specific trace elements, may speed up the recovery of an individual with infectious disease (Molin, 2010).

Taken together, these findings indicate that infection of HBMECs with *T. gondii* is associated with alterations in the levels of Zn, Fe, Mg, Cd and Co. Further studies are needed to confirm the results of our study by using advance available tools in cell biology, molecular genetics and array technology. Our findings also warrant further investigations to evaluate the potential benefits of supplementation or reduction programmes with trace elements status as cost-effective means of reducing the risk of infectious diseases. Zn, Fe, Mg, Cd and Co have immunomodulatory functions and thus influence the susceptibility of a host to various parasitic infections. Zn has anti-parasitic activity, while others may alter the genome of the parasites, enhancing their virulence. Further study is needed to assess the possible role of malnutrition in the emergence of parasitic diseases.

In conclusion, *T. gondii* can interact with HBMECs such that it promotes the growth and replication of the parasite. This interaction possibly leads to several physiological responses of both the parasite and host cells. These responses include (i) multiple morphological changes in the relocation of the host cell organelles around the PV and (ii) changes in the levels of host cell metabolites, fatty acids, and trace elements.

Although HBMECs are considered a good model to study *T. gondii* infection *in vitro*, co-culture with astrocytes and neurons may enhance the predictive power of the model to recapitulate more features for studying the developmental and pathophysiological processes of the BBB in response to infection. Furthermore, HBMECs co-cultured with astrocytes and neurons may provide a more realistic BBB model than the mono-culture model. In addition, the use of other strains, such as

strain II or III, to infect HBMECs is recommended. Labelling host cell or parasite membranes with probes specific for proteins and lipids is also recommended to identify the composition, abundance and distribution of lipids in *T. gondii*-infected cells by using fluorescence confocal microscopy as lipid accumulation in infected cells can be attributed to fatty acid synthesis in *T. gondii* tachyzoites or to the uptake of this fatty acid from HBMECs or both (Vial et al., 2003). Furthermore, label-free imaging to identify the composition, abundance and distribution of metabolites in *T. gondii*-infected cells using Raman micro-spectroscopy is also recommended, because metabolic accumulation in infected cells can be attributed to the synthetic /metabolic abilities of parasites or uptake of these compounds from the HBMECs or both. Such future studies may provide new insights that may facilitate detailed understanding of the impact of *T. gondii* infection on the host cell.

8. References

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