# The relationship between the infection of *Toxoplasma gondii* and Alzheimer's disease

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# Abbreviations

| | |
|---|---|
| Aβ | Amyloid-beta |
| AD | Alzheimer's diseases |
| AChE | Acetylcholinesterase |
| ATCC | American Type Culture Collection |
| BBB | Blood–brain barrier |
| BDNF | Brain-derived neurotrophic factor |
| CCR5 | C-C chemokine receptor type 5 |
| CNS | Central Nervous System |
| $CO_2$ | Carbon dioxide |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMEM- high glucose | Dulbecco's Modified Eagle's Medium - high glucose |
| DC | Dendritic cell |
| DG | Dentate gyrus |
| DNA | Deoxyribonucleic acid |
| DMSO | Dimethyl sulphoxide |
| dNTP | Deoxyribonucleotide triphosphate |
| ECACC | European Collection of Cell Cultures |
| EMEM | Minimum Essential Medium Eagle |
| FBS | Fetal bovine serum |
| FCS | Fetal calf serum |
| HFF | Human fibroblast cells |
| $H_2O_2$ | Hydrogen peroxide |

| | |
|---|---|
| GAP-43 | Growth- associated protein |
| IDO | Indoleamine 2,3-dioxygenase |
| IFN-γ | Interferon gamma |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IF | Immunofluorescence |
| OS | Oxidative stress |
| MAP | Microtubule Associated Protein |
| MEM | Minimum Essential Medium |
| MDBK | Madin-Darby bovine kidney |
| mgcl2 | Magnesium chloride |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MyD88 | Myeloid differentiation primary response gene 88 |
| NeuN | Neuronal nuclei |
| NFTs | Neurofibrillary tangles |
| NK cells | Natural killer cells |
| NO | Nitric oxide |
| NSE | Neuron specific enolase |
| PAMPs | Pathogens Associated Molecules Patterns |

| | |
|---|---|
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PNS | The peripheral nervous system |
| SAG | Surface Antigen |
| SV2 | Synaptic vesicle protein II |
| SYN | Synaptophysin |
| RA | Retinoic acid |
| ROS | Reactive oxygen species |
| TDO | Tryptophan 2,3 Dioxygenase |
| TE | Toxoplasmic encephalitis |
| *T. gondii* | *Toxoplasma gondii* |
| TGF-β1 | Transforming growth factor |
| Th | T helper |
| TLRs | Toll-Like Receptors |
| TNF-α | Tumor necrosis factor alpha |
| WBCs | White blood cells |
| µl | Microliter |
| °C | Degrees Celsius |

# Declaration:

I hereby declare that this thesis was composed by myself and has not been accepted in any other previous application for a degree. The research presented is my own work, except where stated. All sources of information have been acknowledged by means of references.

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# Abstract

*Toxoplasma gondii* is a zoonotic parasite that commonly causes infections in a variety of warm-blooded animals including humans. This parasite causes a disease referred to as toxoplasmosis. *T. gondii* infections are known to have a significant impact on the host brain since the parasite can penetrate the blood-brain barrier and extensively colonize the central nervous system (CNS) for an extended period. Attacks of the brain tissue by the parasite can cause both neurological and structural damage. The parasite infects the brain, forms a cyst and affects several parts of the brain, including the amygdala, hippocampus, other cortical regions, and cerebellum; this can result in alterations in the hosts' behaviour. Studies have proposed that infections caused by *T. gondii* pose a risk in the development of several neuropsychiatric disorders like Alzheimer's disease, Schizophrenia, and Parkinson's disease. The aims of this study were to conduct a pilot study on the association between *T. gondii* infections and Alzheimer's disease using tissue samples from the Manchester Brain Bank (MBB) and to investigate the effect of the parasite on oxidative stress in cultured human brain cells

As a pilot study, previously prepared slides from 124 brain sections from Alzheimer's patients and controls were examined for the presence of *Toxoplasma gondii* cysts. Despite a 10% prevalence of *T. gondii* in humans in the UK, no cysts were detected in any of the sections. It was concluded that unsustainably large tissue samples would be required from MBB to pursue an investigation using this approach.

Instead, a line of investigation was pursued that explored the interactions between *T. gondii* infections and oxidative stress (a feature of neurological diseases such as Alzheimer's) using the development of a model system. SH-SY5Y cells were used as culture models for neuronal cells and flow cytometry and MTT assays were conducted to determine cell viability. To evaluate the effects of oxidative stress, differentiated and undifferentiated SH-SY5Y cells, were examined for cell viability and the effects of infection by *T. gondii* type I and II were

investigated. When high doses of $H_2O_2$ [250 and 500$\mu$M) were used for 24 hours in undifferentiated SH-SY5Y cells, there was a reduction in the cell viability to 8.66% and 4.66% respectively. Conversely, when SH-SY5Y cells (differentiated) were subjected to high doses of $H_2O_2$ (250 and 500$\mu$M) a much reduced decline in cell viability, to 64.6% and 39.33% respectively, occurred. The effects of *T. gondii* strain I and II infections on oxidative stress in both undifferentiated and differentiated cells were also investigated. The outcome showed that pre-treatment of the undifferentiated and differentiated cells with *T. gondii* weakened the $H_2O_2$ effect on the viability of SH-SY5Y cells such that cell viability (85.75%) was significantly higher than in cells treated with only $H_2O_2$ (25.25%) or cells post-treated (28%) by infection with *T. gondii*. Further results demonstrated that pre-treatment of the undifferentiated SH-SY5Y cells using *T. gondii* strain I and II CM (conditioned media) led to a significant decrease in the $H_2O_2$ -induced cell damage and apoptosis.

In conclusion, this study demonstrates that differentiated SH-SY5Y cells show higher resistance towards oxidative stress that induces cell damage and death as compared to undifferentiated SH-SY5Y cells. Interestingly, infection with *T. gondii* results in anti-oxidative effects on neuronal cells which may protect against cell damage. These results suggest that infection with this parasite could protect against neurological diseases such as Alzheimer's disease.

# 1 Chapter 1: General introduction.

## 1.1 Introduction:

### *1.1.1 Toxoplasma gondii:*

*Toxoplasma gondii* is recognized to be a common parasite that causes a disease called toxoplasmosis. It generally infects the cat as the definitive host and all warm-blooded animals as intermediate hosts, including humans. It is estimated that 30-70% of the *T. gondii* parasite exists in the human population in different regions of the world (Parlog, Schlüter, and Dunay 2015; Xiao et al. 2016). The usual mode of transmission of this infection is through ingestion of the parasite oocysts and tissue cysts in contaminated food and undercooked meat. It can be transmitted transplacentally, where an infected mother passes the infection to its fetus through the placenta (Ferguson et al. 2013; Montoya and Liesenfeld 2004; Weiss and Dubey 2009; Weiss and Kim 2000). Humans act as intermediate hosts that harbor the inactive stage of the infection in the lifecycle of the parasite. The host tissues mostly include the heart, liver, lymph nodes, lungs, eyes, and brain. Infection of the brain tissue by the *T. gondii* parasite has been proposed as a risk in several brain disorder conditions such as schizophrenia, anosmia, and bipolar cases (Dickerson et al. 2014; Prandota 2014). It takes seven days for the *T. gondii* infection to spread to the brain tissue and penetrate the blood-brain barrier (Robert-Gangneux and Dardé 2012). Usually, the CNS maintains a strong immunity making it very complicated for the infection to thrive. However, in immunosuppressed individuals, *Toxoplasma* infection progresses to fatal states such as encephalitis when the latent infection is revived (Parlog et al. 2015). Studies show a significant increase in the amounts of *T. gondii* antibodies among patients with Alzheimer's and Parkinson's diseases. However, these studies showed indirect relationships between *T. gondii* infection and the possibility of having brain disorders and how

the *T. gondii* infection may affect the brain or nerve network, causing several conditions to remain undetermined (Passeri et al. 2016).

#### 1.1.1.1 Morphology.

*Toxoplasma gondii* is a protozoan parasite that exists as a unicellular organism. It undergoes three infectious stages which are tachyzoite stage refers to the parasite's rapidly growing form found during the acute phase of toxoplasmosis. Bradyzoite (tissue cyst), which happens in chronic infection and it is slowly dividing. Sporozoites (oocysts) this stage occurs in the environment (Figure 1. 1) (Dubey 1998). The acute phase involves the rapid division of tachyzoites during which there are lysis and destruction of the host cell, and the released parasites infect new cells. The tachyzoites slowly undergo conversion to bradyzoites. This occurs due to environmental stress prompting the formation of tissue cysts that remain in latency, thus the chronic phase (Halonen and Weiss 2013). Most hosts can contain the inflammatory processes preventing the trigger of the infection reactivating. However, reactivation of the dormant phase occurs in an immunosuppressed host causing rupture of the cysts and a change to tachyzoites (Halonen and Weiss 2013). For the third stage which occurs in the environment, the oocyst wall is tough with several layers enabling the parasite to survive any mechanical and chemical damage in the environment. Giving it a longer shelf life even years in the environment (Mai et al. 2009).

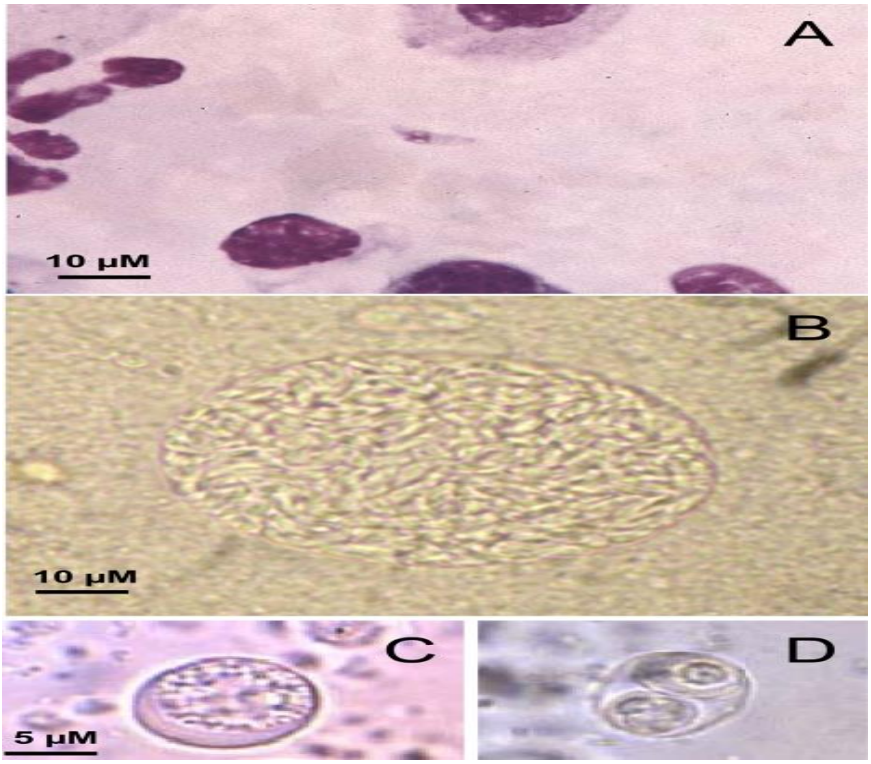



**Figure 1-1** *Toxoplasma gondii* stages. (A) Tachyzoite stage. (B) Bradyzoite stage. (C) Unsporulated oocysts. (D) Sporulated oocysts. Taken from Robert-Gangneux and Dardé (2012).

#### 1.1.1.2 The life cycle of the *Toxoplasma gondii.*

The tachyzoites, bradyzoites and sporozoites are the three infectious stages of *Toxoplasma gondii*. This parasite can be transmitted between intermediate hosts (asexual cycle) or even between definitive hosts, also, between intermediate and definitive hosts (sexual cycle) (Figure 1. 2) (Robert-Gangneux and Dardé 2012). A feline species plays a role as a definitive host where the sexual cycle takes place. Upon consumption of the cysts by the cat, these cysts undergo breakdown of their cysts wall through enzymatic processes in the gut and small intestines (Dubey 1998). Releasing bradyzoites settle inside the cells within the intestines becoming trophozoites. Here asexual multiplication occurs, leading to the growth of merozoites

and the formation of schizonts (Figure 1. 2) (Dubey 1998). The sexual development initially happens with the forming of female and male gametes (Mai et al. 2009). Fertilisation occurs then oocytes are released to the environment through the cats' faeces. Once released meiosis begin which produces eight haploid offspring or sporozoites which are double-walled oocysts (Dubey, J.P., Lindsay, D.S. & Speer 1998). In this stage, the oocysts persevere in the harsh conditions of the environment and maintain being highly infectious to intermediate host through the consumption of contaminated food (Figure 1. 2) (Dubey 2004).

In the intermediate host, any asexual cycle occurs. This happens after the ingestion of *T. gondii* sporulated oocyst in contaminated food or undercooked meat harboring the *T. gondii* cysts by the intermediate host (Dubey 2004). Bradyzoites are released from tissue cysts, while sporozoites are released from sporulated oocysts. Sporozoites or bradyzoites penetrate the intestinal epithelium and actually differentiate to form tachyzoites. Which instantly replicates by endodyogeny inside the cells in the infected organism. Then the tachyzoites convert to bradyzoite (to avoid host immunity) in the tissue cyst as early as 7-10 days after acquiring the infection and may remain latent in the host mostly in the brain or musculature (Robert-Gangneux and Dardé 2012).

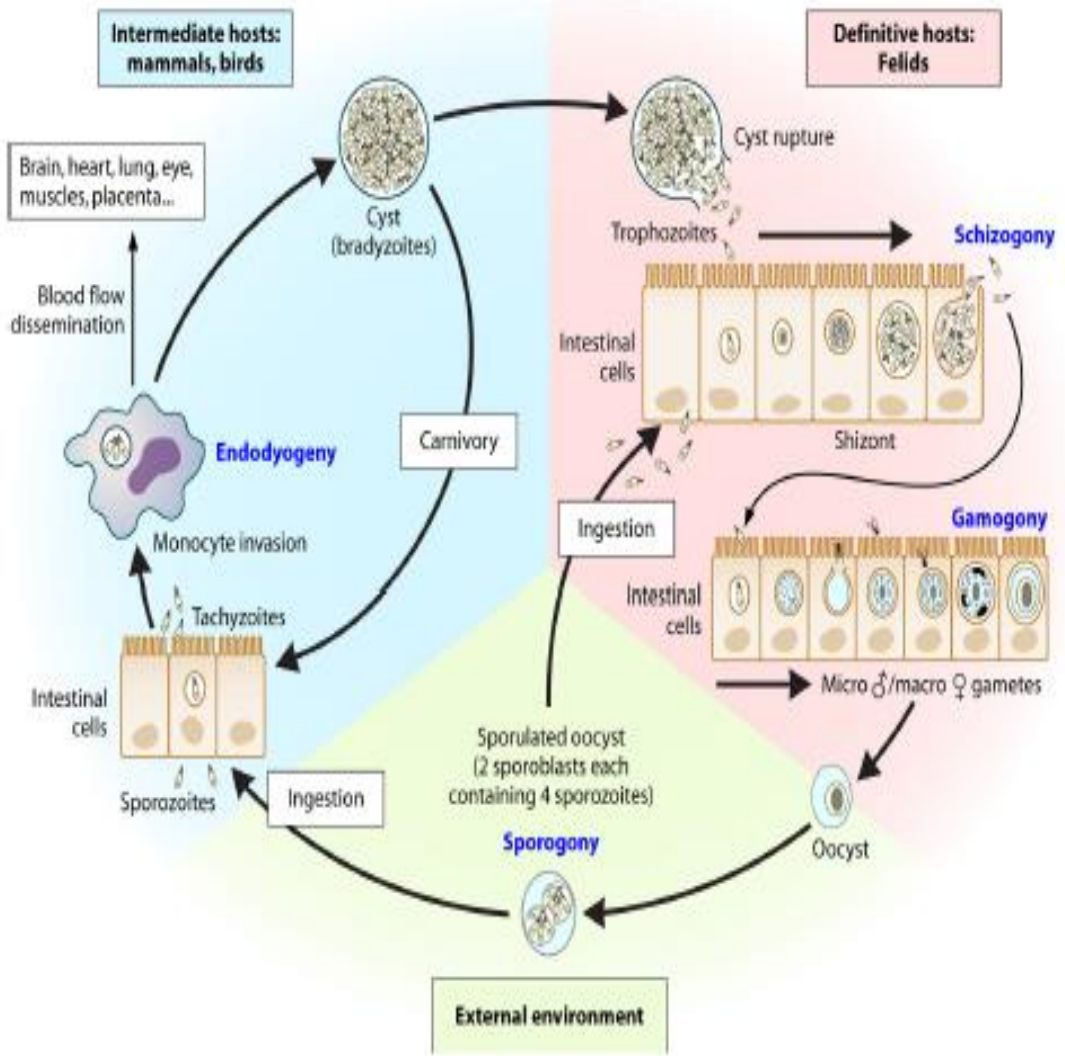



**Figure 1-2** Life cycle of *Toxoplasma gondii*. This schematic indicates the infection, biology and replication of the three infective stages of *T. gondii* in their intermediate and definitive hosts. Taken from Robert-Gangneux and Dardé (2012).

#### 1.1.1.3 Transmission.

There are three main infectious stages of this parasite, they include tachyzoites, bradyzoites and oocysts, and are all acquired through various routes of infection transmission (Figure 1.3). These routes of infections include 1] Horizontal transmission – it involves direct consumption of the oocysts from the environment. 2] Horizontal transmission – here, the bradyzoites are ingested from infected meat that is taken raw or undercooked. 3]. In vertical transmission, the tachyzoites are passed to the foetus in utero through the placenta causing congenital toxoplasmosis (Tenter, Heckeroth, and Weiss 2000) (Figure 1. 3). There are familiar sources of transmission that have been identified; they include the ingestion of undercooked meat harboring the cysts and consumption of food that has been contaminated with oocysts with feline faeces. It has been established that oocysts are more infectious for the intermediate hosts than for definitive feline host while, in contrast, tissue cysts are more infectious for the definitive feline host (Dubey 2006). Also, *T. gondii* infection among rodents produces changes in their behaviour reducing their fear of cats and leading to enhance transmission of the parasite (Berenreiterová et al. 2011). In mothers, *Toxoplasma* is vertically transmitted to the foetus via the placenta during pregnancy. This route of transmission is considered to be very significant among animal species and humans (Weiss and Dubey 2009). In the acute stage of *T. gondii* infection, the tachyzoites cross the placenta to the foetal circulation (Tenter et al. 2000).

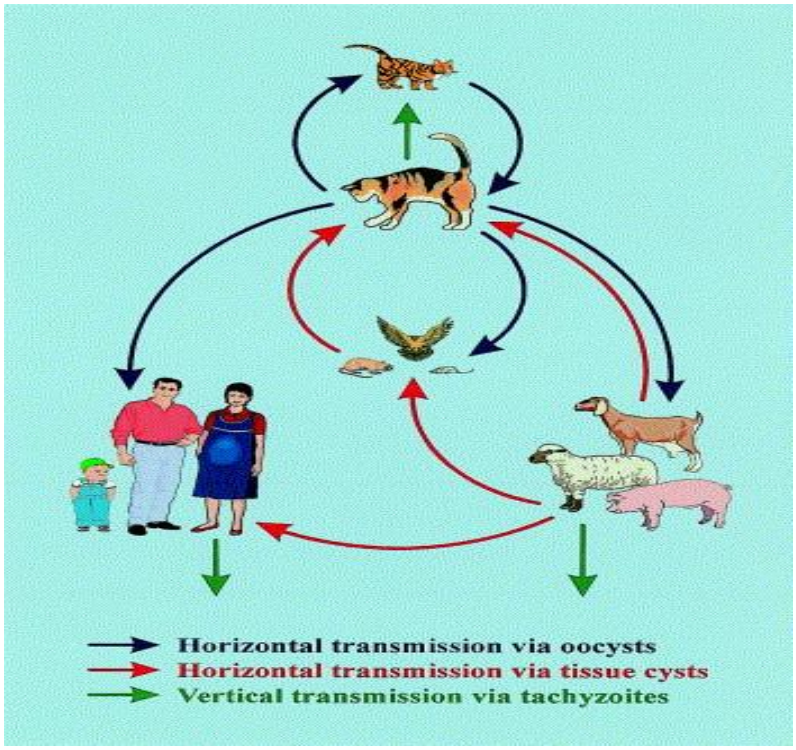



**Figure 1-3** Major routes of transmission of *T. gondii*. Taken from Tenter, Heckeroth and Weiss (2000).

#### 1.1.1.4 *T. gondii* strains.

This parasite infection is prevalent worldwide; it has at least five clone lineages (Type I-V). Among the clones, there is a little different in their genetic makeup and the disease symptoms that each clone represents (Wendte, Gibson, and Grigg 2011). The type I strain (RH) is considered to be the most virulent, causing severe and sudden onset of the disease in both humans and rodents. Type II (Me49) and type III (VEG) are classified as non-virulent strains, thus causing *T. gondii* chronic infection (Asis and Grigg 2016). The capacity of strain type I to

replicate rapidly compared to types II and III strains has enabled it to breach various biological barriers like the blood-brain barriers, intestinal epithelium lining and the placenta (Barragan and David Sibley 2002). With this ability, it links strain type I to its high levels of virulence. For the type II strain which mainly shows minimal virulence, this strain exists in the brain cells for a substantial more extended period through the suppression of the immunity in the CNS (Jung et al. 2012). The other Type IV-V strains are derived from the types I, II, and III forming atypical alleles that are commonly found among patients in South America and Africa (Saeij, Boyle, and Boothroyd 2005).

#### 1.1.1.5 Humoral response.

Upon acquiring *T. gondii* infection, some antibodies are produced. They include IgM, IgG and IgA (Correa et al. 2007). Though these antibodies pose no risk since an extensive part of the parasite's lifecycle occurs intracellularly; thus, the parasite is not predisposed to the attacks by antibodies (Correa et al. 2007). The latest studies have demonstrated that IgA antibodies play a significant role in the case of an oral infection the IgA antibodies produced by mucosal immunity prevents recurrence (Correa et al. 2007). The IgM mediates in the acute stages of the infection, restricting the parasite from disseminating and invading the host cells (Couper et al. 2005). During the infection, IgG is produced, which activates the antibody-dependent cytotoxicity (ADCC) (Correa et al. 2007).

#### 1.1.1.6 Immune response to *T. gondii.*

The parasite actively evades the host immune response to increase its ability to survive. Upon contracting the infection, the host cell immunity is triggered through PAMPs (pathogen associated molecules patterns). These are molecules that activate the hosts' resistance they bind to the hosts' receptors, specifically CCR5 and TLRs (toll-like receptors) (Egan et al. 2009). The cells that get involved first include dendritic cells and neutrophils. They get involved in mechanisms that produce cytokines like interleukin-12 (IL-12) as well as IL- 18. It is known that IL-12 stimulates and activates additional immune cells like macrophages that release tumor necrosis factor-alpha (TNF-α) and the Natural killer cells (NK cells). These are involved in releasing IFN-γ that are essential cytokines that protect the host cell from the *T. gondii* infection (Miller et al. 2009; Pittman and Knoll 2015). Many activities of the infection caused by *T. gondii* are activated and controlled by IFN-γ. IFN-γ directs the transition from the acute to the chronic stages of infection, as well as suppressing the transformation from bradyzoites to tachyzoites during the chronic stage of infection (Jones, Bienz, and Erb 1986). Other important cytokines include IL-1, 6, 12, 17, and 23. These are also known as pro-inflammatory cytokines and are formed by the brain cells like astrocytes and microglial cells in parasitic infections (Carruthers and Suzuki 2007; Wilson and Hunter 2004). The cytokines are released by neurons and cells responsible for the immunity, resulting in a change in the hosts' behaviour due to the disruptions caused by the range of these cytokines produced (Parlog et al. 2015; Webster and McConkey 2010). Other cytokines involved are IL-4, 10, 13, and the IFN-α as well as transforming growth factor-β (TGF-β), which possess the ability to inhibit pro-inflammatory cytokines (Cavaillon 2001).

#### 1.1.1.7 Diagnosis of *Toxoplasma gondii*

*T. gondii* infections can be diagnosed directly through the use of the Polymerase Chain Reaction (PCR), histology, isolation and hybridization (Cermakova, Ryskova, and Pliskova 2005). Serological methods are also used for indirect diagnosis. At first, the indirect approach detects the number of host antibodies like IgG, IgM and IgA, which are against the *T. gondii* parasite. IgG antibody against *T. gondii* is detected in 1-2 weeks after acquiring the infection and is known to persist for an extended period (Halonen and Weiss 2013). Various tests such as ELISA, immunofluorescent antibody test (IFA), Sabin-Feldman dye test, and IgG avidity test are used to detect IgG antibody in *T. gondii* infections (Cermakova et al. 2005; Passeri et al. 2016). IgM antibodies that emerge within the first week of infection, rapidly increase, and then decline and disappear at highly variable rates can be detected using the double-sandwich IgM ELISA and the IgM immunosorbent agglutination assay (ISAGA) (Naot, Desmonts, and Remington 1981; Siegel and Remington 1983). For the identification of IgA antibody tests like IgA ELISA has been used and has shown high levels of sensitivity than the identification of IgM antibodies (Montoya and Liesenfeld 2004). The use of serological test methods shows no links between *T. gondii* infections and Alzheimer's' disease (Mahami-Oskouei et al. 2016). However, serology methods are not accurate in the detection of *Toxoplasma* infection since they are mostly referred to as general methods; thus, there is a need to employ more sensitive methods to assist in diagnosis. Further diagnosing is directly done using molecular methods that are more accurate and precise like PCR and histology (immunohistochemistry). In this case, nested PCR was the method of choice that was used to detect *T. gondii*. This method identified surface antigen genes of the parasite which were SAG1, SAG2, SAG3 and B1 genes from the tissues that were infected (Bajnok et al. 2015; Gashout et al. 2016). Immunohistochemistry (IHC) technique is mostly used to analyze and study the morphology of tissues, identify malignancies and tumours. IHC is an antibody-based method that involves

identifying and analyzing proteins and ensures the structure, composition and cellular make-up of local tissues is maintained (Kaliyappan et al. 2012). This method is regarded as a good technique that helps one to comprehend the pathological processes that interfere with the function of the brain resulting in unusual behaviour (Hermes et al. 2008). Therefore, previous studies indicate that direct methods are more preferred over indirect methods in the diagnosing of the *T. gondii* infection due to its increased accuracy and sensitivity (Gashout et al. 2016; Montoya and Liesenfeld 2004).

## 1.1.2 Brain cells.

Brain cells such as microglia, astrocytes and neurons are commonly infected by the *T. gondii* tachyzoites. These cells acquire the ability to control the parasite when the immunity is stimulated, particularly by T-cell derived IFN-γ. Astrocytes and microglia are able to clear the parasites when infected by *T. gondii*. For the parasites to evade elimination in the neurons, it converts to a cyst form called bradyzoites. Each brain cell involved is discussed below:

### 1.1.2.1 Neuron cells:

Neuron cells contain a cell body, dendrites and axon, which are electrically conducting cells that pass information through chemicals and electrical signals to the brain (Markram et al. 1997). The glial cell joins the neural components and regulates the homeostasis in the brain. Also, glial cells give support and protection to the neurons (Sadigh-Eteghad et al. 2016).

### 1.1.2.2 Glial cells:

Glial cells are subdivided into various categories, namely; microglia, ependymal cells, astrocytes and oligodendrocytes (Figure 1. 6). Each of these categories has specific features and roles. The microglia offers the CNS immunity against microorganisms, by acting as macrophages for the brain tissue. Astrocytes provide a linkage between blood vessels and neurons. This assists in the transfer of nutrients to the neurons and toxins are carried away from the neurons to avoid accumulation in the neurotransmitters (Sadigh-Eteghad et al. 2016). The oligodendrocyte cells provide a myelin shield in the axon regions and the entire neuron (Dupree et al. 2004).

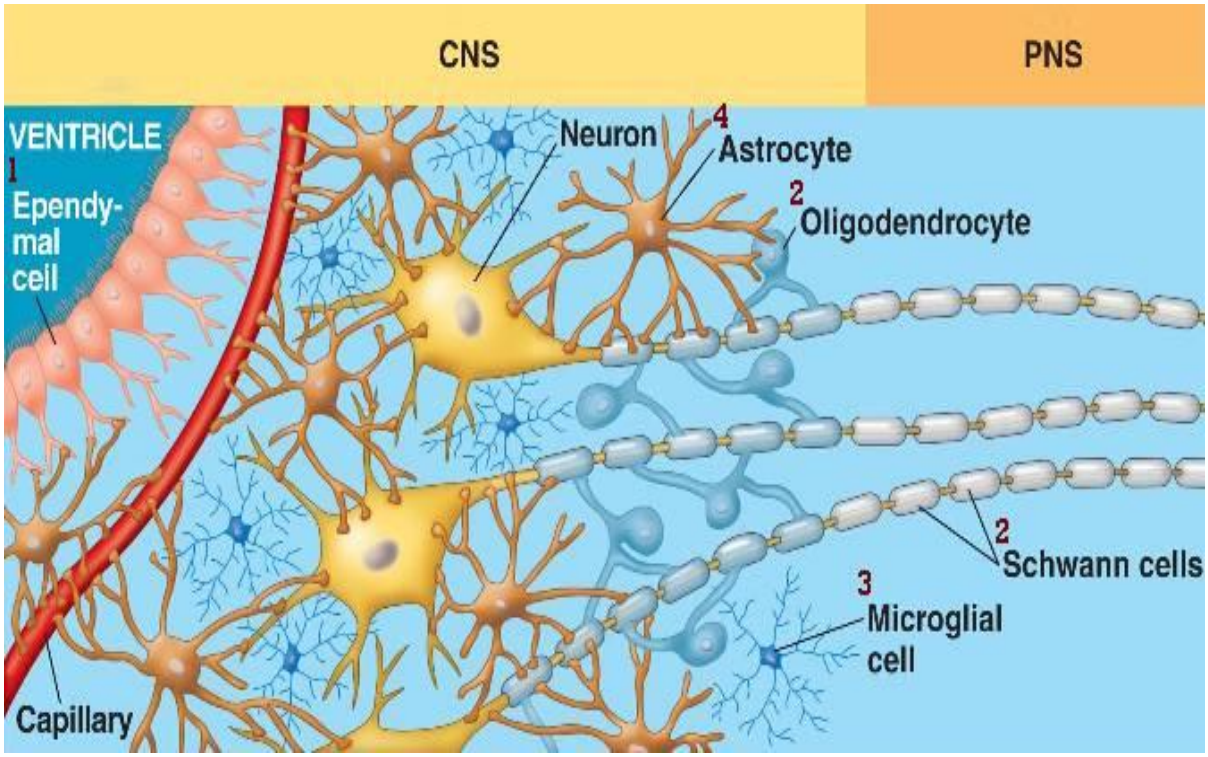



**Figure 1-4** Different type of glia cells: 1) ependymal cell, 2)Oligodendrocyte 3) Microglial cells 4) Astrocyte. Taken from http://bio1152.nicerweb.com/Locked/media/ch49/glia.html.

### 1.1.3 *T. gondii* in brain tissue

After upon ingestion *T. gondii* parasite can be detected in the mesenteric lymph nodes in 18 hours and the blood circulation within 24 hours (Dubey 1997). The parasites can penetrate impermissible biological barriers. The tachyzoites can cross the placenta and intestinal epithelium through the process of paracellular transmigration and enter circulating body cells invading dendritic cells and macrophages. The parasite is also known to breach the blood-brain barrier (BBB) gaining entry to the brain tissues (Masocha and Kristensson 2012). Previous studies have shown that the extracellular form of *T. gondii's* adhesin MIC2 interacts with host ICAM-1 to allow the free tachyzoites to cross epithelial barriers. Then the chemokine receptor CCR5 of host cells interacts with cyclophilin which is secreted by *T. gondii.* This attracts white blood cells (WBCs) to the site of infection. Then immune cells such as monocytes are invaded by *T. gondii* which facilitates the migratory activities from blood vessels to deliver parasites into the brain extravascular space (Masocha and Kristensson 2012). On activation, rhoptries release a cluster of proteins that are necessary for the infiltration and defense of the parasitophorous vacuoles. It is estimated that ten days after contracting the infection, the tachyzoites differentiate into bradyzoites, which gradually develop into cysts. This form of the parasite can avoid the immune system (Carruthers and Suzuki 2007).

Tachyzoites attack a variety of the cells in the brain including neurons, microglial cells and astrocytes as well as the medulla Purkinje cells. *T. gondii* can quickly proliferate in neurons and astrocytes, while activated microglial cells, which are highly phagocytic brain macrophages, effectively inhibit parasite growth and can serve as important inhibitors of *T. gondii* spread in the CNS. These brain cells cause the production of multiple cytokines that can stimulate or subdue the inflammatory responses (Carruthers and Suzuki 2007; Lüder et al. 1999). When the microglial cells are activated, they perform a significant role in the protection

of the CNS against infections, thus hindering the *T. gondii* from replicating through IFN-γ and nitric oxide (NO) production mechanisms.

### 1.1.4 *T. gondii* infection and inflammation.

A type of macrophage residing in the spinal cord and brain are the microglia. The roles performed by these cells include regulation of homeostasis, inflammatory reactions, and tissue injury in the central nervous system. The immune environment of the CNS gives them the plasticity required to accomplish these roles (Orihuela, McPherson, and Harry 2016). Several pro-inflammatory cytokines are secreted by the microglial cells during active infection of *T. gondi* that aid in inhibiting the growth of the *T. gondii*. These include tumor necrosis factors like TNF-α and interleukins like IL-2 and IL-12. In addition to this, anti-inflammatory cytokines are also released during *T. gondii* infection including TGF-β, IL-4, and IL-10. The anti-inflammatory cytokines downregulate the immune response in the intracerebral region and promote the growth of *T. gondii* that can cause the development of toxoplasmic encephalitis (TE) (Sarciron and Gherardi 2000). The progression of *T. gondii* infection from the acute stage to the chronic stage is characterized by cyst formation 2 months after infection. In the acute phase, tachyzoite replication occurs and in the chronic stage, dormant cysts are formed (Hester et al. 2012; Nguyen et al. 1998). When the infection becomes latent, the risk of life-threatening sequelae from TE also reduces gradually (Sarciron and Gherardi 2000). The rise in the level of pro-inflammatory cytokines after brain infection with *Toxoplasma* is modest, hence the infection is controllable and the damage to the host is minimal. IFN-γ is an important cytokine in late infection as it inhibits the proliferation of tachyzoites in the CNS and thus plays a significant role in preventing TE (Mordue et al. 2001). Anti-inflammatory cytokines TGF-β

and IL-10 are released during the chronic infection stage playing a part in reducing the exaggerated inflammatory response arising in the brain pertinent to the chronic infection (Harris et al. 2010; Sarciron and Gherardi 2000). These anti-inflammatory cytokines, in turn, cause a reduction in the level of expression of effector molecules like IFN-γ, they also reduce nitrous oxide production. Both these mechanisms are essential for the establishment of latency of *T. gondii* infection (Harris et al. 2010; Rozenfeld et al. 2003, 2005). A harmonious balance of these cytokines, processes, and strategies enables the parasite to survive in the host for a long time causing latency without being expelled by the brain tissue.

### 1.1.5 Toxoplasmosis and Psychosis.

Brain disorders including depression, dementia, epilepsy, Alzheimer's and stroke affect almost 2 billion people. In Europe, the cost of brain disorders in 2010 was estimated to be €798 billion (Gustavsson et al. 2011). Once parasite enters the body, *T. gondii* tachyzoites cross the intestinal epithelium as free parasites (tachyzoites), and then *T. gondii* tachyzoites spread across the bloodstream, using dendritic cells and macrophages as transporters (Trojan horses) to cross the BBB and penetrate the CNS (Carruthers and Suzuki 2007). *T. gondii's* ability to cross the blood-brain barrier and colonize brain cells including microglia, astrocytes, and neurons is the primary cause of infected hosts' neuropathogenesis (Elsheikha, Büsselberg, and Zhu 2016). The damaging of the neurological structure happens when the tachyzoites invade, grow, lyse, burst, and exit the infected host cells during the acute infection. While in the chronic phase, the formation of the tissue cysts causes damage to the neurologic structure. These cysts become latent in the cortical regions, olfactory bulb, amygdala and cerebellum (Berenreiterová et al. 2011; Haroon et al. 2012). The infected hosts can experience hormonal and behavioral changes as a result of this parasite. (Elsheikha et al. 2016). Three potential mechanisms occur in *T. gondii* infection which may link between *T. gondii* infection and behavioral changes in the infected host. The first chronic infection causes degenerative loss in neurons. Infected neurons may die or have their processes atrophied as a result of parasites within them, and inflammation may lead to the death of nearby neurons. (Hermes et al. 2008). For the second mechanism, there is a change in the amounts of interferon-gamma (IFN-γ) and indoleamine 2, 3 dioxygenases (IDO) (Webster and McConkey 2010). This leads to efficiency and turning over of neuromodulators which include serotonin, dopamine, and glutamate. In the third mechanism, it shows the involvement of the *T. gondii* influence on the neurotransmitter levels that in the increased dopamine levels and dormant *T. gondii* infections exist (Berenreiterová et al. 2011). Recent research shows that *T. gondii* infection poses a risk for some brain disorders

like Alzheimer's disease, schizophrenia, and Parkinson's disease (Fabiani et al. 2015). On the other hand, some research has shown that some disorders are not associated with *T. gondii* infection such as postpartum depression (Gao et al. 2019).

#### 1.1.5.1 Neurodegenerative disease.

Health care improvements have contributed significantly to the increase in life expectancy over the last decade. However, such improvements have also been associated with the rise in the number of people who have neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease. These diseases are described as involving the continuous loss of specific neuronal cells that are related to individual protein aggregates. Evidence suggests that oxidative stress (OS) may be a common factor in this group of conditions when OS is responsible for the damage to or death of the neuronal cells, resulting in the pathology. OS happens because of the uncontrolled release of ROS such as hydrogen peroxide ($H_2O_2$), nitric oxide (NO) and superoxide as well as highly reactive hydroxyl radicals. The brain tissue becomes prone to oxidative damage when exposed to increased levels of oxygen use, low anti-oxidants levels and lower regenerative capacities (Barnham, Masters, and Bush 2004). Among the neurodegenerative disorders, there is a common pathological characteristic: the loss of subsets of neurons. This causes a variety of types of neural cell death that includes necrosis and apoptosis. These forms of cell death can be due to different cellular insets including excitotoxicity and OS. However, the primary risk factor for neurodegenerative disease is increasing age (Chin-Chan, Navarro-Yepes, and Quintanilla-Vega 2015; Md et al. 2018).

#### 1.1.5.2 Alzheimer's disease.

This disease is known to be a neurological disorder and it is known to progress leading to dementia among older adults (Md et al. 2018). It is approximated that over 35 million people have Alzheimer's in the world (Montacute et al. 2017). Its symptoms include cognition impairment which, in advanced cases, leads to functional impairment. The early stages of the disease present with signs memory loss, problems in planning, inability to solve problems, depression and lack of interest (Lee et al. 2010). The moderate signs of AD in later stages include long-term memory loss, irritability, aphasia (lack of fluency in speech) and disorientation (Lee et al. 2010). In most advanced stages, there is emaciation, immobilization and ultimately death commonly caused by pneumonia. In the year 1906, Alois Alzheimer, a German neuropathologist defined two variations in the brain of a woman who has had dementia (Lee et al. 2010). The senile plaques include the extracellular fibril aggregates and the unstructured accumulation of β-amyloid peptide (Aβ). Also, the neurofibrillary tangles (NFTs), as well as intracellular fibrillary deposits of the microtubule-associated protein tau are oxidized and hyperphosphorylated (Montacute et al. 2017). The neurofibrillary tangles and senile deposits are mostly identified in the brain areas such as the hippocampus, basal forebrain and the cortex in the AD case (Mattson, Maudsley, and Martin 2004). There are two kinds of Alzheimer's disease. The early-onset (EOAD) or the familial which is linked with certain gene modifications which occur in amyloid precursor protein as well as presenilin genes 1 and 2. These are all associated with amyloid-beta peptide synthesis (Piaceri, Nacmias, and Sorbi 2013). EOAD starts as early as 65 years, and accounts for approx. 5% of the cases. The second form of the disease, which is of late-onset or sporadic (LOAD), contributes to 95% of AD cases and is the most common. Several risk factors have been identified numerous genetic factors like polymorphism in ApoE (encoding for apolipoprotein E) and SORL1 (encoding for a neuronal receptor of ApoE) as well as GSK3 (encoding for glycogen synthase kinase 3 beta)

genes contribute to this form of AD. For LOAD, the ApoE is the most dominant inherent risk factor, however, is not fully adequate in explaining the existence of the disease (Godfrey, Wojcik, and Krone 2003). Thus it is still uncertain about the factors that contribute to its etiology; hence it is proposed that there are multifactorial causes of LOAD.

#### 1.1.5.3 Oxidative stress (OS).

Oxidative stress (OS) is defined as a condition where there is an imbalance between cellular pro-oxidant and anti-oxidant defenses and which is induced by reactive oxygen species (ROS), causing different effects depending on the cellular type and the cell context (Chandra, Samali, and Orrenius 2000; Fatokun, Stone, and Smith 2008). ROS, which include superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide ($H_2O_2$), hydroxyl radicals(•OH) and singlet oxygen($^1O_2$), are generated as metabolic by-products by biological systems (Sato et al. 2013). An increase and accumulation of reactive ROS inside a cell may occur in both physiological and pathological conditions and can result in cell damage (Gutteridge and Halliwell 2000). Excessive amount of ROS damage mitochondrial deoxyribonucleic acid (mtDNA) and has a negative impact on Ca2+ homeostasis, membrane permeability, and mitochondrial defense systems (Dincel and Atmaca 2016). It is crucial to remove the ROS when it has finished its action on its target; this prevents cellular damage that is due to the heightened reactivity of the ROS with biomolecules. Anti-oxidant systems work together to eliminate free radicals. Enzymes including superoxide dismutase (SOD), glutathione reductase (GR), catalase, and glutathione peroxidase support these anti-oxidant systems (Fang, Yang, and Wu 2002). However, when the anti-oxidant systems and the DNA damage repair are insufficient, cellular dysfunction and eventually

apoptosis occur as a result of the large amount of ROS (Roos and Kaina 2006). In the case of increased ROS, biomolecule oxidative damage can occur to molecules that include DNA, lipids and proteins. In the brain, ROS cause neuronal damage by negatively affecting neuronal and glial cells. These cells are mainly sensitive to free radicals that lead to neuronal damage and are known to cause apoptosis, which in turn is known to be a pathological cause of different neurodegenerative diseases (Wang et al. 2013).

#### 1.1.5.4 Antioxidants.

Anti-oxidants are substances that search for molecules that have the ability to slow down and prevent oxidation of further molecules by changing the ROS to water and therefore stopping overproduction of the ROS. Anti-oxidants act to hinder any kind of oxidative stress process that can adversely affect the cells (Aouache et al. 2018; Uttara et al. 2009). This is achieved by inhibiting the ROS chain reaction, and the elimination of intermediates of free radicals then follows the blocking of any other oxidative responses (Siddiqui et al. 2010). Anti-oxidants exist in two forms: non-enzymatic and enzymatic. The enzymatic anti-oxidants are also called natural anti-oxidants. These include SOD, hemoxygenase, and glutathione reductase, and others are catalase and thioredoxin as well as glutathione peroxidase (Aouache et al. 2018; Lopes et al. 2017).

The non-enzymatic anti-oxidants include dietary supplements and artificially processed anti-oxidants such as vitamin A, vitamin C, and vitamin E. Others include minerals such as zinc, glutathione, and the nicotinamide adenine dinucleotide (NAD) system as well as flavonoids (Aouache et al. 2018). Anti-oxidants can be used as anti-inflammatory drugs during the onset of neurological diseases to calm the immune system, specifically in situations where the

immune system is greatly provoked because of the oxidative stress that is triggered. The application of non-steroidal anti-inflammatory drugs shows inhibition of the neurotoxicity that is caused by Aβ protein (Lee *et al.*, 2010). The involvement of enzymes and anti-inflammatory medications in the anti-oxidant therapy determines that the increased practice of the treatment in the ROS generation results in the prevention of pathologies in neurodegenerative disorders (Uttara et al. 2009).

#### 1.1.5.5 Alzheimer's disease and Oxidative stress.

As described above, oxidative stress happens when there is a discrepancy between the produced ROS and the rate at which the biological system clears the reactive intermediates or repairs the cell damage. This discrepancy occurs due to a variety of issues like advanced age, stroke, hyperglycemia, injury and toxins (Guglielmotto et al. 2010). Other researchers have examined the connection between Aβ and oxidative stress. Aβ causes *in-vivo* and *in-vitro* oxidative stress which increases Aβ production (Figure 1. 7) (Tabner et al. 2005; Tamagno et al. 2002; Tong et al. 2005). For the transition of healthy aging to dementia, oxidative stress plays a role as it is shown to occur in the early phase of the AD (Lee et al. 2010). Patients with AD present with reduced ability to counter the oxidative stress due to decreased antioxidant levels and antioxidant enzymes activities (Rinaldi et al. 2003). These outcomes elaborate that oxidative stress is a critical pathological event in AD since it results in damage to the membranes, cytoskeletal changes and death of cells (Smith et al. 2000; Zhu et al. 2007). The increase in ROS causes damage in most of the cell components that include the mitochondrial DNA, nucleus and membranes as well as cytoplasmic proteins (Figure 1.7) (Harman 1992).

It is suggested that mitochondria have a significant function in a particular process since it is the leading site for the production of ROS (Corral-Debrinski et al. 1992; Harman 1992). It further is shown that the ROS is a by-product of the cell metabolism and it is produced through mitochondrial oxidative phosphorylation. This process also leads to release of molecules like hydrogen peroxide or molecules that have electrons that are not paired like superoxide. The susceptibility of the brain to OS is due to the high uptake of oxygen, polyunsaturated fatty acids high level, as well as low levels of antioxidants (Floyd and Hensley 2002). It is evident that high ROS levels act as synaptic loss essential mediators and ultimately promoting the development of senile plaques and neurofibrillary tangles (Kern and Behl 2009). Thus, this relation between ROS and buildup of Aβ enhances the advancement of AD.

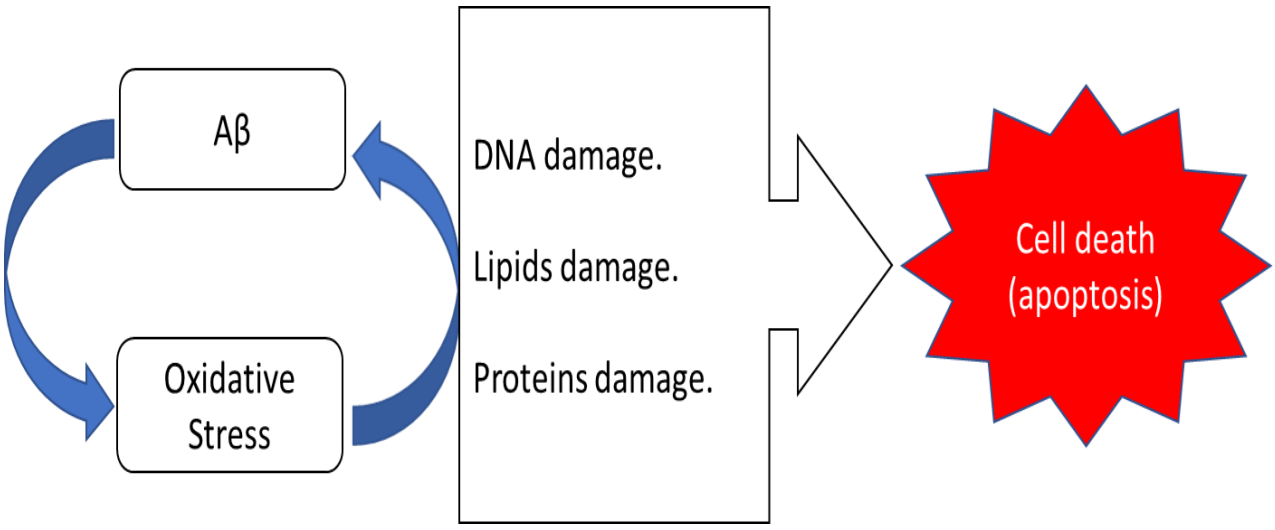



**Figure 1-5** Proposed cycling mechanism between β-amyloid peptide (Aβ) deposition and oxidative stress**.**

#### 1.1.5.6 Alzheimer's disease and *T. gondii.*

In developed countries, Alzheimer's represents the most prevalent neurodegenerative disease, which is age-related. It is estimated to affect 7% of individuals aged over 65 years and 40% of individuals aged above 80 years of age respectively. Several clinical presentations of cognitive disorders include language and memory impairment. These signs of AD illustrate that there is an abnormal accumulation of β-amyloid peptide (Aβ) in the form of senile plaques and phosphorylated tau protein which appears as neurofibrillary tangles and the loss of neurons in the brain parenchyma (Lai and McLauring 2012). Although studies show, there are other risk factors considered to cause Alzheimer's disease. This extensive form of AD is regarded as a multifactorial process that involves viruses and bacteria which were isolated from the brain tissue of individuals who have Alzheimer's disease (Kusbeci et al. 2011).

It is reported that bacterial and viral infections can induce ROS and pro-inflammatory cytokine productions, which results in Alzheimer's disease (Bu et al. 2015; Shima, Kuhlenbäumer, and Rupp 2010). Thus, a parasitic infection like *T. gondii* could be a significant risk factor in neurodegenerative diseases such as AD. Various reports suggest that neuronal degenerations are a result of the production of proinflammatory cytokines that include IFN-γ, IL-1β and TNF-α (Kusbeci et al. 2011). Concerning the previous studies, the connection between the infection with *T. gondii* and AD is indirectly related to enhanced release of anti-inflammatory cytokines such as IL-10 and TGF-β, in the brain issues also causing minimal neuronal cell death, amyloid plaque aggregation and neurodegeneration (Jung et al. 2012; Möhle, Israel, Paarmann, Krohn, Pietkiewicz, Müller, Inna N. Lavrik, et al. 2016).

### 1.1.6 SH-SY5Y cells.

SH-SY5Y cells have been extensively used in various studies that relate to oxidative stress, which plays a role in human pathologies like neurodegenerative diseases, cancer, and diabetes (Akki et al. 2018). For further understanding of AD, the SH-SY5Y cells have been used as *in vitro* models of AD (Jazvinšćak Jembrek, Hof, and Šimić 2015; Sutinen et al. 2012). The neuronal cell line SH-SY5Y acquired from the bone marrow biopsy of four year old girl suffering from metastatic neuroblastoma; this cell line is a SK-N-SH line sub-clone (Forster et al. 2015). There are two phenotypes of the SH-SY5Y cell line and they include N-type as well as substrate adherent (S-type) cells. Most cultures contain the N-type cells while only a lesser number of the S-type cells (Kunzler et al. 2017). The cell line can undergo differentiation from neuroblast like phase to mature neurons by several mechanisms.

There are clear variances between differentiated and undifferentiated SH-SY5Y cells. The undifferentiated cells present as being non-polarized having limited short projections. They also grow into clumps rapidly and show indicators of immature neurons. However, the differentiated cells seem to be polarized, longer and show slow growth (Figure 1.8). The differentiated cells display more mature neurons displaying mature neuronal markers of differentiation like growth-associated protein (GAP-43), microtubule-associated protein (MAP), neuronal nuclei (NeuN), and synaptophysin (SYN). Others are synaptic vesicle protein II(SV2) and neuron-specific enolase (NSE) (Figure 1. 9) (Gimenez-Cassina, Lim, and Diaz-Nido 2006; Shipley, Mangold, Kuny, et al. 2017; Xie et al. 2010).

SH-SY5Y differentiated cells can be induced by all-trans-retinoic-acid (RA), cholesterol (CHOL), dibutyryl cyclic AMP, brain-derived neurotrophic factor (BDNF), 12-o-tetradecanoyl-phorbol-13-acetate, vanadate, nerve growth factor, vitamin D3, and neuregulin

beta1 (Fan *et al.*, 2016). Retinoic acid has widely been used in SH-SY5Y cell differentiation. It is reported that a wide range of promoter sites found in the neuronal and glial cells are affected by RA nuclear receptors. The use of RA for the differentiation results in alteration of the sodium conductance, thus increasing the acetylcholinesterase (AChE) activity, enhancing the recycling of synaptic vesicle, changing of sodium conductance and inhibiting cell division.

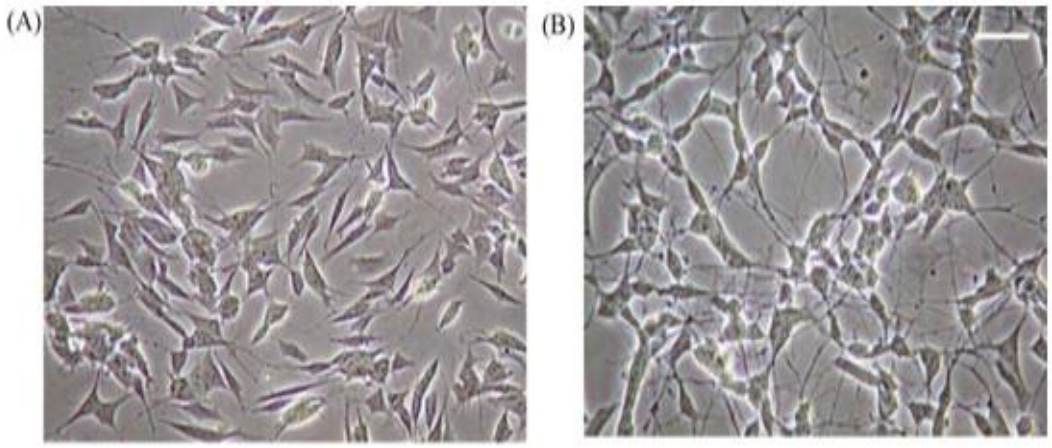



**Figure 1-6** Morphological appearance of undifferentiated and differentiated SH-SY5Y cells(A) Undifferentiated SH-SH5Y cells cultured in 10% complete medium for 3 days. (B) Differentiated SH-SY5Y cells were cultured with RA (10 µM) for 7day.Scale bar = 50 µm. Taken from Cheung et al. (2009).

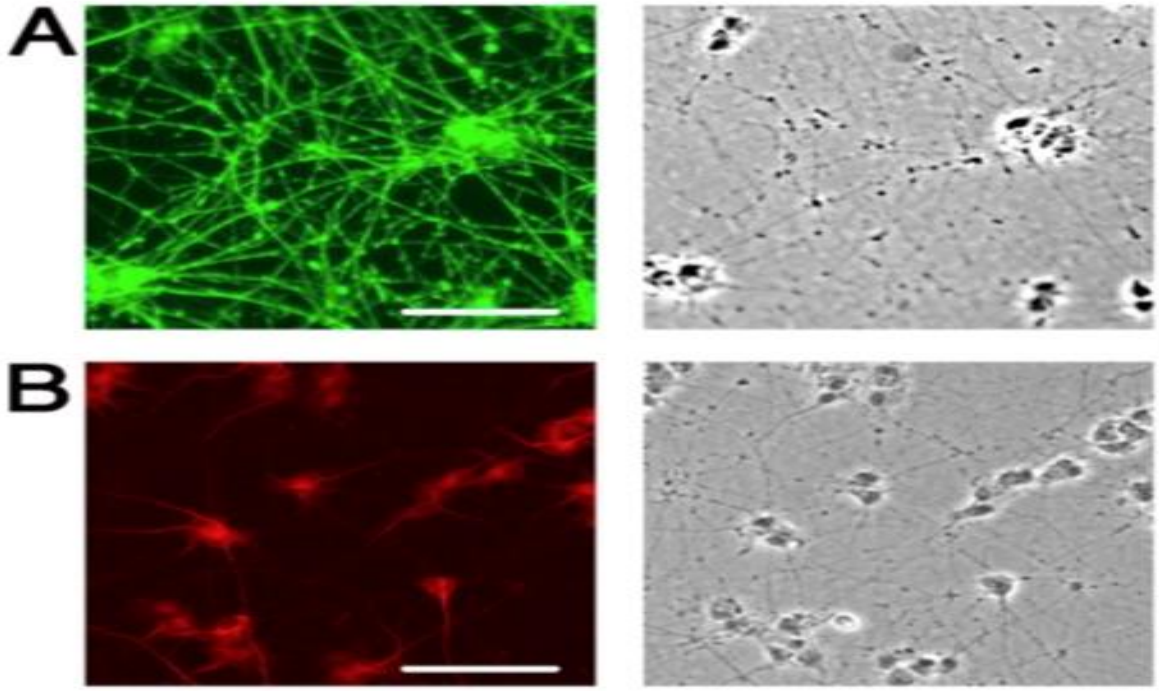



**Figure 1-7** Markers of neuronal differentiation, by using immunofluorescence staining for differentiated SH-SY5Y cells. (A) Anti-SMI31 (green) stains the phosphorylated neurofilament H in the extensive network of neurites. (B) Anti-MAP2 (red) labels microtubule-associated protein 2, these images were obtained at 10X magnification using an inverted epifluorescence microscope. Scale bar, 100 µm. Taken from Shipley, Mangold and Szpara (2017).

## 1.2 Aims.

The *T. gondii* parasite affects multiple organs in the human body including the brain. Lifelong persistence in the neurons within the central nervous system, it could modify their structure and function, which could result in behavioral changes in the host. Previous studies conclude that *T. gondii* infection plays a vital role in the development of neuropsychiatric disorders, specifically schizophrenia and may have a link with other neurobiological diseases such as Parkinson's disease and Alzheimer's disease (Fabiani et al. 2015). The general aim is to develop a model brain cell culture system that can be used to explore the question as to whether there are any relationships between *T. gondii* infection and cellular process that are involved in neurological diseases like Alzheimer's disease and others. The focus of these investigations will be around the relationships between *Toxoplasma* infection and oxidative stress.

There are three main objectives of this project:

- Analysis of the frequency and distribution of *T. gondii* infection in a collection of samples from Alzheimer's patients and control subjects to determine the feasibility of using human brain samples from the Manchester Brain Bank to investigate the possible roles of infection.
- Development of cell culture model systems for the investigation of the effects of *Toxoplasma* infection on brain cells.
- Investigation of the effects of oxidants on neuronal cell viability and morphology.
- Investigation of the effects of *Toxoplasma* infection on oxidative stress in a model brain cell culture system.
- Investigation of the effects of *Toxoplasma* conditioned media on cell growth associated with oxidative stress in a model brain cell culture system.

# 2 Chapter 2 Materials and Methods.

## 2.1 Materials.

### 2.1.1 Culture reagents.

- Dulbecco's Modified Eagle's Medium-high glucose (D5796), Laminin obtained from Engelbreth-Holm-Swarm murine sarcoma basement membrane (l2020-1MG), Retinoic acid (R2625), Trypan blue solution 0.4 % (v/v; T8154) from Sigma-Aldrich Chemical Company, Poole, UK.
- Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM / F-12) (31330038), N-2 Supplement (100X) (17502048), Penicillin Streptomycin 100X, Bovine Serum Albumin (BSA)(BP9710-100) obtained from Fisher Scientific UK.
- Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30-2002™), obtained from American Type Culture Collection (ATCC), UK.
- Minimum Essential Medium (21090022), MEM Non-Essential Amino Acids Solution (100X) (11140050), L-Glutamine (200 mM) (25030081), Dimethyl Sulfoxide ( DMSO - D/4121/PB08), sodium pyruvate (100 mM) (11360039), Annexin V APC Ready flow reagent (R37176) Invitrogen, Annexin-binding buffer 5X-(V13246) Invitrogen, obtained from Thermo Fisher Scientific, UK.
- 1x phosphate-buffered saline, Dulbecco's formula, without magnesium and calcium (0918604) obtained from MP Biomedicals Life Sciences Division, USA.
- Vectashield mounting medium with DAPI (ZB1130) obtained from Vector Laboratories Ltd, Peterborough, UK.
- Fetal calf serum (CA-115) obtained from Labtech international, UK.
- Fetal bovine serum.

- Thiazolyl Blue tetrazolium bromide, 98%(L11939) obtained from Alfa Aesar, UK
- 
- Hydrogen peroxide ($H_2O_2$) 30% w/v obtained from Scientific Laboratory Supplies, UK.
- RIPA Buffer 100 ml (89900) obtained from Thermo Scientific, UK
- Giemsa Stain Rapid (HS300-1L), obtained from TCS Biosciences Ltd, Buckingham, UK.

### 2.1.2 Plasticware.

- Disposable Serological Pipettes (5ml, 10ml,25ml, cryovial (Greiner Bio-One™ 122280), Mr. Frosty™ Freezing Container (10110051) obtained from Fisher Scientific UK.
- 30 ml Polystyrene Universal, 7ml polystyrene bijou containers (E1412-0710) obtained from Starlab Ltd, UK.
- TC Plate 6 Well, Standard (83.3920), TC Plate 12 Well, Standard TC Plate 24 Well, Standard, TC Flask T75, Standard (83.3911), TC Flask T25, Standard (83.3910), Tube 15ml, 120x17mm, PP (62.554.502), Tube 50ml, 114x28mm, PP 62.547.254), Filter tip, 1000 µl (70.762.211) Filter tip, 2-200 µl 70.760.211 obtained from SARSTEDT AG & Co. KG, Germany.

### 2.1.3 Specialized equipment.

- Centrifuges Mega Star 1.6, VWR International, Leicestershire, UK.
- CellGard class II microbiological safety cabinet, NuAire, Plymouth, MN 55447 USA
- Esco class II biosafety cabinet, Infinity, Esco Micro Pet Ltd, Singapore.
- Nikon Digital Sight Microscope Camera, Nikon UK Limited, Surrey, UK.
- Grant waterbath JB Academy, Grant Cambridge, UK
- Zeiss microscope primovert, Carl Zeiss Ltd, Cambridge, UK.
- (BD FACS Verse flow cytometer
- BioTek Cytation 3 Cell Imaging Reader
- Multi-mode microplate reader (MTT Assay), FLUOstar® Omega, BMG-LABTECH.

### 2.1.4 Specialized laboratory reagents:

#### 2.1.4.1 Antibodies:

The ideal staining dilutions and conditions were reached for the antibodies (see table 2. 1 and 2.2). All samples would be exposed to similar conditions; this included both the positive and negative controls for every antibody.

##### 2.1.4.1.1 Primary antibodies.

The following are the primary antibodies and working dilutions used in the study for the immunofluorescence technique (Table 2. 1).

- Monoclonal anti-human Neurofilament H & M Antibody was obtained from Sigma Aldrich. Neurofilaments (NF) represent a type of filament in neurons. They are the main element of the neuronal cytoskeleton and work basically to offer structural sup-

port for the axon and control axon diameter. The SH-SY5Y cells start to express neurofilaments when undergoing differentiation.

- The monoclonal anti-human MAP2 (2a+2b) antibody was obtained from Sigma-Aldrich. Microtubule-associated protein 2 (neuronal protein). It is implicated in the microtubule assembly that is an essential step in neurogenesis.

- Monoclonal anti-human Neuron-specific enolase (NSE) antibody obtained from Proteinech Group. NSE is also considered as a cell-specific isoenzyme of the enzyme glycolytic enolase that can be involved in cell differentiation.

#### 2.1.4.1.2 Secondary antibodies.

- Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) preadsorbed Product code: ab150117 obtained from Abcam, Cambridge, UK.
- Goat Anti-Mouse IgG H&L (Phycoerythrin) Product code: ab97024 obtained from Abcam, Cambridge, UK.
- .

**Table 2-1** Primary Antibodies for Immunofluorescence. Table of optimal antibody dilutions and staining conditions for each antibody.

| Primary Antibody | Host species | Cat no | Brand | Dilution | Incubation Time |
|---|---|---|---|---|---|
| **Map2** | Mouse | M1406 | Sigma-Aldrich | 1:500 | 1 hour at RT |
| **NF** | Mouse | MAB1592 | Sigma-Aldrich | 1:500 | 1 hour at RT |
| **NES** | Mouse | 66150 | Proteintech | 1:200 | 1 hour at RT |

RT= Room temperature.

**Table 2-2** Secondary Antibodies for Immunofluorescence. Table of optimal antibody dilutions and staining conditions for each antibody.

| Secondary Antibody | Host species | Target species | Cat no | Brand | Dilution | Incubation Time |
|---|---|---|---|---|---|---|
| **Alexa Fluor® 488** | Goat | Mouse | ab150117 | Abcam | 1:200 | 1 hour at RT in the dark |
| **Phycoerythrin** | Goat | Mouse | ab97024 | Abcam | 1:200 | 1 hour at RT in the dark |

RT= Room temperature

### 2.1.5 Brain samples.

Dr. Gemma Lace kindly provided slides from a previous project containing sections from the hippocampus and frontal lobe of the brain. The samples were already stained by other PhD students. The staining technique used was immunohistochemistry staining with Beclin-1 or AT8 antibodies specifically for the previous Alzheimer's disease project. Haematoxylin stain was used to stain the background for the previous project and this will also show up potential *T. gondii* cysts. Slides were examined by light microscopy. The author examined the slides in detail for the presence of *T. gondii* cysts.

## 2.1.6 Cell lines.

Three different cell lines and two strains of *T. gondii* as listed below were employed through this study:

### 2.1.6.1 HFF cells.

Dr. Gemma Lace provided the human fibroblast cells (HFF) cell line. It was used for the development of tissue cultures and controls.

### 2.1.6.2 MDBK cells.

Madin-Darby bovine kidney cell line (MDBK) was obtained from American Type Culture Collection (ATCC), UK. This cell line was explicitly used to produce *T. gondii*.

### 2.1.6.3 SHSY5Y cells.

SH-SY5Y cell line was a kind gift by Dr. Sara Rollinson from the University of Manchester, these cell lines were models of neuron cells.

### 2.1.6.4 Parasites.

- *T. gondii* strain I (RHΔKU80) was kindly provided by Dr. Paul Denny, University of Durham.
- *T. gondii* strain II (ME49) was obtained from American Type Culture Collection (ATCC), UK.

## 2.2 Methods:

### 2.2.1 Microscopy.

Light microscopy (LM) was used in this project for examining slides, using the magnification power of light ranging from x4, x10, x40 and x100. Brain tissues collected from two sites, the frontal lobe and the hippocampus tissue sections (4-5 µm in thickness) were used. They were obtained from the brains of Alzheimer's patients and controls. A systematic examination of the whole slide from each sample was done to search for *T. gondii* cysts, using light microscopy using low and high- power magnification. Pictures were taken of the most interesting fields of the examined samples.

### 2.2.2 Cells counts.

Trypsinisation and centrifugation of these cell lines were done and resuspension of the 1 ml of the media. The suspension of 20µl was added to 20µl of trypan blue and mixed; 10µl of the mixture was then transferred to the two chambers of the haemocytometer. These chambers were filled using the capillary action, then counting of the cells was done in each of the haemocytometer square (2 squares). Determination of the number of cells was done as follows:

Then cells at a particular density and capacity (typical 20µl) of the cell suspension were thinned 1:10 in Trypan blue solution with a concentration of (0.4% v/v). Counts of the viable cells in the five fields of the Neubauer chamber was done under the light microscope (Figure 2. 1 A B). Calculation of the cell density was done as follows:

$$\textbf{Average of cell count} = \frac{\textbf{viable cells}}{\textbf{number of squares}}$$

$$\textbf{Cell count per milliliter} = \textbf{Average of cell count} \times \textbf{Dilution} \times 10000$$

Preparation of the cell solutions of a particular cell concentration was obtained from the predetermined concentration in the original flask using the equation above.

$$\textbf{Vol of cell solution required from flask} = \frac{\textbf{Concentration of cell required} \times \textbf{Vol of cell suspension required}}{\textbf{Concentration of cell solution in flask}}$$

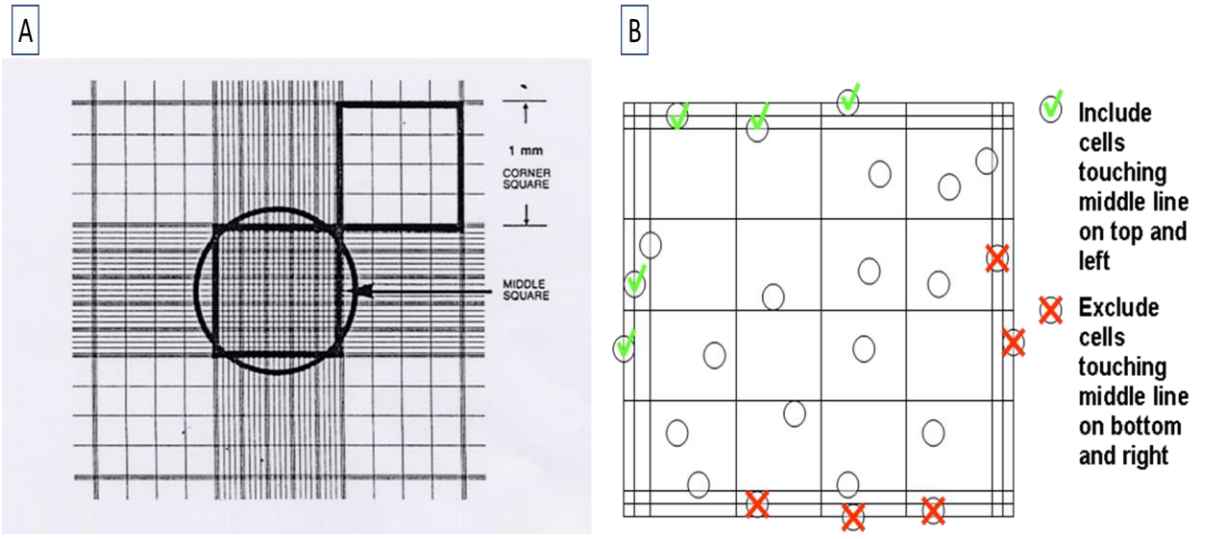



**Figure 2-1 The diagrams show the cell count method with Haemocytometer. (A)** The appearance of the haemocytometer grid visualised under the microscope. (B) Counting system to ensure accuracy and consistency. Count the cells within the large square and those crossing the edge on two out of the four sides.

### 2.2.3 Routine Cell Culture Methods.

Cells were cultured in complete growth medium in T75 flask and incubated at 37˚C and 5 % $CO_2$ for 48 hours or until a confluency of 90 % was achieved. A serological pipette was used to remove the growth medium then the cell monolayer was washed by phosphate-buffered saline (PBS) twice. Trypsinization (0.25%Trypsin/0.03%EDTA) technique was used to separate the cells from the plates (growth surface) and this was done at 37˚C and 5 % $CO_2$ for a maximum of 2 minutes. 10 mls of complete growth medium (contains 10% FBS) was added to then this suspension was moved to a centrifuge tube and centrifuged at 150 g for five minutes. After this, the supernatant was discards then 10mls of fresh culture complete growth medium (based T75 flask) was used to re-suspend the pellets. The suspension was properly mixed and then 1ml added to individual flasks. 14 mls warmed complete growth media was added in each of these flasks then placed into an incubator at 37ºC and 5 % $CO_2$. The culture media was replaced every 2 days until a confluency of 90 % was attained. Once this confluency was obtained the cells were ready for use in future studies or split every grown on.

#### 2.2.3.1 Cryo-preservation of cells.

During cryo-preservation, the cryopreservation media that was utilized included the 90 % (v/v) FBS and sterile dimethyl sulphoxide (DMSO) 10 % (v/v). The cells were collected through trypsinization as illustrated in section 2.2.3. The cells were then suspended again using a cryopreservation medium (1ml) and then the contents laid on a cryovial. To prevent crystal formation and enable slow cooling, the cells were set on isopropanol- containing Mr. Frosty. Before being placed on long-term storage under liquid nitrogen, the cells were kept overnight at a temperature of -80°C.

#### 2.2.3.2 Thawing of cryo-preserved cells.

A water bath with a temperature of 37$^{o}$C was used to defrost the cells after taking the cryovials from the liquid nitrogen under an ice pack. 70% ethanol was then sprinkled on the thawed cryovials and then transferred to a hood (class II microbiological safety cabinet). They were later shifted to a sterile universal tube holding a fresh culture of 20mls in each medium. The suspension was then centrifuged at 125 g for 5 minutes. Thereafter the supernatant was taken out and the pellets re-suspended using a new culture media (1ml) and later relocated to the T25 sterilized flask holding 5mls per-medium of growth. Subculturing and incubation of these cells were carried out as discussed in section 2.2.3.

#### 2.2.3.3 Maintenance of HFF cells.

The HFF cells were grown in Minimum Essential Medium (MEM) containing: 15% (v/v) heat-deactivated fetal bovine serum (FBS), 2Mm L-glutamine, 100 units/ml/penicillin/streptomycin, and 1% (v/v) of non-essential amino-acids. The HFF cells were maintained as a monolayer and incubated at 37°C and 5%$CO_2$. This medium was replaced at least every two days until 90 % confluent. The cells were then split and seeded as described in section 2.2.3.

#### 2.2.3.4 Maintenance of MDBK cells.

The MDBK cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing: 10% (v/v) heat-deactivated fetal bovine serum (FBS) and 100 units/ml/penicillin/streptomycin. MDBK cells were maintained as a monolayer and incubated at 37°C and 5%$CO_2$. The medium had to be replaced at an interval of two days until 90% confluent. The cells were then split and seeded as described in section 2.2.3.

#### 2.2.3.5 Maintenance of SH-SY5Y cells.

The SH-SY5Y cells were grown in Dulbecco's Modified Eagle's Medium with high glucose (DMEM-high glucose) containing: 10% (v/v) foetal calf serum (FCS), 4mM L-glutamine and 100 units/ml penicillin/streptomycin. SH-SY5Y cells were maintained as a monolayer and incubation were at 37°C under a 95% humidified atmosphere (v/v) air/5 % (v/v) $CO_2$. The medium was supposed to be changed every two days until 90 % confluent. Once this confluency was obtained the cells were ready for use in future studies or split every grown on. as described in section 2.2.3.

#### 2.2.3.6 SH-SY5Y Differentiation:

##### 2.2.3.6.1 Coverslip coating and preparation.

Glass coverslips were rinsed using ethanol and left to dry before being placed in the six-well plates. Then the coverslips were coated by adding 2 ml of the dilution of laminin (1:20) in PBS without Mg and Cl. The plates were then covered and incubated overnight in the fridge. After that, the laminin was discarded, and the coverslips were washed with PBS without Mg and Cl twice before use.

##### 2.2.3.6.2 Retinoic acid preparation.

Retinoic acid (RA) is essential for *in-vitro* SH-SY5Y differentiation. As per the distributor (R2625-Sigma-Aldrich Chemical Company), the RA can be stored at -20°C for one year in powder form. Since it becomes more sensitive to light, heat and air when in solution form. It can be dissolved in DMSO and stored as stock for six weeks at -20°C this can be done before using it in a differentiation medium. RA can be diluted in 95-100% ethanol, and then added to the differentiation medium depending on the concentration requested.

##### 2.2.3.6.3 Ten-day differentiation protocol.

The SH-SY5Y human neuroblastoma cell line was cultured in a growth medium, as discussed in the previous sections (2.2.3.5). Upon reaching the 90% confluence harvesting was done through trypsinization then counted as described. Undifferentiated SH-SY5Y cells were seeded at $1x10^5$ cells/ ml on coated sterile coverslips (2.2.3.6.1) then placed in an incubator at 37° C and 5% $CO_2$ for two days or when 60% confluence was reached. The growth medium was removed then the differentiation medium containing DMEM/ F-12 medium, 1% (v/v) N-2 Supplement (100X), 2mM L-glutamine and 100 units/ml penicillin/streptomycin. This dif-

ferentiation medium supplemented with 10µM retinoic acid (RA) was added and then incubated at 37°C in 5% $CO_2$. These cells were preserved in these conditions for ten days with a change of the medium every two days. Images were taken on days 0, 2, 4, 6 and 10.

#### 2.2.3.6.4 Twenty-one-day differentiation protocol.

The culturing of the SH-SY5Y cells was done on coated sterile coverslips and then placed in an incubator at a temperature of 37° C under 5% $CO_2$ for two days or when 60% confluence was reached. Differentiation medium which described above was added with 1µM RA then allowed to incubate at 37°C and 5% $CO_2$. These cells were preserved under these conditions for 21 days a medium change every two days. Images were taken on days 0, 5, 10, 15 and 21.

#### 2.2.3.6.5 Immunofluorescence assay.

These cells were left to differentiate for the next 21 days, as discussed (2.2.3.6.4) in a 6 well plate onto coverslips or without coverslips, for the immunofluorescence studies. Washing the cells once with PBS for 3 minutes and fixing with cold methanol or 4% paraformaldehyde (pH7) for 10minutes at room temperature. After which these cells were rinsed three times using PBS for 5 minutes every time. The cells were permeabilized in PBS that had 1% Triton X-100 plus 5% bovine serum album for an hour at room temperature. Primary antibodies and secondary antibodies were performed in 1% BSA; the cells were incubated with the primary antibodies: Anti-Neurofilament H&M Antibody (1:500), anti-human MAP2 (2a+2b) antibody (1:500) and anti-human NSE antibody (1:200) in 1 hour at room temperature. These cells were then washed three times for five minutes in PBS. Incubation was at room temperature for one hour using Goat Anti-Mouse IgG H&L Alexa

Fluor® 488 (1:200) as the secondary antibody. They were then washed three times for five minutes using PBS. During mounting, a Vectashield medium that contains DAPI was used. The images were taken on the Cytation 3 imaging reader. The magnification that was used included 10x and 40x.

#### 2.2.3.7 Maintenance and growth of *T. gondii.*

Culturing of the parasites was done in a Class II biosafety cabinet (Esco class II biosafety cabinet, Infinity) where the aseptic technique was applied. MDBK cells were then cultured on a T25 flask as elaborated in sections (2.2.3.4). When the cells attained 70-90%, it was important to exchange the growth medium (GM). with a new *T. gondii* medium TM (Dulbecco's Modified Eagle Medium containing: 1% (v/v) foetal bovine serum (FBS). Then the MDBK cells were infected with the *T. gondii* tachyzoites, at ratios of 5:1 (parasite: cell). A day after the flask inoculation. The cells were washed twice removing non-infective parasites using PBS then exchanged with fresh *T. gondii* medium TM. Within 48-72 hours after inoculation, the tachyzoites were harvested, through scraping the monolayer of cells using a cell scraper (BD Falcon, Le Pont de Claix, France). Then pushed along a 25-g plastic needle twice connected to a 5-ml syringe. It was then centrifuged at 1,000 g at room temperature (RT) for 10 minutes. The supernatant was discarded then the suspension of the pellets in fresh medium (TM) before the tachyzoites produced were counted using the haemocytometer (2.2.2). There was further sub-culturing of the parasites in new T25 $cm^2$ flasks that contains a confluent monolayer of MDBK cells, in the medium (TM) and incubated at a temperature of 37°C and 5% $CO_2$.

#### 2.2.3.8 Cryo-preservation of *T. gondii* cells:

The freezing medium was used contained 60% growth medium, 30% FBS and 10% DMSO. Two methods were used to freeze these parasites this includes intracellular and extracellular freezing.

##### 2.2.3.8.1 Intracellular freezing.

This involved taking the T25 flask infected with *T. gondii* strain from the incubator and examine under the microscope to ascertain infection. For the ideal conditions that ensure the survival of the *T. gondii* strain during preservation, those flasks with ~70% MDBK cells having *T. gondii* vacuoles should contain 8 parasites per vacuole. Harvesting was done through trypsinization, as discussed in section (2.2.3.7) then centrifuged at 300 g for 5 minutes. The supernatant was disposed of and the pellets re-suspended in 1 ml of the cryopreservation medium then put in a cryovial. These cells were then placed in Mr Frosty and placed at -80°C overnight before transferring to liquid nitrogen for long-term storage.

##### 2.2.3.8.2 Extracellular freezing.

The T25 flask containing *T. gondii* parasite was taken from the incubator and examined under the microscope to confirm that 70% of the host cell monolayer has been lysed. Harvesting of the Tachyzoites in 48-72 hours after inoculation. The scraping of the monolayer of cells was conducted using a cell scraper then the cells were lysed by passing through a 25-g needle two times and centrifuged at 1000 g at RT for 10 minutes. Then follows the discarding of the supernatant and the pellets from the deposits were re-suspended in 1 ml of a freezing medium before placing it in a cryovial. The cells were then placed in Mr Frosty and stored at a temperature of -80°C overnight before transferring to liquid nitrogen for long-term use storage.

### 2.2.3.9 Thawing of cryo-preserved cells *T. gondii.*

Cryovials were obtained from liquid nitrogen and transported on the ice. The cells were thawed in a water bath at 37°C. After thawing these vials were sprayed with 70% ethanol, then put in the hood and the cells transferred to a T25 flask with a confluent monolayer of MDBK cells containing 5ml of DMEM with 1% FBS. The flask was incubated at 37°C, 5% $CO_2$ for 24 hours. To remove the dead parasites, the medium was aspirated from the T25 flask and placed in a biohazardous waste container. In the T25 flask new 5-7 ml warm medium was added then follows incubation at 37°C with 5% $CO_2$. The flasks were examined daily to monitor vacuole formation and tachyzoite growth within the MDBK cells.

### 2.2.3.10 Comparison of type I and type II *T. gondii* strains.

#### 2.2.3.10.1 The growth rate of *T. gondii* strains into undifferentiated SH-SY5Y cells.

The undifferentiated SH-SY5Y cells were cultured in 25-$cm^2$ T-flasks ($1x10^6$ cells / 1ml) in DMEM supplemented with 10% FBS / for 24 hours at a temperature of 37°C and 5% of $CO_2$. DMEM supplemented with 1% FBS defined as *T. gondii* medium TM was used to wash the cells before the addition of either type I or II *T. gondii* parasites as a ratio of 5 tachyzoites to 1 cell. Control cells were prepared using the *T. gondii* medium TM only. After 12 hours, the cells were washed with medium to remove non-adherent parasites. The cultures were then incubated for 3 and 4 days at a 37°C and 5% $CO_2$.Tachyzoite were harvested as previously described (2.2.3.7), then after 3 or 4 days of inoculation, the tachyzoites produced were counted using the haemocytometer (2.2.2).

**2.2.3.10.2 The growth rate of *T. gondii* strains into differentiated SH-SY5Y cells.**

The undifferentiated SH-SY5Y cells were cultured using 25-$cm^2$ T-flasks ($1x10^6$ cells/ 1ml) in DMEM supplemented with 10%FBS / for 24 hours at a temperature of 37°C and 5% $CO_2$. These cells were left to differentiate for the next 21 days, as discussed (2.2.3.6.4) After which the differentiated medium was removed and differentiated medium supplemented with 1% FBS defined as *T. gondii* medium TM was used to wash the cells. Then this experiment was performed as described above (2.2.3.10.1).

**2.2.3.10.3 The infection rate of *T. gondii* strains into undifferentiated SH-SY5Y cells.**

The seeding of the undifferentiated cells was done at a concentration of $5x10^4$ cells per 1 ml on coverslips placed into a 6-well plate in DMEM and left to incubate at of 37°C and 5% $CO_2$ for 24 hours. DMEM supplemented with 1% FBS defined as *T. gondii* medium TM was used to wash the cells before the addition of ether type I or II *T. gondii* parasites as a ratio of 5 tachyzoite to 1 cell. Control cells were prepared using the *T. gondii* medium TM only. After 12 hours, the cells were washed with medium to remove non-adherent parasites. The cultures were then incubated for 3 and 4 days at a 37°C and 5% $CO_2$. Three or four days later, the cells were washed with PBS, then fixed in 4% paraformaldehyde (pH7) for 10 minutes. The cells were then rinsed in PBS, stained using Giemsa stain for 40 minutes, washed in PBS and mounted on a glass slide for analysis under the light microscope.

**2.2.3.10.4 The infection rate of *T. gondii* strains into differentiated SH-SY5Y cells.**

The seeding of the undifferentiated cells was done at a concentration of $5x10^4$ cells per 1 ml on coverslips placed into a 6-well plate in DMEM and left to incubate at of 37°C and 5% $CO_2$ for 24 hours. These cells were left to differentiate for the next 21 days, as discussed (2.2.3.6.4).

After which the differentiated medium was removed and differentiated medium supplemented with 1% FBS defined as *T. gondii* medium TM was used to wash the cells. Then this experiment was performed as described above (2.2.3.10.3).

## 2.2.4 Effect of $H_2O_2$ toxicity and *T. gondii* infection on SH-SY5Y cells.

### 2.2.4.1 Cell viability measurement (MTT assay).

The ability of the SH-SY5Y cells to survive in a culture media was determined by MTT assay (Alfa Aesar-L11939) This is formed from the degradation of the yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into the crystals of violet formazan through extra-mitochondrial dehydrogenases and mitochondrial enzymes. The formation of formazan from the MTT assay is the exact depiction of the viable cells. Seeding of the SH-SY5Y cells was done at a concentration of $5x10^4$ cells per 1 ml in a flat-bottomed 24-well plate. Incubation was done overnight using a growth media. Following treatments, incubation of the SH-SY5Y cells was done for 2 hours in MTT solution 500µl (5mg/ml) (dissolve in PBS) at 37°C and 5% $CO_2$. In each well, the culture medium was extracted without interfering with the insoluble formazan blue crystals. The dissolution of the formazan crystals was attained through the addition of 500µl of DMSO (Fisher Scientific, UK). This extent of reduction was assessed through monitoring the absorbance of the soluble formazan product a 540 nm and background at 690 nm using the plate reader (FLUOstar® Omega, BMG-LABTECH, UK). This MTT data was expressed as a percentage of the untreated cells which were the control.

#### 2.2.4.1.1 Effect of $H_2O_2$ on undifferentiated SH-SY5Y cells and induced cell death determined by MTT assay.

The undifferentiated SH-SY5Y cells were seeded in DMEM (growth medium) at a concentration of $5x10^4$ cells per 1 ml using a 24-well plate and then incubated at 37°C and 5% $CO_2$ for 24 hours. the medium was removed and treated with various concentrations of $H_2O_2$ in DMEM (50$\mu$M, 100$\mu$M, 150$\mu$M, 250$\mu$M and 500$\mu$M) for 24 hours and 48 hours at 37°C and 5% $CO_2$. The controls, the cells were incubated with DMEM where $H_2O_2$ was not added. An MTT assay was performed as described previously (2.2.4.1).

#### 2.2.4.1.2 Effect of $H_2O_2$ on differentiated SH-SY5Y cells and induced cell death determined by MTT assay.

The undifferentiated SH-SY5Y cells were seeded in DMEM (growth medium) at a concentration of $5x10^4$ cells per 1 ml using a 24-well plate and then incubated at 37°C and 5% $CO_2$ for 24 hours. These cells were left to differentiate for the next 21 days, as discussed above (2.2.3.6.4). After which the differentiated medium was removed and treated with various concentrations of $H_2O_2$ in differentiated medium (50$\mu$M, 100$\mu$M, 150$\mu$M, 250$\mu$M and 500$\mu$M) for 24 hours and 48 hours at 37°C and 5% $CO_2$. The controls, the cells were incubated with DMEM where $H_2O_2$ was not added. An MTT assay was performed as described previously (2.2.4.1).

#### 2.2.4.1.3 Effect of infection of *T. gondii* strain type I and II on $H_2O_2$ toxicity in undifferentiated SH-SY5Y cells determined by MTT assay.

The seeding of the undifferentiated cells SH-SY5Y cells was done using DMEM (growth medium) at a concentration of $5x10^4$ cells per 1 ml in a 24 well plate then incubated at 37°C and 5% $CO_2$ for 24 hours. DMEM supplemented with 1% FBS defined as *T. gondii* medium TM was used to wash the cells before the addition of ether type I or II *T. gondii* parasites as a ratio of 5 tachyzoite to 1 cell. Control cells (non-infected cells) were prepared using the *T. gondii* medium TM only. All cells were then incubated for 24 hours at 37°C and 5% $CO_2$. Treatment was then done using 150µM of $H_2O_2$ for 24 hours at 37°C and 5% $CO_2$. The control samples of non-infected and infected cells were then incubated using the medium alone. After 24 hours, an MTT assay was performed as described previously (2.2.4.1).

#### 2.2.4.1.4 Effect of infection of *T. gondii* strain type I and II on $H_2O_2$ toxicity in differentiated SH-SY5Y cells as determined by MTT assay.

The seeding of the undifferentiated cells SH-SY5Y cells was done using DMEM (growth medium) at a concentration of $5x10^4$ cells per 1 ml in a 24 well plate then incubated at 37°C and 5% $CO_2$ for 24 hours. These cells were left to differentiate for the next 21 days, as discussed above (2.2.3.6.4). After which the differentiated medium was removed and differentiated medium supplemented with 1% FBS defined as *T. gondii* medium TM was used to wash the cells before the addition of ether type I or II *T. gondii* parasites as a ratio of 5 tachyzoite to 1 cell. Control cells (non-infected cells) were prepared using the *T. gondii* medium TM only. All cells were then incubated for 24 hours at 37°C and 5% $CO_2$. Treatment was then done using 150µM of $H_2O_2$ for 24 hours at 37°C and 5% $CO_2$. The control samples of non-infected and infected cells were then incubated using the medium alone. After 24 hours, an MTT assay was performed as described previously (2.2.4.1).

#### 2.2.4.1.5 Effect of conditioned media (CM) of *T. gondii* strain I and II on $H_2O_2$ toxicity in undifferentiated and SH-SY5Y cells as determined by MTT.

The undifferentiated SH-SY5Y cells were cultured in a growth medium using 24 well plates for 24hours. The growth medium was removed and replaced with conditioned media (CM) taken from 5 different experimental scenarios. The first group of cells was incubated with conditioned media (CM) either for the strain I and II for 24 hours to 48 hours. The second group of cells was incubated using hydrogen peroxide (150$\mu$M) for 24-48 hours. The third group cells were incubated using hydrogen peroxide (150$\mu$M) and (CM) either for strain I and II for 24-48hour (this is known as co-treatment). The Fourth group of cells was incubated using hydrogen peroxide (150$\mu$M) for 24 hours then CM was added either from the type I or II strains for 24hour (this is referred to as the post-treatment). The fifth group of cells was incubated with CM either with strain I or II for 24 hours then supplemented with hydrogen peroxide (150$\mu$M) for 24hour (this is referred to as the pre-treatment). Control cells were incubated with DMEM without $H_2O_2$. At the end of experiment an MTT assay was performed as described previously (2.2.4.1).

### 2.2.4.2 Effect of $H_2O_2$ on undifferentiated SH-SY5Y cells and induced cell death determined by DAPI stain.

The seeding of the undifferentiated cells was done at a concentration of $5x10^4$ cells per 1ml in a 6-well plate in DMEM and left to incubate at a temperature of 37°C for 24 hours. Thereafter, the medium was removed and different concentrations of $H_2O_2$ in the growth medium (50$\mu$M, 100$\mu$M, 150$\mu$M, 250$\mu$M and 500$\mu$M) were used as the treatment of these cells for 24 hours at a temperature of 37°C and 5% $CO_2$. The cells used as controls were incubated

with a growth medium without $H_2O_2$. Washing was done once with PBS for 3 minutes and then fixing with 4% paraformaldehyde (pH7) for 10 minutes at RT. These cells were again rinsed three times using PBS for five minutes each time. Staining was done for five minutes using a Vectashield medium that contains DAPI, after which the cells were washed three times with PBS. Pictures were taken using the Cytation 3 imaging reader.

#### 2.2.4.3 Effect of $H_2O_2$ on undifferentiated SH-SY5Y cells and induced cell death determined by morphology change.

This experiment was performed as described above (2.2.4.2). The treated cells and un-treated cells (control) were left to incubate at 37°C and 5% CO2 for 24 hours. These cells were rinsed three times using PBS. Pictures were taken using the Cytation 3 imaging reader.

#### 2.2.4.4 Effect of $H_2O_2$ on differentiated SH-SY5Y cells and induced cell death determined by morphology change.

The undifferentiated SH-SY5Y cells were seeded in growth medium at a concentration of about $5x10^4$ cells per 1 ml using a 6 well plate and then incubated at 37°C and 5% $CO_2$ for 24 hours. These cells were left to differentiate for the next 21 days, as discussed above (2.2.3.6.4). After which the differentiated medium was removed and treated with various concentrations of $H_2O_2$ in the growth medium (50$\mu$M, 100$\mu$M, 150$\mu$M, 250$\mu$M and 500$\mu$M) for 24 hours at 37°C and 5% $CO_2$. In the controls, the cells were incubated with a growth medium where $H_2O_2$ was not added. These cells were rinsed three times using PBS. Pictures were taken using the Cytation 3 imaging reader.

### 2.2.4.5 Effect of infection of *T. gondii* strain type I and II on $H_2O_2$ toxicity in undifferentiated SH-SY5Y cells as determined by Giemsa stain.

The seeding of the undifferentiated cells was done at a concentration of $5x10^4$ cells per 1 ml on coverslips placed into a 6-well plate in DMEM and left to incubate at of 37°C and 5% CO2 for 24 hours. DMEM supplemented with 1% FBS defined as *T. gondii* medium TM was used to wash the cells before the addition of ether type I or II T. gondii parasites as a ratio of 5 tachyzoite to 1 cell. Non infected cells were prepared using the *T. gondii* medium TM only. All cells were then incubated for 24 hours at 37ºC and 5% $CO_2$. Treatment was then done using 150µM of $H_2O_2$ for 24 hours at 37°C and 5% $CO_2$. The control samples of non-infected and infected cells were then incubated using the medium alone. After 24 hours, the cells were stained with Giemsa stain as previously described (2.2.3.10.3). Then washing and mounting on a glass slide for analysis under the light microscope was done.

### 2.2.4.6 Effect of CM of *T. gondii* strain I and II on $H_2O_2$ toxicity in undifferentiated SH-SY5Y cells as determined by FACS.

Analysis by flow cytometry was illustrated using Annexin V APC Ready flow reagent-R37176 (Invitrogen, Thermo Fisher Scientific, UK). The SH-SY5Y cells were inoculated at a concentration of $3x10^5$ per 1 ml in 24-well plates and then incubated under 37°C and 5% $CO_2$ for 24 hours. The first group of cells was treated using 150$\mu$M $H_2O_2$ for 24 hours at a temperature of 37°C and 5% $CO_2$. the second group of cells was treated with CM of *T. gondii* strains (I and II) for 24 hours. The third group of cells was pre-treated with the CM of *T. gondii* strains (I and II) for 24 hours following the addition of 150$\mu$M $H_2O_2$ for 24 hours under 37°C and 5% $CO_2$. The cells used as controls were incubated with growth medium only. Harvested cells were then centrifuged at 1000g for 10 minutes then washed two times with PBS and the cell pellet

re-suspended in 500µl binding buffer that contains 5µl Annexin V-FITC and 5µl propidium iodide (PI). They were then incubated for 15 min in a dark room at room temperature. Samples were kept on ice and acquired immediately on a BD FACS Verse flow cytometer, using the BD FACSuite V1.0.6 software. The PI and Annexin V-APC emissions were identified in the channel APC-A and PE-A, respectively. In every plot, the lower left (LL) quadrant denoted viable cells while the upper left (UL) quadrant exhibited necrotic cells. The lower right (LR) quadrant revealed early apoptotic cells while the upper right (UR) quadrant revealed late apoptotic cells. This experiment was repeated three times.

#### 2.2.4.7 Data analysis

All graphs were prepared using Graph Pad Prism. Analysis of statistical data was performed by both One-way ANOVA followed by " Tukey's Multiple Comparison Test“, Dunnett comparison test" and Paired t-test. The results represented mean ±S.D., as well as the p-value $< 0.05$, which was regarded as statistically significant.

# 3 Chapter 3: Analysis of the frequency and distribution of *Toxoplasma* infection in a collection of samples from Alzheimer's patients.

## 3.1 Introduction.

This chapter aimed to undertake a preliminary study to establish whether samples obtained from the Manchester Brain Bank could be used as a source of material to determine whether there was any association between *Toxoplasma gondii* and Alzheimer's disease. This pilot study was carried out using previously prepared haematoxylin and eosin (H and E) stained slides from Alzheimer's patients which were used for another study.

*Toxoplasma gondii* is known to be an intracellular protozoan parasite with many secondary hosts, but it only reproduces sexually in the gut of its definitive host, the cat. Intermediate hosts constitute all warmblooded animals and include humans. The infection usually occurs orally where the cysts of the parasite or its oocysts are ingested and on reaching the intestines, they change to tachyzoites. These rapidly multiply and spread all over the body infecting various organs including the central nervous system where it infects neurons, microglia as well as astrocytes. Due to the non-conducive environment created by the immune system, the intra-neuronal tachyzoites later change to bradyzoites which have slower replication rates. Cysts are then formed from these bradyzoites (Figure 3. 1). Persistent infection with *T. gondii* does not usually present with any clinical symptoms and it is estimated about 30% of the human population have chronic *T. gondii* infection. Studies have, however, recently revealed that the human susceptibility to neurological diseases as well as behavioral changes can be increased by *T. gondii* infection (Haroon et al. 2012). The development of Parkinson's and Alzheimer's diseases have lately been connected with *T. gondii* infection. This was demonstrated by the

elevated anti-*T. gondii* antibodies in the serum of Alzheimer's patients (Passeri et al. 2016). These findings, alongside others, suggest the possibility that there is an association between the *T. gondii* infection and the development of neuropsychiatric disorders. Nevertheless, it has not been demonstrated by the serological studies that there are any neuronal cellular or molecular changes. Alzheimer's disease has been associated with damage to the brain's frontal lobe area and hippocampus by past studies (Lehtovirta et al., 1995; Shammi and Stuss, 1999). In past studies, murine models have been used to examine the effects that *T. gondii* has on host behavior. The outcomes have shown that this parasite alters the behavior of rodents with regards to prey-predator interaction. This happens by causing rodents to lack fear and be unable to small cat's urine (Berdoy, Webster, and MacDonald 1995; Webster 2001). These changes in rodents' behavior imply that this parasite alters the rodents' behavior, making them at risk of being captured by the definitive feline host (Hermes et al. 2008; Parlog et al. 2015). This parasite has also been shown to affect the activity of the human brain. Several research studies have identified *T. gondii* infection as one of the risk factors for certain neuropsychiatric disorders, especially schizophrenia (Torrey and Yolken 2003). It has been demonstrated that among individuals with chronic schizophrenia, there is usually a high percentage of anti-*T. gondii* antibodies compared to control groups. Serological data has also shown a relationship between psychiatric disorders and *T. gondii* infection among people with bipolar disorders (Fabiani et al. 2015; Passeri et al. 2016). An elevated level of anti-*T. gondii* antibodies have also been detected in sera from Alzheimer's disease patients, which also points to a possible relationship between Alzheimer's disease and *T. gondii* infections (Kusbeci et al. 2011). Even though these studies have shown an indirect association between *T. gondii* infection and the chances of developing brain disorders, the impact of *T. gondii* on neuronal networks and the human brain leading to these conditions has not been established (Passeri et al. 2016). In this chapter a preliminary investigation on two human brain regions will be carried out for

*Toxoplasma* presence and any association with Alzheimer's disease established. The samples, used in this study, were sourced from the Manchester Brain Bank (MBB), and were used for another study by colleagues at Salford University. The MBB was established by Professor David Mann in 1986. This brain bank has supported much clinical and basic research on frontotemporal dementia for more than thirty years. This Brain Bank stores about 1,000 brains obtained from elderly, healthy individuals and patients with different neurodegenerative disorders. Many of these brains have frozen and formalin-fixed tissues that can be used in approved research. In order to get access to samples and ethical approval to use the samples for specific studies, proof of principle is required. This is the subject of this chapter.

In these studies, samples from the frontal lobe area and hippocampus stored at the Manchester Brain Bank were obtained. The brain tissue samples were provided by Dr. Gemma Lace-Costigan from a previous AD study and the tissues were previously with H&E and IHC for specific AD proteins (but not for *T. gondii*). However, H&E staining should enable us to potentially pick out *T. gondii* cysts (Figure 3.1). Light microscopy was used to examine these samples with the aim of determining the presence of *T. gondii* cysts. The hypothesis of this project will be to consider whether there is an association between the presence of the *T. gondii* cysts in these samples and samples from Alzheimer's patients.

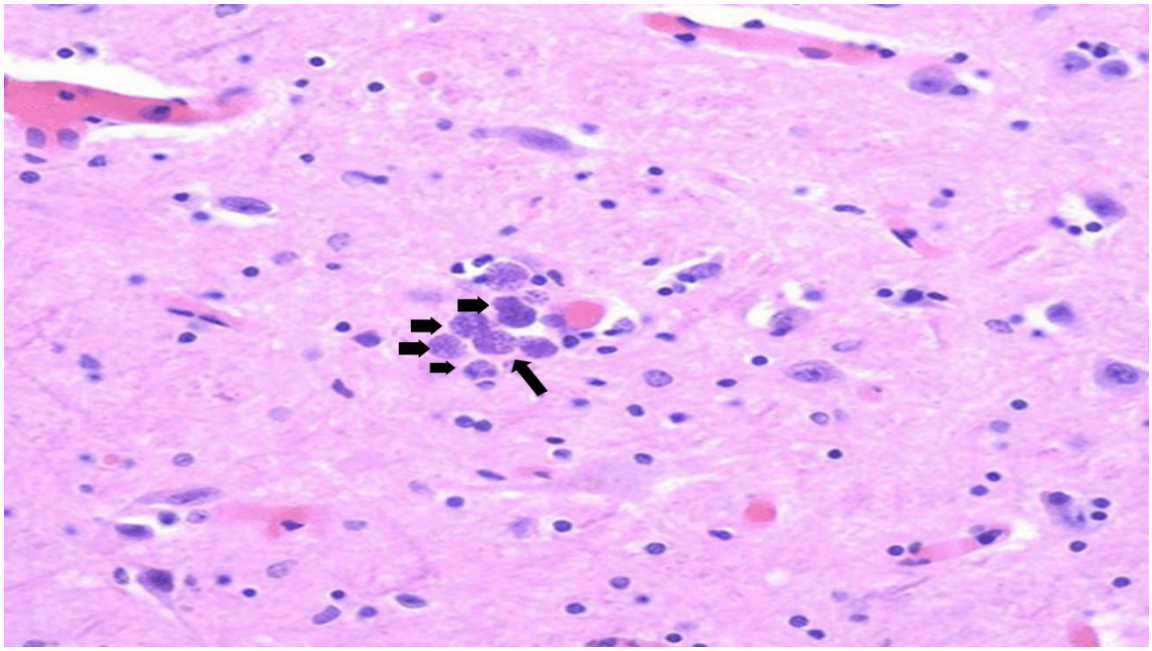


**Figure 3-1** The cysts of *T. gondii* in the brain of humans. *T. gondii* cysts in the midbrain tissues were stained using hematoxylin and eosin (H&E) staining. 400X magnification. The arrows indicate examples of cysts. Images adapted from (Hamz, Anderson, and Al-Khafaji 2017).

### 3.1.1 Objectives.

This chapter aims to analyse the frequency and distribution of *Toxoplasma* cysts in a collection of samples from patients suffering from Alzheimer's disease. The purpose is to determine the feasibility of using human brain samples from the Manchester Brain Bank to investigate the relationship between infection and disease. The objectives are as below;

- Use visualization by light microscopy to evaluate the presence of *Toxoplasma* cysts in human frontal brain tissue.
- Use visualization by light microscopy to evaluate the presence of *Toxoplasma* cysts in human hippocampal brain tissue.

## 3.2 Methods:

A total of 124 brain samples from humans Alzheimer's patients (63% female and 37% male, mean age at death 84.2 years old). These samples were taken from two brain regions: hippocampus (35 samples) demonstrating an early stage of AD and frontal lobe (89 samples) indicating the advanced disease progression. The samples were pre-stained through immunohistochemistry for the purpose of another Alzheimer's disease project and kindly supplied by Dr Gemma Lace. The samples were analyzed by light microscopy under different objective including 4x, 10x, 40x and 100x for the presence of *T. gondii* cysts as described in (2.2.1). All samples were examined using 100 random fields of view per slide ensuring coverage of most of the sections.

## 3.3 Results.

### 3.3.1 Detection of *T. gondii* in brain tissue.

To determine the presence of *T. gondii* cysts in the frontal lobe (89) and region, 89 H&E stained slides were examined. On examining the slides, the outer cerebral cortex layer showed the gray matter with neuronal cell bodies, glial cells as well as blood vessels (Figure 3. 2 A). There were also axons and glial cells in the white matter (Figure 3. 2 B). Additionally, in the inside of the neuronal cell body, there was a build-up of β-amyloid senile plaques (Figure 3. 2 C) in patients with AD. All samples (89 slides) were examined, using 100 random fields of view per slide, to find *T. gondii* cysts. *T. gondii* cysts were undetectable in any samples of the frontal lobe (Table 3.1). Also, 35 slides from the hippocampus were examined as described in (2.2.1). On examining the slides, the cortex outer layer revealed the white and grey matter. Other hippocampus constituents such as dentate gyrus and the brain blood vessels were also visible and showed the build-up of neurofibrillary tangles within the neuronal cell bodies (Figure 3. 3 A, B, C). *T. gondii* cysts were undetectable in the samples from the hippocampus (Table 3. 1).

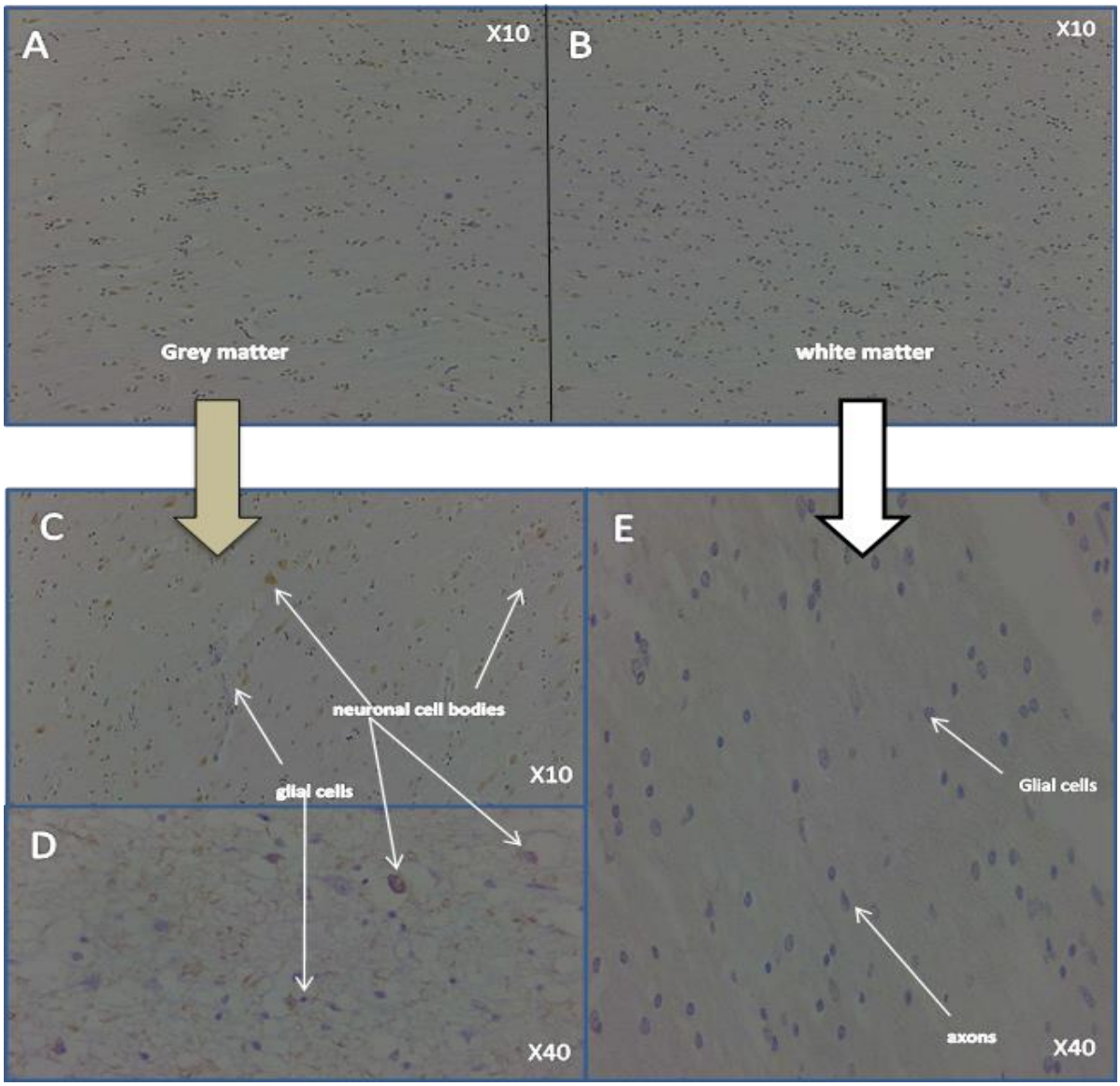



**Figure 3-2** Microscopic anatomy of the human Frontal lobe. The samples were stained by Immunohistochemistry with antibodies against Beclin-1 and AT8. (A), (C) and (D) show the outer layer of the cortex - the grey matter - which consists of neuronal cell bodies and glial cells. (C) This tissue was stained with Beclin-1 and shows the accumulation of β-amyloid as senile plaques inside the neuronal cell bodies, and in (D) shows neurofibrillary tangles inside the neuronal cell body. No *T. gondii* cysts were visualized in these sections. (B) and (E) show the white matter which contains glial cells and axons that are parts of the neuronal cell. X10 and X40 indicate the magnification of the slides. The staining and slide preparation was carried out by Dr. Gemma Lace as part of a previous study. (Slide number - 09/22).

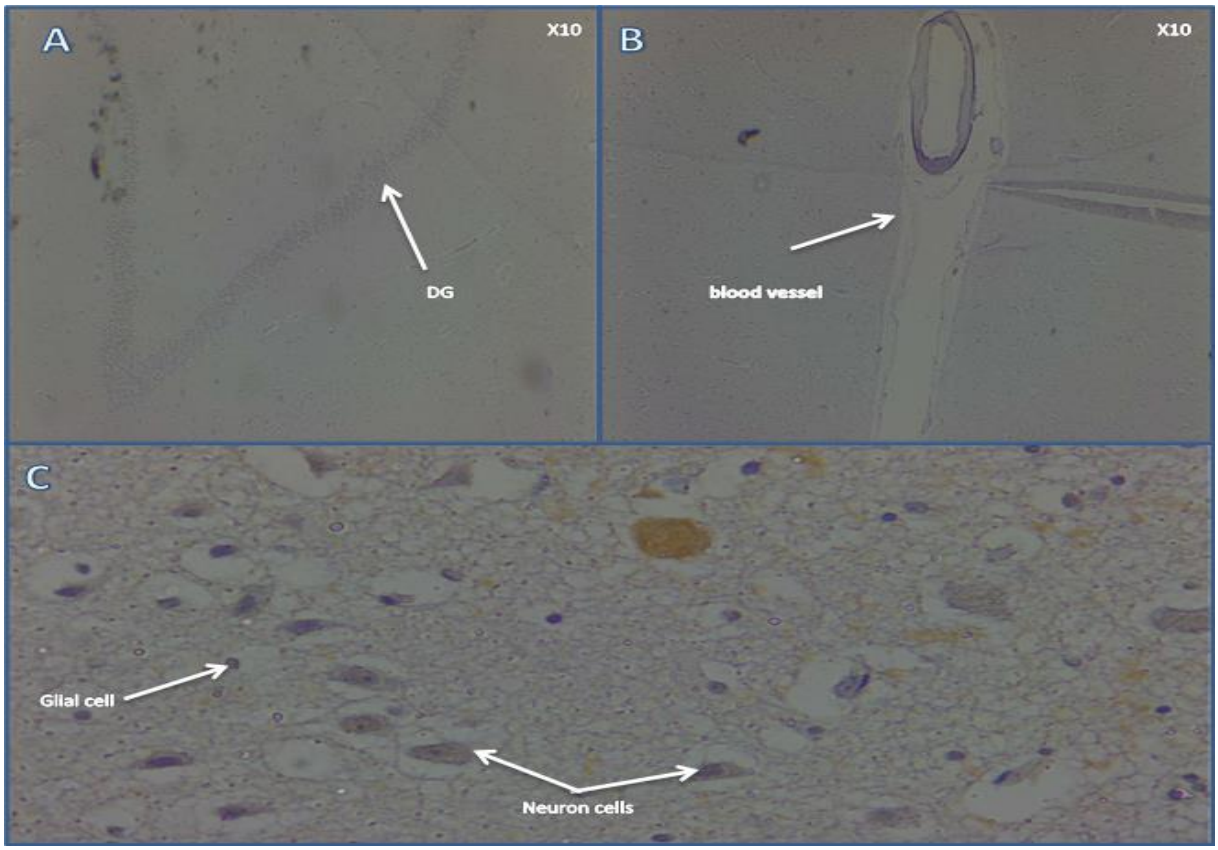



**Figure 3-3** Microscopic anatomy of the human hippocampus. The samples were stained by Immunohistochemistry with antibody AT8. (A) the hippocampal dentate gyrus (DG). (B) Blood vessel. (C) shows different glial cells and the body of neuronal cells. Magnification: X10 and X40. The staining and slide preparation were carried out by Dr. Gemma Lace as part of a previous study. (Slide number-14/09).

**Table 3-1** Summary of the examination of 90 slides of samples of tissues from two parts of the brain: frontal lobe and hippocampus. No detection of *T. gondii* cysts was observed.

| **Brain region** | **No. samples** | | **Average sample size** | **Percentage of *T. gondii* infection** |
|---|---|---|---|---|
| **Frontal lobe** | 89 | | 0.0005 $cm^3$ | 0% |
| **Hippocampus** | 35 | | 0.0005 $cm^3$ | 0% |

## 3.4 Discussion.

In this study, A total of 89 human brain frontal lobe samples and 35 hippocampal samples were investigated for the presence of *T. gondii* infection. In both sets of samples, *T. gondii* cysts were undetectable. The hypothesis that *T. gondii* infection is associated with Alzheimer's Disease (AD) cannot be addressed using these samples since we were unable to detect cysts. A secondary purpose of this study was to investigate whether this analysis could provide proof of concept data to inform a more substantive application to the Manchester Brain Bank for a wider study on the association between *T. gondii* infection and AD. These data suggest that this approach could not be used to investigate this question. This raises the question as to why cysts were not detected in these samples. In the UK, it is estimated that 10% of the human population are infected with *T. gondii* (Pappas, Roussos, and Falagas 2009) and the global infection rate is 30%. One possibility is the sample number is too small to detect this level of infection. If the UK figure is used (10%), 12 samples would have been expected to be positive and contain cysts. There is a statistically significant difference ($p$ = 0.0004) between the expected 12 positive samples and the observed positive samples (0). It is therefore unlikely that the sample number was too small. A second possibility is that the physical sample size of each section is too small to ensure the detection of cysts. In this study, small samples with a volume of about 0.063cm$^3$ from patients with Alzheimer's disease were. The whole volume of these samples was therefore tiny compared with the total volume of the human brain which is about 1260 in men and 1130 cm$^3$ in women (Cosgrove, Mazure, and Staley 2007). These samples, therefore, represented only 0.0049% of the human brain volume. As discussed above, all the sample parts were closely analyzed for the presence of *T. gondii* cysts using high and low power magnification. A series of pictures of the sample fields were then taken for comparison; the results show that *T. gondii* cysts were undetectable. It is likely, therefore, that there was insufficient brain tissue to detect cysts even if they were there. These samples are

therefore not large enough to give accurate results. In another study, published during the course of the work carried out in this chapter, human brain samples weighing about 3g were analyzed using a molecular technique (PCR) (Burrells et al. 2016). This was a relatively larger sample when compared with ours – in fact, approximately 47x times larger based on weight. According to the findings of this study, *T. gondii* was present in about 17.9% of the samples. It seems very likely, therefore, that our failure to detect cysts was based on the tiny amount of tissue we had available. Therefore, to develop a future strategy to overcome this problem it may be necessary to request 3 grams of brain tissue for each sample from the Manchester Brain Bank. This is likely to be a big ask when tissue is restricted. Furthermore, the finding indicates that a more sensitive technique be used – such as PCR or a parasite specific immunohistochemical technique. It should be noted that the use of appropriate immunohistochemistry staining techniques with special anti-bradyzoite antigen 1 (anti-BAG-1) antibody as well as the anti-tachyzoite surface antigen 1 is important because they can easily detect the presence of *T. gondii* (Passeri et al. 2016; Tomita et al. 2017). The demonstration of the lack of *T. gondii* presence in the brain tissues in this study is certainly significant from the point of evaluating the viability of utilizing the Manchester Brain Bank's human brain samples. Since this resource is designed for multiuser projects, the large amount of brain tissue required (3g) would seem unsustainable - especially if different areas of the brain are to be sampled separately. Other substitute approaches could include cell culture or animal methods. These alternative approaches can be utilized because, for instance, small animals like mice and rats have comparatively smaller brains and this increases the chances of *T. gondii* detection when looking at a relatively small tissue section. Another approach that can be utilized in this study is the cell culture method where commercially available human cell lines can be used. SH-SY5Y cells are an example of a human cell line, which has been utilized as a prototype of human cell neurons. These cells can be transformed into neurons and used to specifically target

interactions with neurons. Numerous studies on neurodegenerative diseases have utilized the *in vitro* SH-SY5Y cells for general studies (Cheung et al. 2009; Pahlman et al. 1990) but none, to our knowledge, have been used to investigate the interaction between *T. gondii* and neural cells.

# 4 Chapter 4: Development of a model system, using cell culture, to investigate the effects of *Toxoplasma* on brain cell pathology.

## 4.1 Introduction.

This chapter aims to develop a model system, based on tissue culture, that can be used to investigate the effects of the parasite *Toxoplasma gondii* on brain tissue. The basic components of this research are first to develop cell culture systems that can be used to mimic brain cells in such a way that their behaviour can be measured in response to parasitic infection. Secondly, to develop cell culture systems that support the growth of *Toxoplasma gondii* for transfer and infection of cultured neural cells.

### 4.1.1 Introduction to tissue culture and development of model systems.

Tissue culture is now regarded as an important approach for cell biological analysis of cells from various multicellular organisms. It offers an *in vitro* prototype of different tissues in an appropriate environment, which can be analyzed and manipulated easily (Diab and El-Bahy 2008; Phelan 2007). In order to acquire a sufficient quantity of cells needed to conduct an analysis, the cells have to be artificially grown for a given period of time using cultures. This process requires stringent utilization of aseptic procedures to deter possible contamination and loss of valuable cell lines. This method has been utilized in various studies on Alzheimer's disease to investigate the causes of the disease and to develop diagnostic methods (Koriyama et al. 2015; Phelan 2007; Teppola et al. 2016).

The development of cell culture is usually done through cell lines that are very specific to this study such as the SH-SY5Y cell line, which is usually used as an *in –vitro* model of neuronal

differentiation and function (Pahlman et al. 1990). In a culture environment, the neuronal SH-SY5Y cell lines retain their ability to differentiate and recess/regress. It has been shown by different studies in the past that cholesterol and trans-retinoic acids can trigger SH-SY5Y cellular differentiation. Therefore, the introduction of retinoic acid (RA) in a cell culture medium has been widely used to trigger SH-SY5Y cell differentiation (Teppola et al. 2016; Xun et al. 2012). The possibility of altering the culture medium containing the SH-SY5Y neuroblastoma cells to differentiate them into different cells that contain comparatively mature phenotypes that resemble neuronal cells has been useful in neuroscience studies. The benefits of this process include the possibility of extensive expansion before differentiation in an easy manner and cost-effectiveness in comparison to primary neurons (Kovalevich and Langford 2013). The ethical issues that are always associated with the culture of human neuronal cells will not arise in this case, as these are a cell line and not primary human cells. Moreover, the SH-SY5Y cells are obtained from humans and they depict protein and other protein variants that are specific to humans and cannot be found in rodent cultures or in *in vivo* rodent experiments. Additionally, the cell cycle is usually harmonized during differentiation to generate a standard population of neuronal cells as this process can vary immensely in SH-SY5Y cells that are undifferentiated and other cell lines (Encinas et al. 2000; Ross, Spengler, and Biedler 1983).

The possibility of generating cell cultures for *T. gondii* host cells comprising the SH-SY5Y, as well as general pathogen culture cells such as human foreskin fibroblast (HFF), and Madin-Darby Bovine Kidney (MDBK) cells, will be explored in this chapter (Figure 4. 1). Furthermore, the possibility of deriving the neuronal cell prototypes from the SH-SY5Y neuroblastoma cells through differentiation will be examined. The SH-SY5Y cell line can be obtained commercially by purchasing them through various organizations such as the American Type Culture Collection (ATCC) and the European Collection of Cell Cultures

(ECACC). In order to grow this cell line, only specific media like DMEM can be used. The conditions required are the temperatures of 37 °C, and 5% $CO_2$ (Di et al. 2018; Shipley, Mangold, Kuny, et al. 2017). As has been demonstrated previously that it is possible to differentiate the SH-SY5Y cells into prototypes of mature neurons. Therefore, the risk of developing Alzheimer's disease through Tau protein phosphorylation and increased levels of beta-amyloid can be investigated by using this as a model system. Additionally, the exploration of the impacts of various environmental elements such as oxidative stress and how they trigger the expression and distribution of Tau proteins that are associated with Alzheimer's disease can be carried out (Agholme et al. 2010; Akki et al. 2018; Chin-Chan et al. 2015; Schneider et al. 2012).

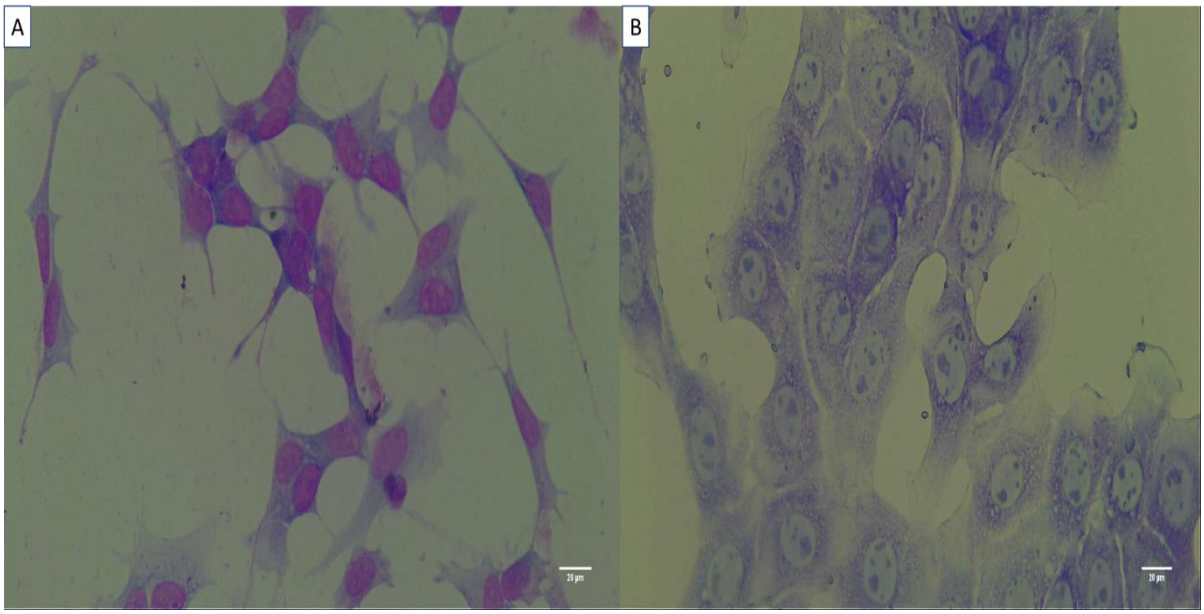



**Figure 4-1** Photomicrographs showing SH-SY5Y and MDBK cells in culture. (A) undifferentiated SH-SY5Y cells after 24 h of culture and stained with Giemsa. (B) MDBK cells after 24 h and stained with Giemsa. Images were taken by light microscopy. Scale bar, 20 μm.

### 4.1.2 Introduction to *T. gondii* culture and development of a model infection system.

*Toxoplasma gondii* is primarily an intracellular parasite and it has the capacity to enter all nucleated cells and reproduce in them. The main route of infection by this parasite is oral where the oocytes or the cysts are ingested when an individual eats a tissue that has been infected (Halonen and Weiss 2013). Inside the host, the tachyzoites, which are known to be the rapidly replicating form of *T. gondii* usually, found in the acute stage of toxoplasmosis, replicate within the nucleated cells. These tachyzoites then differentiate to form the bradyzoites, which develop to form the tissue cysts. Continuous multiplication and differentiation of these tachyzoites within the cell causes pressure in it and the cell eventually raptures to release them. This causes their spread through the bloodstream to various parts of the body (Wilson and Hunter 2004). There are three main genotypes of *T. gondii*, which are Type 1, Type II, and, Type III (Boothroyd and Grigg 2002). The type I strain is known to be vastly infectious and harmful in rats and mice however some strains do not form cysts in the brain (e.g. type I- RH). Nevertheless, other strains, which include types II and type III are less infectious and are generally associated with chronic infections in humans (Angeloni et al. 2009) and are also capable of producing cysts (e.g. type II- Me49). This parasite is, therefore, emerging as one of the ideal systems for intracellular parasitism studies. This is due to its various features, which makes genetic analysis of its make up possible. Furthermore, this parasite can infect almost all the nucleated mammalian cells, which makes it possible to perform *in vitro* propagation through cell culture methods. Nevertheless, the kind of primary cell line infected by the *T. gondii* parasite usually defines its *in-vitro* rate of growth (Figure 4.2) (Evans et al. 1999). Studies have shown that different genotypes of strains of *T. gondii* exhibit different *in-vitro* growth rates where type I strains grow more rapidly compared to other

types (II and III) (Radke and White 1999). However, in SH-SY5Y cells, the *T. gondii* growth rate has not been established by any studies in the past.

This chapter will explore the ability to maintain the type I and type II strains of *T. gondii in-vitro*. Furthermore, the rate of host infection and growth of this parasite in the differentiated and undifferentiated SH-SY5Y cells will be investigated. Type I and type II (RH and Me49) of the *Toxoplasma* strains will be utilized in this experiment.

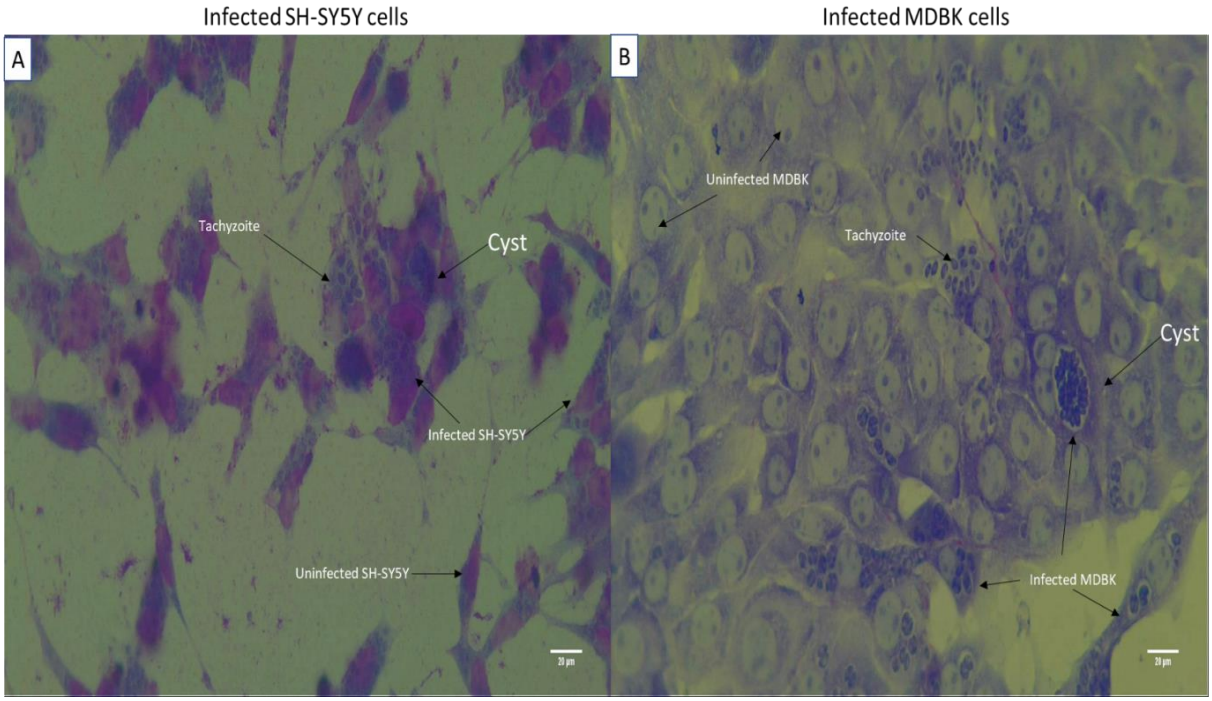



**Figure 4-2** Photomicrographs showing undifferentiated SH-SY5Y and MDBK cells in culture and their interaction with *T. gondii*: (A) SH-SY5Y cells after 48h of culture and after 24 h of infection with *T. gondii* (arrow), stained with Giemsa. (B) MDBK cells after 24 h of culture and after 48h of infection with *T. gondii* (arrow), stained with Giemsa. Numerous intracellular and extracellular *T. gondii* tachyzoites and cysts (arrows). images were taken by light microscopy. Scale bar, 20 μm.

### 4.1.3 Objectives.

The aim of this chapter is to develop a model system, using cell culture, to investigate the effects of *Toxoplasma* on brain cell pathology.

There are four main objectives:

- Development of cell culture methods for growing the host cells for *T. gondii* using MDBK, HFF, and SH-SY5Y cells.
- Development of methods for differentiation of SH-SY5Y cells into neuronal cells.
- Infection of MDBK cells with *T. gondii* and maintenance of *Toxoplasma.*
- Development of a model system for infection of SH-SY5Y cells and differentiated neuronal cells with *Toxoplasma.*

## 4.2 Methods.

In section (2.2.3), the technique of culturing the SH-SY5Y, MDBK, and HFF was described in detail. Appropriate environments were created when culturing these cells and upon reaching 70-90% confluent cells, the trypsinization method was used to collect/harvest them. Also, in section (2.2.3.6), the technique of SH-SY5Ycell differentiation was discussed in detail. Using coated 6 well plates, these cells were grown and upon reaching 60-70% confluent cells, or after 48 hours, the culture medium was replaced every 2 days by adding 10 µm RA up to day 10 or by adding 1 µm RA up to day 21. Images were also taken on specific days to note the morphological changes that occur in SH-SY5Y cells during the process of differentiation. For immunofluorescence staining, the differentiation and growth of the cells were carried out as described (2.2.3.6.4). Following fixation, permeation and blocking, the cells were incubated with primary antibodies. The visualisation was achieved using Alexa fluorophore secondary antibodies. Cells were then mounted using media containing DAPI and imaged using a Cytation 3 imaging reader (2.2.3.6.5). For *T. gondii* growth, the culturing and conservation of the *T. gondii* into MDBK cells was described in the methods section (2.2.3.7). MDBK cell culture was carried out for 24 hours and then tachyzoites were then introduced. 72 hours after inoculation, harvesting of the tachyzoites was done and, using a tachyzoite to cell ratio of 5:1, the tachyzoites were maintained through a sequential relocation into new MDBK cells after every 48 and 72 hours. Remaining tachyzoites were stored and frozen at -80°C. The long-term preservation of the *T. gondii* requires the cells to be frozen using liquid nitrogen at about -196$^{o}$C as discussed in the methods section. As previously described, (2.2.3.8), two techniques of *T. gondii* freezing were used: intracellular and extracellular freezing. Determining the growth rate of *T. gondii* strains type I and II (RH$\Delta$KU8 and ME49 strain) was carried out. Here, undifferentiated and differentiated SH-SY5Y cells were grown in the T25 flasks at a concentration of 1x10$^{6}$ cells per mL for 24 hours. *T. gondii* tachyzoites type I and II were then

introduced at a ratio of 5 parasites in 1 cell at 37ºC and 5% $CO_2$, incubation of the cultures was done for a period of 3 and 4 days. After the third and fourth-day post-inoculation, tachyzoites were harvested and counted using a hemocytometer. Moreover, determining the infection rate of *T. gondii* strains type I and type II was carried out. The undifferentiated and differentiated SH-SY5Y cells were grown on coverslips into 6-well plates with a concentration of $5x10^4$ cells per mL for 24 hours. *T. gondii* tachyzoites type I and II were then introduced at a ratio of 5 parasites in 1 cell at 37ºC and 5% $CO_2$. Incubation of the cultures was done for a period of 3 and 4 days, respectively. Giemsa was then used to stain the infected cells and then a light microscope used to examine them.

## 4.3 Results.

### 4.3.1 Development of cell culture for Human foreskin fibroblast (HFF) cells.

The aims of the initial set of experiments were to develop cell culture methods for growing Human Foreskin Fibroblast (HFF) cells. These cells are good host cells for growing *T. gondii* and are also good cells for use as controls and as cell lines for developing techniques. Using a growth media, the HFF cells were cultured and incubated under 37ºC and 5% $CO_2$. Every 2 days, the cell medium was changed and on reaching a confluence of 85–90%, subculturing of the cells was done. From the outcomes, the cells grew adequately and were passaged every six days as seen in (Figure 4. 3). These cells also were frozen by using a freezing medium comprising 90 % (v/v) FBS and 10% DMSO as is described in chapter 2 (2.3.1). When these cells were successfully recovered, the experiments showed that most cells remained alive after thawing and recovery of the frozen cells.

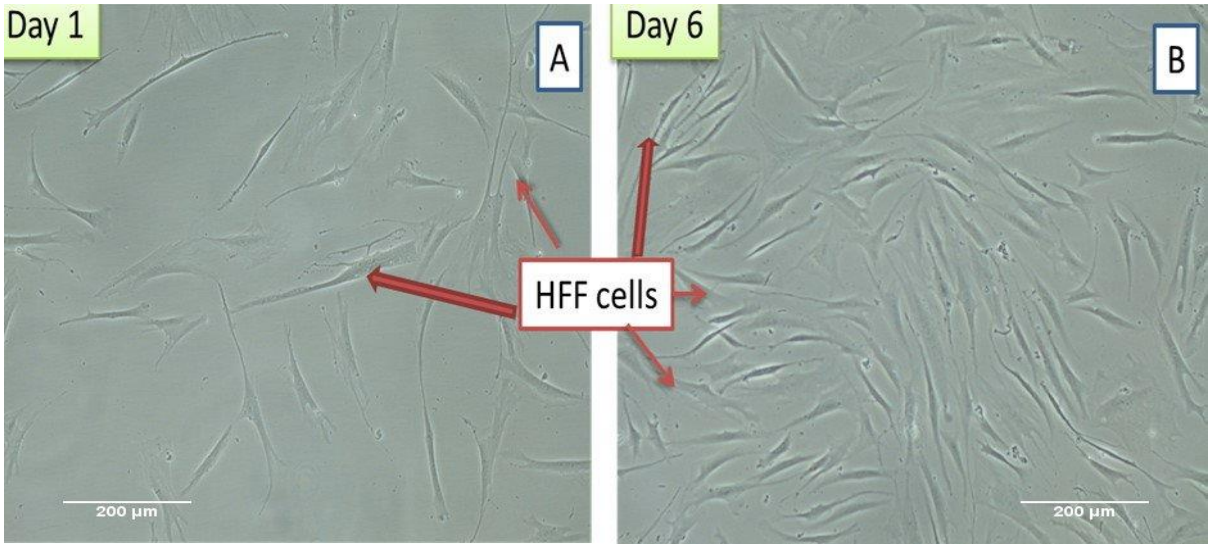



**Figure 4-3** Morphology and growth of HFF cells. The cells were used as a control for the tissue culture methods. These pictures show that the successful growth of HFF in the recommended medium for 6 days. (A) Shows the cells confluent at 40 % after 24h of culturing and (B) shows the cells confluent between 80-90 % after 6 days of culturing. Images were obtained at 10X magnification, scale bar, 200 µm.

### 4.3.2 Development of cell culture for Madin-Darby Bovine Kidney (MDBK) cells.

The aims of the second stage of the process were to develop cell culture methods for growing Madin-Darby bovine kidney (MDBK) cells. Also, these cells are good host cells for growing *T. gondii* and are also good cells for use as controls and as cell lines for developing techniques. In later experiments, the culture and conservation of the *T. gondii* parasites were done using MDBK cells. The cryovials holding the frozen MDBK cells were thawed and recovered. Using a growth media, the MDBK cells were cultured and incubated under 37°C and 5% $CO_2$. These cells grew optimally and were passaged every four days and the remaining cells were frozen slowly to -80°C and stored for future use.

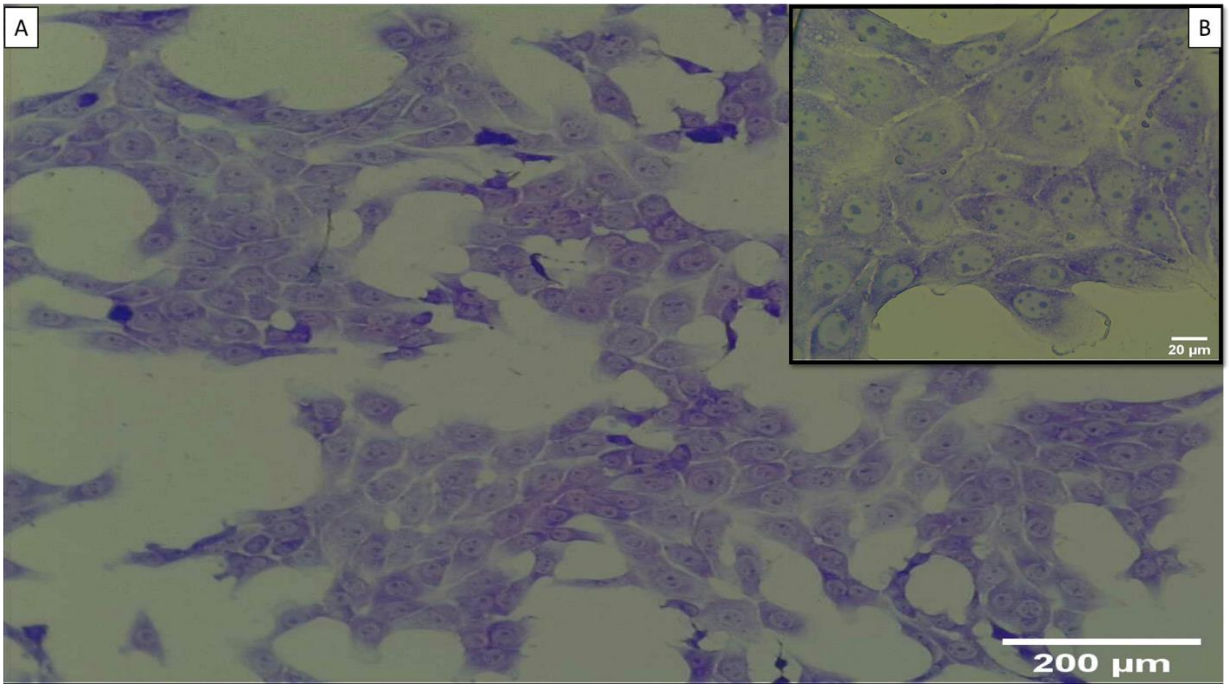



**Figure 4-4.** Morphology and growth in MDBK Cells. This image shows the successfully recovery of cells from frozen storage and subsequent growth. (A) and (B) MDBK cells after 24 h incubation and stained with Giemsa. Images were taken by light microscopy. (A) Scale bar, 200µm. (B)Scale bar, 20 µm.

### 4.3.3 Development of cell culture for SH-SY5Y cells.

The aims of the next stage of the setup of a model system were to develop cell culture methods for growing SH-SY5Y cells. These cells are often used as *in vitro* models of neuronal function and differentiation. In the study of Alzheimer's disease, SH-SY5Y cells have been commonly used. Culturing and subculturing of these cells were conducted up to passage 20 and all the passages were kept in liquid nitrogen and frozen for use in future studies. Following the optimization of growth conditions, these cells grew well. They were passaged every six days and fresh SH-SY5Y cells were frozen at -80°C. As shown in Figure 4.5, SH-SY5Y cells grew well and showed cells with characteristic phenotypes: a flat, large, epithelial-like phenotype with numerous short processes extending outward.

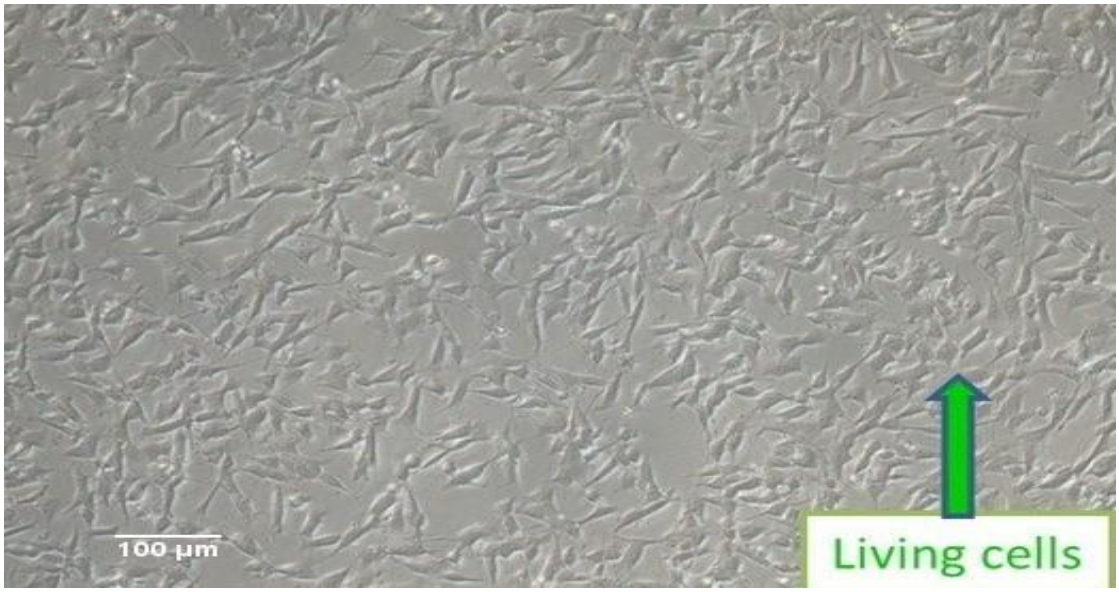



**Figure 4-5** Morphology and growth in SH-SY5Y cells. This image shows the successful recovery of cells from frozen storage and subsequent growth. Images were obtained at 10X magnification, scale bar 100 µm.

## 4.3.4 Differentiation of SH-SY5Y cells into neuronal cells.

### 4.3.4.1 Optimization of differentiation of the SH-SY5Y cell into neuronal cells.

The aims of these experiments were to optimize differentiation methods for the differentiation of SH-SY5Y cells. These cells can differentiate into neuronal cells *in vitro*. Retinoic acid (RA) is an essential inducer for SH-SY5Y differentiation however, it is highly sensitive to light and heat. As shown in figure 4.6, the differentiation medium was prepared by adding 10 μM of RA to the medium then this medium was kept in the fridge as usual (without a foil covering – i.e. stored in the light). Also, the differentiation of SH-SY5Y was done on plates, then incubated at 37$^{o}$C and 5 % $CO_2$ as was described in the methods section. In figure 4.6 B, the results showed that on the second day of the differentiation, the cells did start to differentiate. The cells began to elongate as the process of differentiation in SH-SY5Y progressed. As shown in figure 4.6 C, on the third day, the cell numbers began to decrease dramatically. And on the tenth day, as shown in figure 4.6 D, all the cells were dead. The reason for cell death is due to the exposure of the RA to light, which makes it toxic to cells and leads to cell death. To address this challenge, the light needs to be prevented from reaching the differentiation medium used for the differentiation of SH-SY5Y cells.

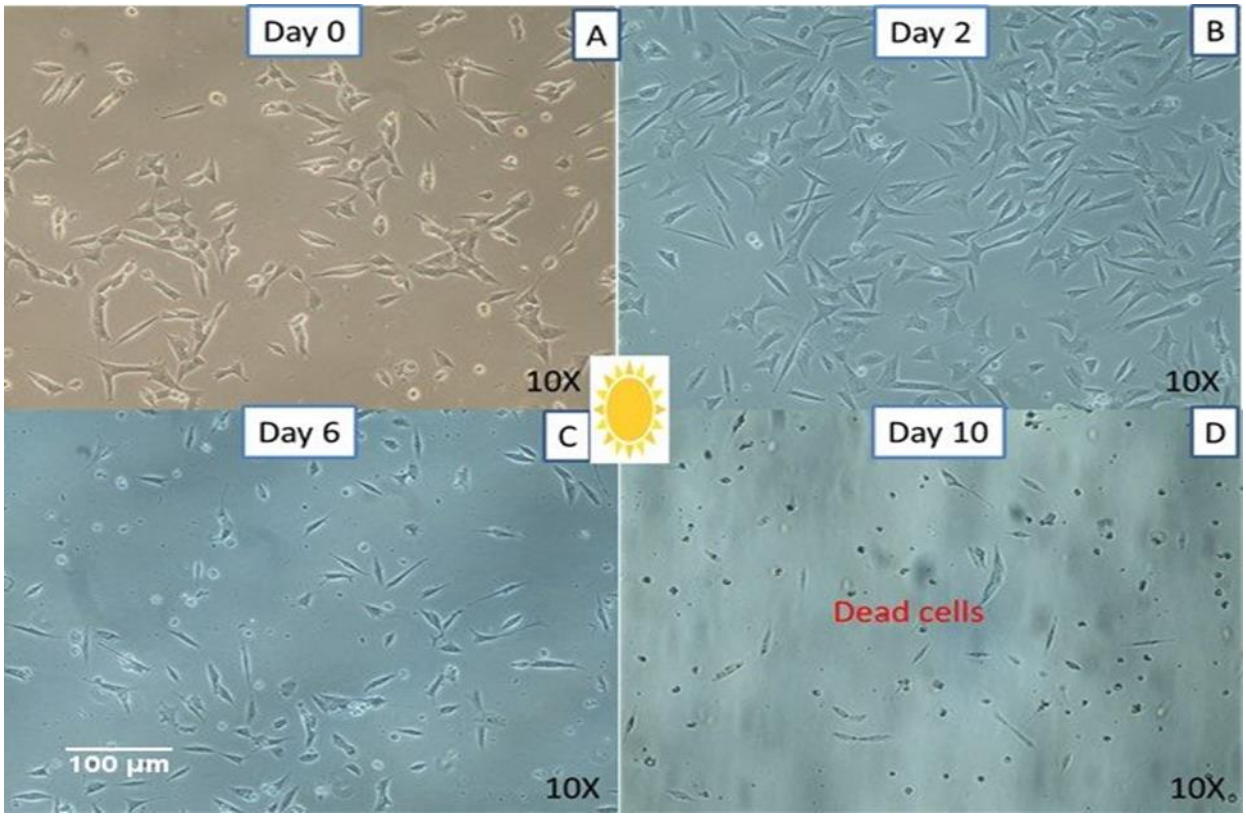



**Figure 4-6** Morphological appearance of differentiated SH-SY5Y cells, this experiment was done with light exposure. (A) On day 0 of differentiation. (B) On day 2 of differentiation. (C) On day 6 of differentiation that shows a decreased number of cells. (D) On day 10 of differentiation that shows most of the cells died. Images were obtained at 10X magnification, scale bar, 100 µm.

#### 4.3.4.2 Differentiation of SH-SY5Y cells.

The aims of this part of the study were to differentiate SH-SY5Y cells into neuronal cells. The ability to differentiate these cells into neuron cells *in-vitro* allows them to be used in Alzheimer's disease research. Differentiation of the SH-SY5Y cells to the characteristic neuronal morphology occurs when they are exposed to retinoic acid. Before establishing whether the cellular bioenergetics are altered through differentiation, the SH-SY5Y cells were seeded at a concentration of $1x10^5$. Thereafter, 10$\mu$M RA was used to differentiate them for 10 days or 21 days using 1$\mu$M of RA. As demonstrated in figure 4.7, on day 0, the cells showed

a very compact morphology, which altered between days 4-10 after being exposed to RA (10$\mu$M). As shown in figure 4.8, the cells formed dendrite like protrusions, axons and the cytoplasm were shrunken. Also, in figure 4.9, the result shows that by using 1 µM RA concentrations, the full differentiation of the SH-SY5Y cells was successful. As demonstrated in figure 4.8 A, there was a rapid growth of the undifferentiated SH-SY5Y cells with large, flat, features of epithelial and several short projections. On the other hand, the differentiated cells did not continue growing and had numerous neuritic protrusions that were joined to the nearby cells (figure 4.8 B). The next stage was to confirm that the differentiation of SH-SY5Y cells was indeed occurring by methods such as immunofluorescence detection of classical neuronal markers.

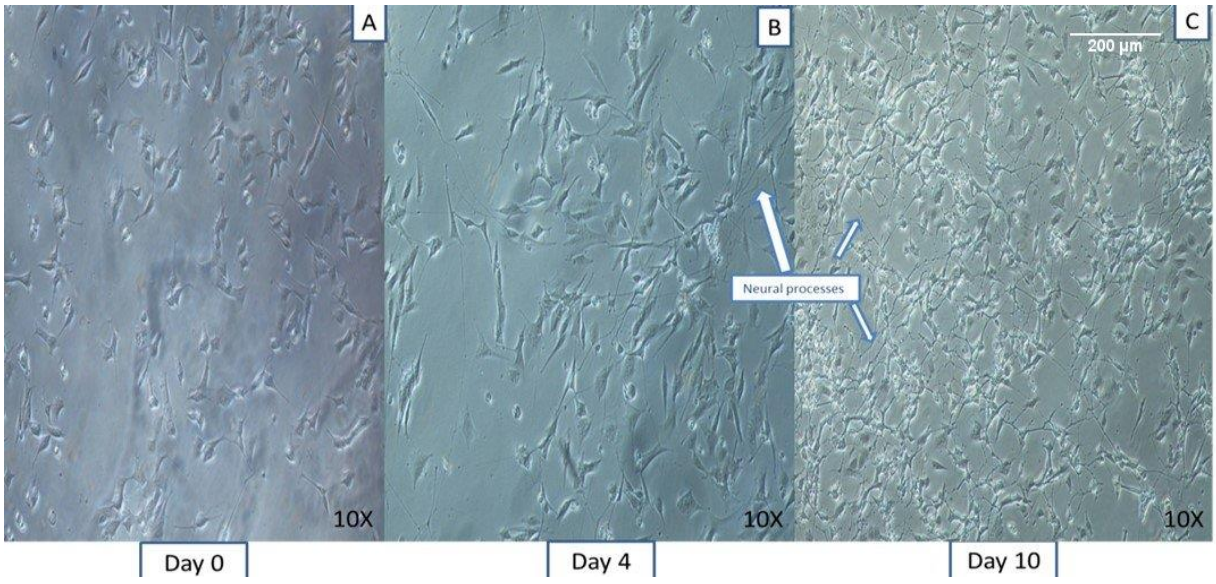



**Figure 4-7** Morphological appearance of differentiated SH-SY5Y cells. The plate was seeded with 1 x$10^5$ of cells then treatment by 10$\mu$M RA for 0, 4 and 5 days. (A) Day 0 of differentiation. (B) Day 4 of differentiation. (C) Day 10 of differentiation. Neurite extension was evident in day 10 cells (arrows). Images were obtained at 10X magnification, scale bar, 200 µm.

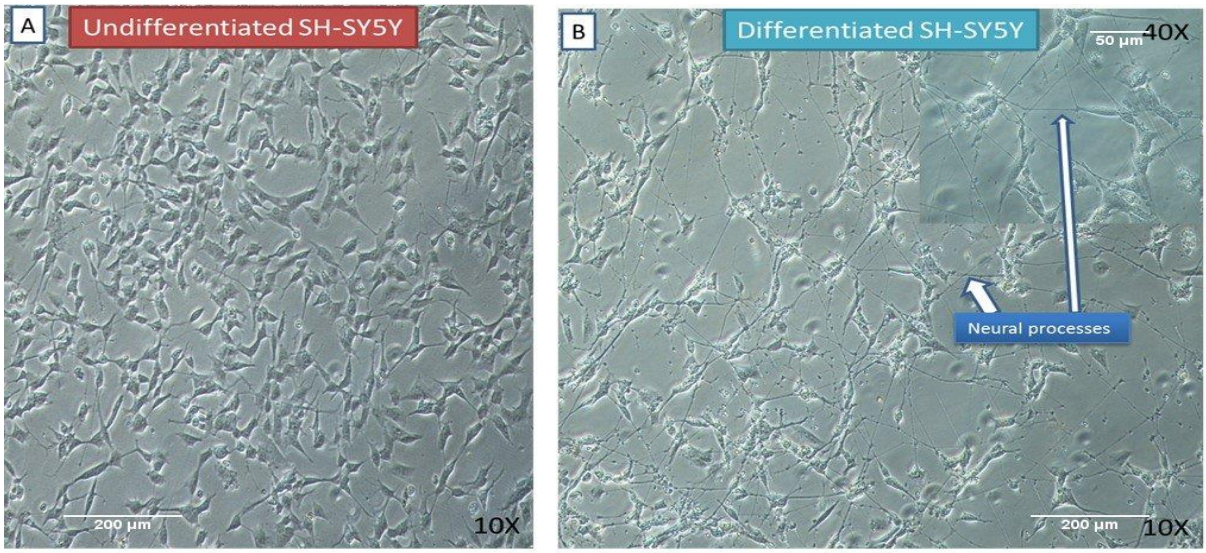



**Figure 4-8** Morphological parameter of human neuroblastoma SH SY5Ycells differentiated with 10$\mu$M of retinoic acid for 10days. The cells extended long neural processes (arrows). (A) The image was obtained at 10X magnification, scale bar, 200 µm. and (B) was obtained at 10X and 40X magnification, scale bar, 200 and 50 µm, respectively.

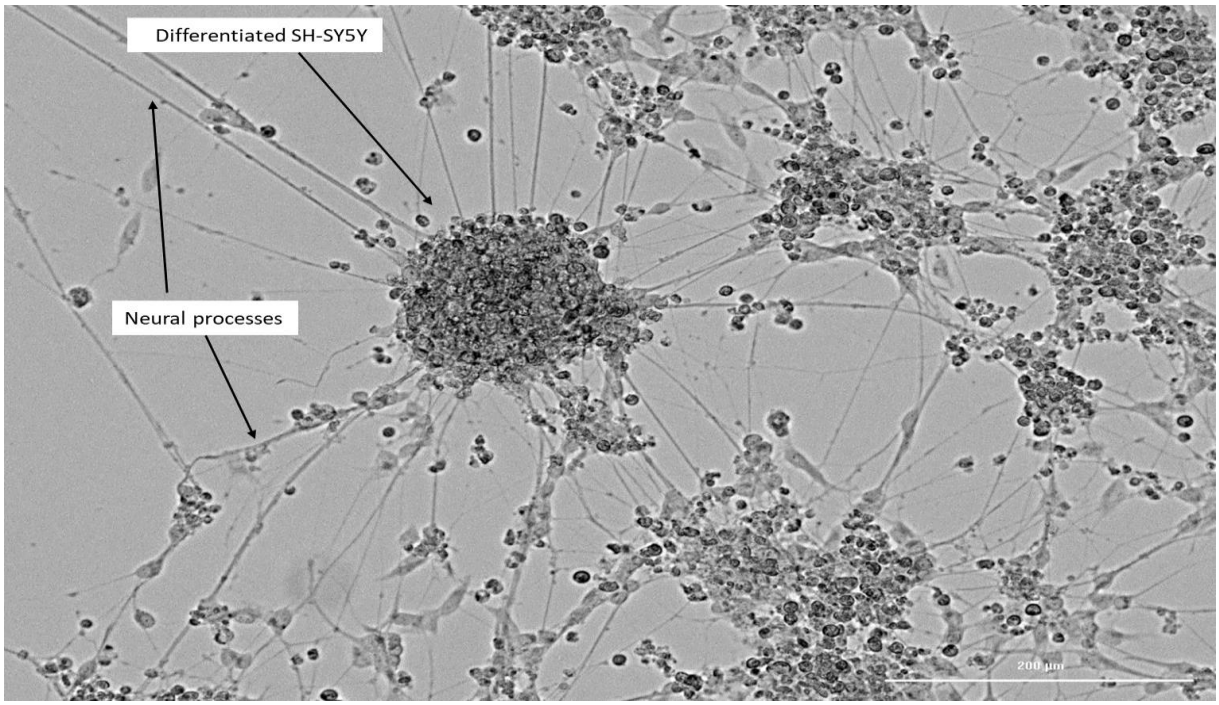



**Figure 4-9** Overview of a neuronal network of fully differentiated SHSY5Y. Morphological parameters of human neuroblastoma SH SY5Ycells differentiated with 1$\mu$M of retinoic acid for 21 days. The cells extend long neural processes and fully differentiated (arrows). Images were obtained by using a Carl Zeiss microscope. Scale bar 200um.

#### 4.3.4.3 Immunofluorescence staining of differentiated SH-SY5Ycells.

The aim of this study is to stain the differentiated SH-SY5Y cells by Immunofluorescence staining. It is known that SH-SY5Y cells are capable of differentiation, so it is important to confirm this differentiation by Immunofluorescence staining using specific antibodies to detect the expression of differentiation markers such as MAP2, NSE, and NF. The ideal results were picked after several wide-ranging optimization experiments described above. The chosen optimal results showed the expression of neuronal markers on the *in-vitro* grown differentiated SH-SY5Y cells. In order to confirm the neuronal marker alterations, the NF, MAP2, and NSE were immunocytochemically analyzed and a comparison made between the undifferentiated and RA-differentiated SH-SY5Y cells. Using the coated coverslip on 6 wells plate, culturing of the SH-SY5Y cells was done for two days. A differentiation medium with 1µm RA was then used to replace the culture medium for 21 days as previously described. Using the specific antibodies, staining of the cells was then done through Immunofluorescence staining to identify the expression of the differentiation markers. In the control, as shown in figure 4.10, the immunoreactivity of the MAP2, NSE, and NF was not identified in the cytoplasm of undifferentiated SH-SY5Y cells. Nevertheless, as shown in figure 4.10 and figure 4. 11, the differentiation marker was detected in the soma and neurites of differentiated SH-SY5Y cells.

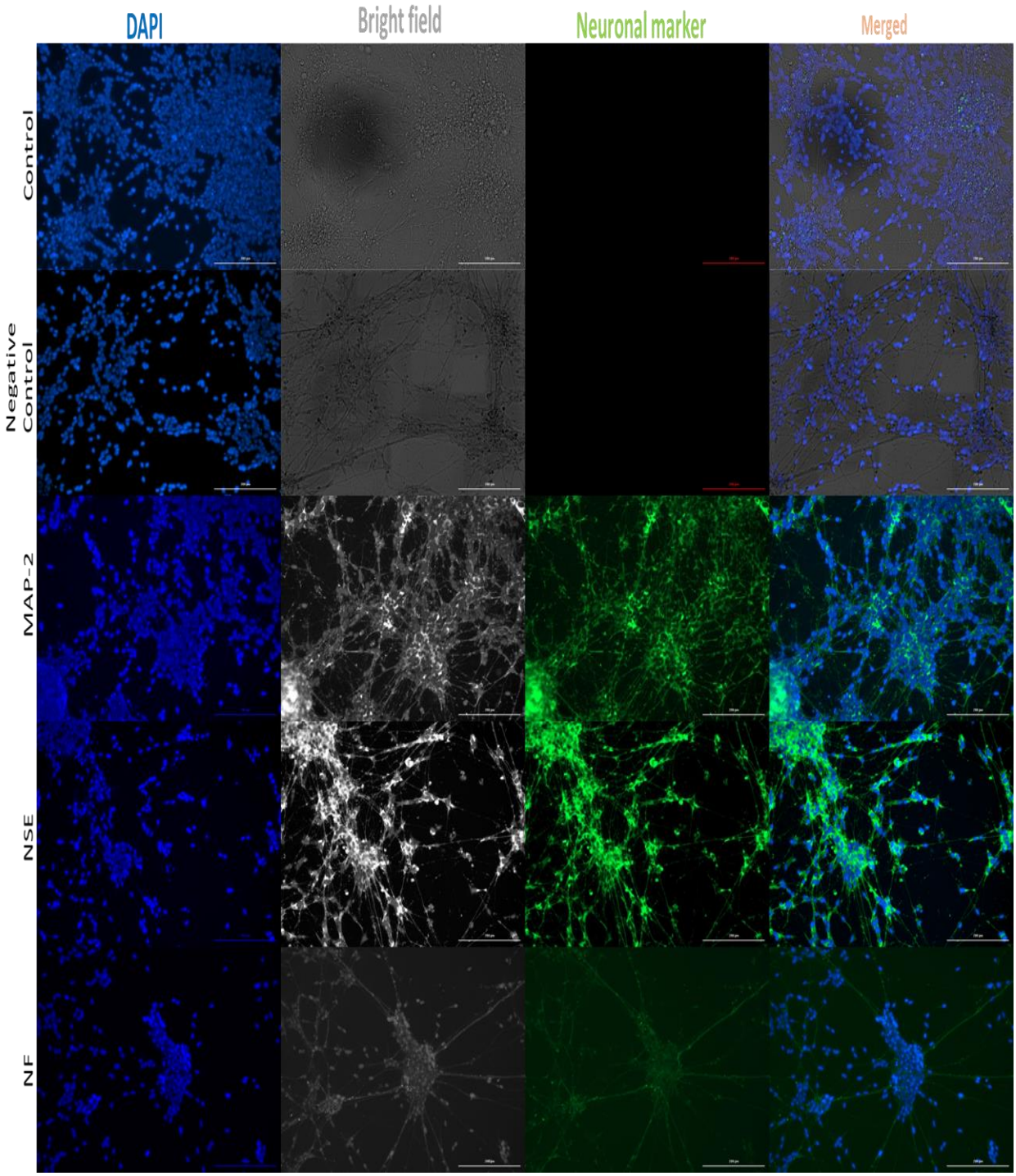



**Figure 4-10** Neuronal differentiation of SH-SY5Y cells with RA treatment (1um, 21 days). Immunofluorescence illuminates neuronal features of fully differentiation SH-SY5Y cells. Immunofluorescence images showing: (1) unlabeled cells (control) (top row). (2) Negative control (second row). (3) cells labeled with MAP-2, microtube associated protein 2 (third row). (4) NSE, Neuron-specific enolase (fourth row). (5) NF, neurofilament (fifth row). Nuclei are shown by DAPI staining (blue); Bright field; neuronal markers are shown by MAP-2, NF and NSE (green); and merged images presented co-localization of specific neuronal markers and DAPI. Images were obtained by Cytation 3 imaging reader. Scale bar, 100 µm.

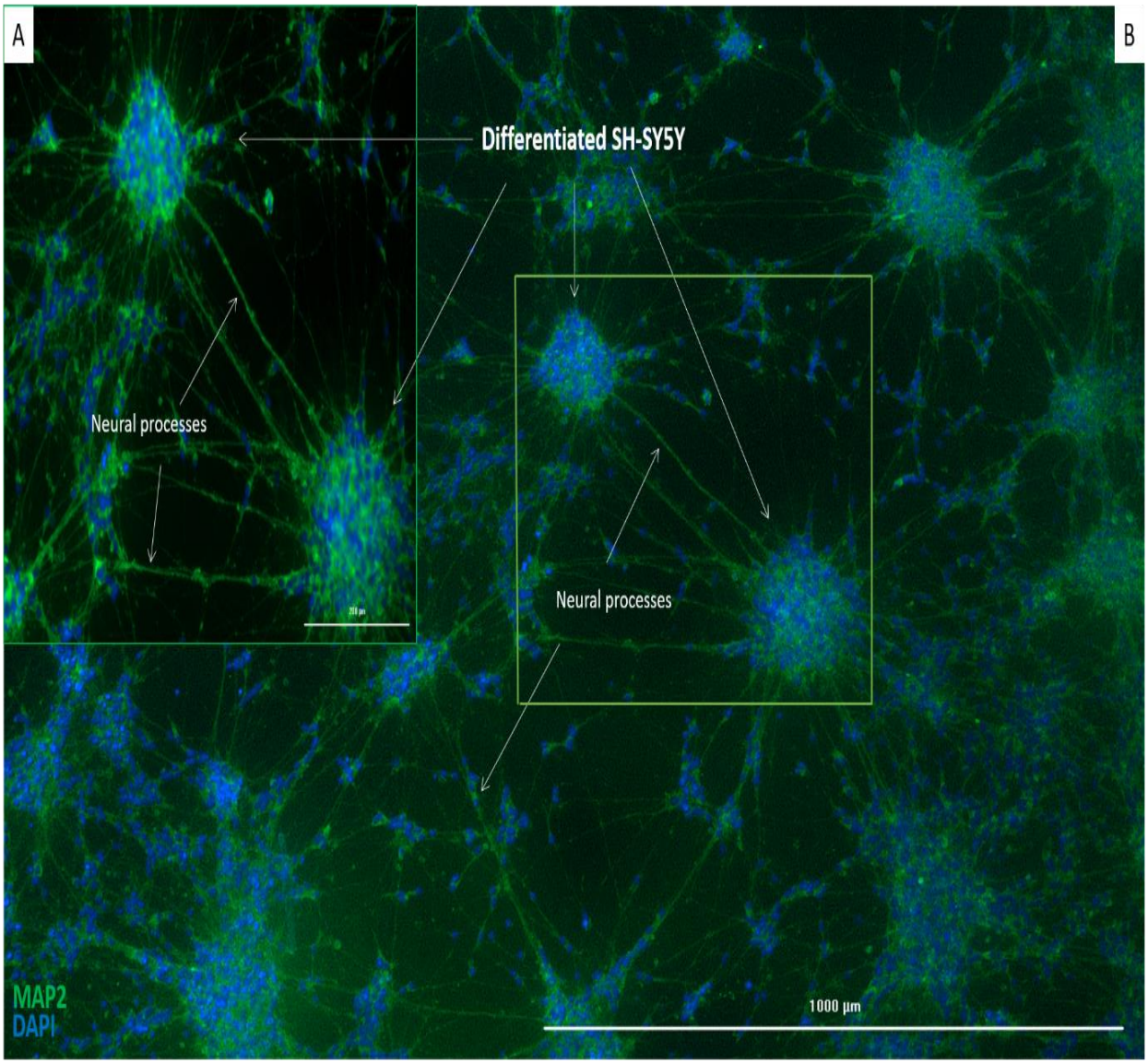



**Figure 4-11** Immunofluorescence staining of neuronal features of fully differentiated SH-SY5Y cells. Neuronal differentiation of SH-SY5Y cells with RA treatment (1µm, 21 days). The images showing SH-SY5Y cell morphology after differentiation, with neurite elongation and branching apparent. MAP2 (green) labels microtubule-associated protein 2, revealing the neuronal soma and proximal portion of neurites; Nuclei are shown by DAPI staining (blue). Images were obtained by Cytation 3 imaging reader. (A) Scale bar, 200µm. (B) Scale bar, 1000 µm.

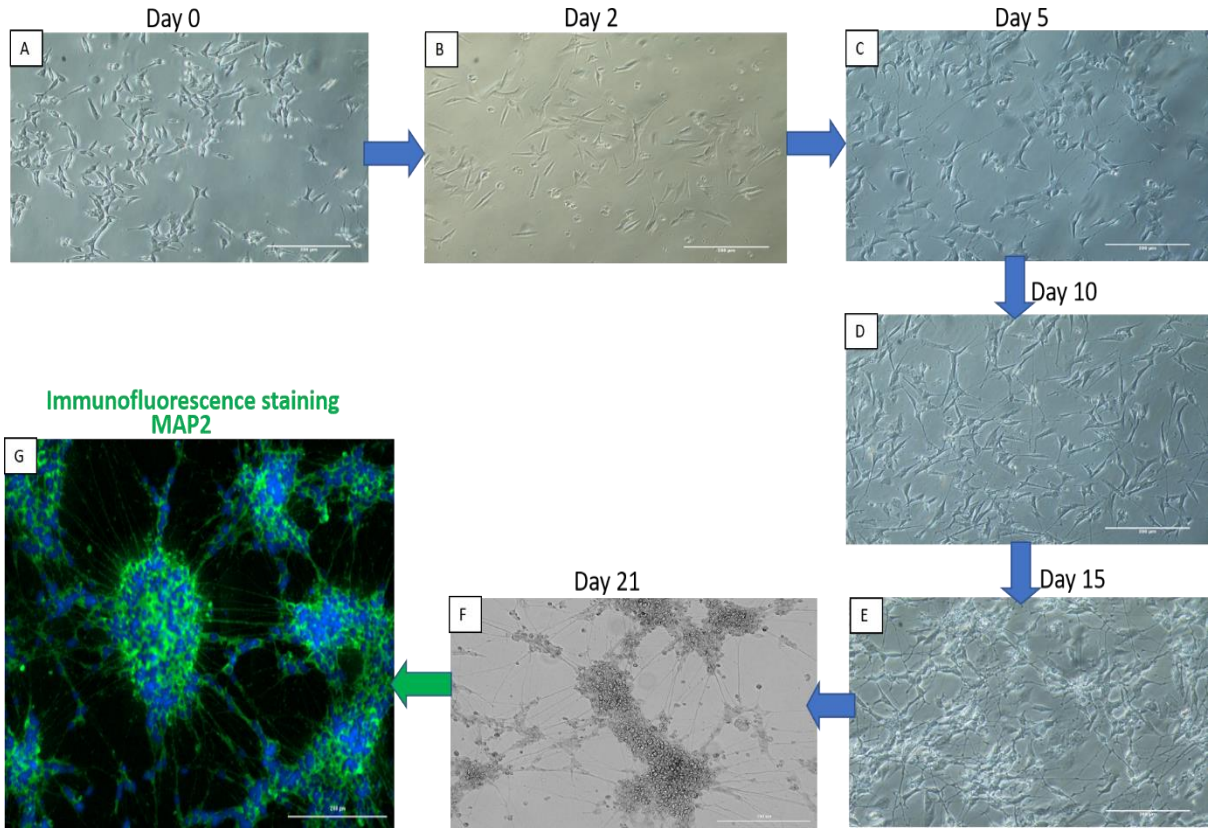



**Figure 4-12** SH-SY5Y cells undergo morphological changes during RA-induced differentiation. (A) Undifferentiated SH-SY5Y cells were cultured in the complete growth medium, day 0. (B) SHSY5Y cells were treated with 1 $\mu$M RA for 2 days. (C) SH-SY5Y cells cultured with 1 $\mu$M RA for 5 days. (D) First signs of initial differentiation in SHSY5Y, day 10. (E) Almost fully differentiated SHSY5Y, day 15. (F) fully differentiated SHSY5Y, day 21. (G) Immunofluorescence images obtained when fully differentiated SHSY5Y cells were stained with MAP2 (green) and DAPI (blue). Images were obtained by using a Cytation 3 imaging reader. Scale bar, 200 µm.

### 4.3.5 Development of a *T. gondii* cell culture system.

#### 4.3.5.1 Thawing and recovering *T. gondii* cells.

Cryopreservation of *T. gondii* was optimised using a freezing medium and included both intracellular and extracellular freezing of the *T. gondii* (2.2.3.8). The *T. gondii* cells grew optimally after thawing and culture on a new flask. From the results, a freezing medium that has a 60% growth medium, 30% FBS and 10% DMSO was the best for frozen intracellular and extracellular cells.

#### 4.3.5.2 Development of cell culture for *T. gondii* cells.

Through a variety of cell lines such as MDBK, it is possible to culture and maintain the *in vitro T. gondii*. In this case, culturing of the MDBK cells was done in T25 flasks for one day thereafter the *T. gondii* tachyzoites from the type I strains were used to infect it. Within 2-3 days after the infection, microscopy was used to examine the MDBK monolayer for lysis and the presence of parasites. The parasites, that were released after the infection of the MDBK cells, were used to infect other cells. The outcome showed that about 60% of host cells were infected after 3 days and 15-20 x $10^6$ tachyzoites were released after complete MDBK monolayer lysis and this process took seven days. To maintain the *T. gondii* tachyzoites, they were sequentially moved to fresh MDBK cells and other tachyzoites were stored at -80°C. Having established conditions for infection, the rate of growth and host infection between two strains of *Toxoplasma* in different cell types can be compared.

#### 4.3.5.3 Comparison of *T. gondii* tachyzoite type I and type II growth rate.

An experiment was conducted to compare the rate of growth between the tachyzoites of the *T. gondii* strains in differentiated and undifferentiated SH-SY5Y cells. Using the T25

flasks, $1x10^6$ cells per 1 mL of undifferentiated SH-SY5Y cells were cultured for 24 hours. Thereafter, they were infected with *T. gondii* tachyzoites strains of type I (RH**Δ**KU8) and type II (ME49) as discussed in (2.2.3.10.1). After four days, $7x10^6$ of the tachyzoites from type I in undifferentiated SH-SY5Y cell cultures were harvested as demonstrated in table 4.1. Nevertheless, from type II, only $3x10^6$ tachyzoites in undifferentiated SH-SY5Y cell cultures were harvested four days after infection (Table 4.1). The examination of the differentiated SH-SY5Y cells was also done. For a period of 21 days, the SH-SY5Y cells were differentiated using the T25 flask. *T. gondii* type I and II tachyzoites were then used to infect them as described above. However, due to technical issues in the lab, no results were obtained. The most important of these technical issues was the frequent breakdown of the incubator. To overcome this problem, the *T. gondii* cells were frozen until the malfunction was fixed. However, the time taken to fix the incubator combined with the arrival of the Covid-19 infection and closed down laboratories meant that this experiment was not able to be repeated.

**Table 4-1** Amount of viable tachyzoites obtained from 25-$cm^2$ T-flask supernatants after SH-SY5Y cell infection with the indicated number of *T. gondii RHΔKU8* or *ME49* strain. The results were expressed by tachyzoite counts per mL of medium obtained on days 3 and 4 (average of three 25-$cm^2$ T-flasks). The experiments were performed in duplicate as mean ± SD. *$p < 0.05$.

| *Toxoplasma gondii* strain tachyzoites added to 25 $cm^2$ T-flasks | Amount of viable *Toxoplasma gondii* strain tachyzoites released $10^6$ /mL (tachyzoite multiplication after initial inoculum) Days post-infection | |
|---|---|---|
| | **3** | **4** |
| RHΔKU80 | 2 | 7 |
| ME49 | 1.2 | 3 |

#### 4.3.5.4 Comparison of *T. gondii* tachyzoite type I and type II infection rate.

An investigation on the rate of infection of the two strains of *T. gondii* was carried out. In this case, $5x10^4$ cells per 1 ml of undifferentiated SH-SY5Y cells were cultured on the 6-well plates with coverslips for 24 hours. Thereafter, they were infected with *T. gondii* tachyzoites strains type 1(RH***Δ***KU8) and type II (ME49) as discussed in (2.2.3.10.2). From the results, infection by the RH***Δ***KU80 was present in 66.6 % of the host cells after four days. Conversely, after four days, the ME49 strain only infected about 37.6 % of the host cells (Table 4. 2). As shown in figure 4.13 A, the differentiated SH-SY5Y cells were also analyzed. These cells were differentiated on the coated 6-well plates with coverslips for 21 days. Strains of *T. gondii* were then used to infect them. However, there was a challenge in determining the differentiated SH-SY5Y cells that were infected. Proper visualization of the tachyzoites *T. gondii* within the differentiated SH-SY5Y cells was also difficult. This is because differentiated SH-SY5Y cells are large and consist of layers that make it difficult to see what is inside the cells (figure 4.13 B).

**Table 4-2** The percentage of infected cells by 2 strains of *T. gondii* stains. The host Cells were cultured on coverslips into 6-well plates for 24h and infected with *T. gondii* (host cell: tachyzoites, 1:5) for 3 and 4 days. Then the cells were washed, fixed, stained with Giemsa stain and analysed under a light microscope**.**

| *Toxoplasma gondii* strain added to each well of 6 wells plate | Percentage of the infected cell (Days post-infection) | |
|---|---|---|
| | 3 | 4 |
| RH***Δ***KU80 | 29% | 66.6% |
| ME49 | 7.3% | 37.6% |

This is the mean of 3 replicates

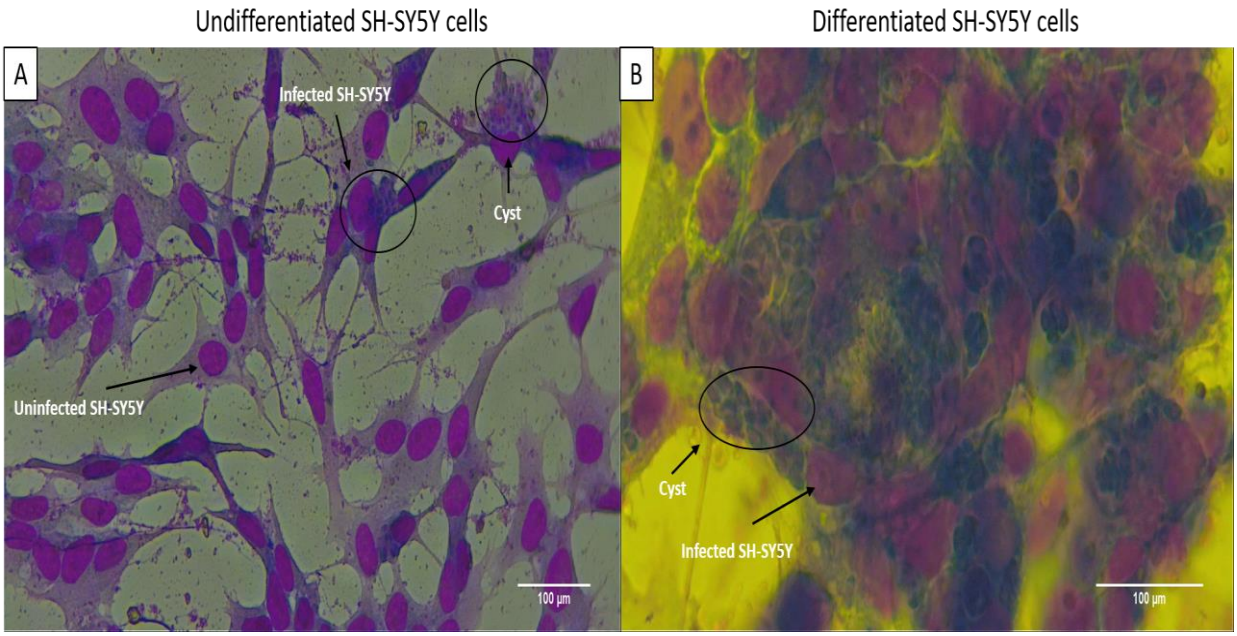



**Figure 4-13** Photomicrographs showing undifferentiated and differentiated SH-SY5Y cells in culture and its interaction with *T. gondii* type I: (A) Undifferentiated SH-SY5Y cells after 24h of culture and after 24 h of infection with *T. gondii* (arrowhead), stained with Giemsa. (B) differentiation of SH-SY5Y cells with RA treatment (1µM, 21 days), then differentiation of SH-SY5Y cells were infected with *T. gondii* for 48h (arrowhead), stained with Giemsa. Numerous intracellular and extracellular *T. gondii* tachyzoites and cysts (arrows). Images were taken by light microscopy. Scale bar, 20 µm.

## 4.4 Discussion.

### 4.4.1 HFF, MDBK and differentiated and undifferentiated SH-SY5Y cell maintenance cultures.

The tissue culture method has been used in the latest studies by biology researchers due to its cost-effectiveness, stability for unlimited studies and easily accessible cells. The HFF cell line was utilized to optimize and develop the tissue culture prior to the actual experiments. The sub-culturing of the HFF cell line, passaging, cryopreservation, and recovery phases were described in the results section. This technique was therefore ratified for use in the experiments with the HFF cells as the control. MDBK cells were also used in this study and this cell line can be used to culture and maintain the *T. gondii*. The MDBK cell line subculturing, passaging, cryopreservation and recovery phases were successful. The third cell line was used SH-SY5Y cell line. SH-SY5Y cells have the capacity to differentiate into neuron cells and their subculturing and passaging. Firstly, Undifferentiated SH-SY5Y cells were well maintained as shown in the results by using appropriate growth medium (DMEM-high glucose). Secondly, undifferentiated SH-SY5Y cells were converted to differentiated SH-SY5Y cells. As shown in (4.3.4.1) several differentiation experiments were conducted but failed due to the toxicity of RA. As previously described, RA is highly sensitive to heat, air, and light and its degradation products are toxic (Shipley, Mangold, and Szpara 2017). The cells maintained in differentiation media, therefore, should be kept in the dark not be exposed to light.

### 4.4.2 Differentiation of SH-SY5Y.

The SH-SY5Y neuroblastoma cells are known to modify their sensitivity to neurotoxins by changing the survival signaling pathways (Cheung et al. 2009). There has been wide use of differentiated and undifferentiated SH-SY5Y cells in neuroscience research; therefore, the

utilization of this cell line in AD studies has a significant implication. The differentiation features caused by RA treatment are well known (Das et al. 2012; Lopes et al. 2010; Schneider et al. 2012; Shipley, Mangold, and Szpara 2017). This study has shown that the *in-vitro* differentiation of the neuroblastoma took not less than seven days by adding 10 µM of RA or 21 days by adding 1 µM of RA and this is confirmed by the results of other studies (Das et al. 2012; Higashi et al. 2015; Shipley, Mangold, Kuny, et al. 2017). The differentiation property of RA in SH-SY5Y cells with a concentration of 1 µM and 10 µM was re-established in this case and was done by analyzing the alterations in neuronal and differentiation markers as well as the morphology. Morphologically, extensive projections of neurites were observed as a characteristic SH-SY5Y cells neuronal phenotype, which is comparable to other reports (Vesanen et al. 1994; Xun et al. 2012). Apart from the morphological changes that supported the attained differentiation, the immunofluorescent technique was also used to confirm it because the technique can identify the differentiation biomarkers (Higashi et al. 2015). In this case, it was established that after treating the SH-SY5Y cells for 21 days using RA, they were successfully differentiated. There was also enhanced expression of the NES, NF and MAP2 biomarkers which are specific neuronal markers. Our data are comparable to other studies, which demonstrated that in differentiation, there is an expression of neuronal markers like NES, NF, and MAP2 (Cheung et al. 2009; Clelland et al. 2009; Schneider et al. 2012; Teppola et al. 2016).

### 4.4.3 *T. gondii* culture.

Through the process of cell culture, the tachyzoites of *T. gondii* can be produced in the lab, which can offer a source of fresh viable tachyzoites (Evans et al. 1999). Continuous cell lines are also inexpensive, reliable and can be easily maintained. Various continuous cell lines including HFF, Vero, Hela, and MDBK have been used to a culture *T. gondii*. These continuous

cell lines have a wide difference in the quality or quantity of the tachyzoites produced (da Costa-Silva et al. 2012; Diab and El-Bahy 2008). in this study, *T. gondii* were cultured and maintained in MDBK cell line, as shown in result (4.3.5.2) tachyzoites were ready to harvest after 3 days of infection. Then *T. gondii* tachyzoites were transferred to fresh MDBK cells and other tachyzoites were stored in liquid nitrogen for long-term storage. In this study, the comparison of *T. gondii* strains was investigated. As mentioned in the literature review, there are three lineages in the *T. gondii* population structure. These include type 1, II and III. The growth rate is often known to be associated with virulence characteristics in protozoan pathogens (Boothroyd and Grigg 2002; Rorman et al. 2006). During the literature review, no information regarding the difference in the growth rate of the *Toxoplasma* strains into undifferentiated and differentiated SH-SY5Y cells was found. The aim of this study was to determine the infection and growth rate in order to identify the differences between type I and II strains of *T. gondii*. In determining the *T. gondii* strains growth rate in undifferentiated SH-SY5Y cells, the results showed that in type I 2 $x10^6$ tachyzoites per ml were harvested while in type II, 1.2 $x10^6$ tachyzoites per ml were harvested. Therefore, it was established that type I has a faster growth rate as compared to type II. The investigation was also conducted in differentiated cells; nevertheless, no results were obtained, as the experiments could not be completed. Again, the results of the *T. gondii* strains infection rate in undifferentiated SH-SY5Y revealed that after 3 days the type I strain (RH**Δ**KU8) infected 29% of host cells while in type II strain (ME49) infected 7.3% of the host cells. From the results, it was shown that type I has a faster rate of infection in the undifferentiated SH-SY5Y as compared to type II. The experiment on the rate of infection was also conducted on differentiated cells; however, no results were shown because the cells could not be identified. This resulted from the formation of layers that made the visualization of the host cell contents difficult (3d structure obscuring view). Generally, the results of our experiment showed that the rate of growth of

type I *T. gondii* strain in undifferentiated SH-SY5Y cells is faster as compared to type II. In harmony with these results, earlier studies have shown that type I *T. gondii* strain multiply three times faster than the type II strain (Asis and Grigg 2016; Boothroyd and Grigg 2002; Switaj et al. 2005).

Future studies on the current topic are therefore recommended that the experiments to determine the growth rate of *T. gondii* strains in differentiated SH-SY5Y cells need to be carried out in line with the previously mentioned experiments. On the other hand, the measurement of infection rate would be enhanced by the use of special staining like immunofluorescence staining, which is able to detect the presence of *T. gondii* in host cells. The sensitivity of immunofluorescence staining in detecting the *T. gondii* tachyzoites and bradyzoites in host cells and the use of special biomarkers such as surface antigen (SAG) and bradyzoite specific antigen (BAG) that are associated with *T. gondii* has been shown in some previous studies (Buchholz et al. 2011; Chang et al. 2015; Ferguson et al. 2013; Rozenfeld et al. 2005; Weiss and Kim 2000; Zhang et al. 2001).

# 5 Chapter 5: Investigation of the effects of oxidants on neuronal cell viability and morphology.

## 5.1 Introduction.

Alzheimer's disease is regarded as a kind of dementia that is characterized by the build-up of the amyloid-β (Aβ) peptides, extracellular plaques, and the tau protein in the intracellular neurofibrillary tangles. These are usually in the brain parts that include the amygdala, cortex, and hippocampus (Agholme et al. 2014; Lee et al. 2010; Md et al. 2018). Many factors are known to contribute to Alzheimer's disease, and they include environmental and genetic factors. Stress, particularly those that are oxidatively related, are known to cause mitochondrial and nuclear damage, inducing apoptosis of the cells. this results in progressing neurodegeneration, explaining the pathogenesis of Alzheimer's disease (Dincel and Atmaca 2016). Oxidation results from the excessive production of reactive oxygen species (ROS), causing lysing of cells, oxidative burst, or excess free transition metals. ROS cause damage to proteins, lipid membranes as well as the deoxyribonucleic acid (DNA) leading to apoptosis (Martindale and Holbrook 2002; Valko et al. 2007).

Hydrogen peroxide ($H_2O_2$) is one of the main of ROS produced during healthy metabolism. When the equilibrium between the antioxidation process and oxidation is disrupted, hydrogen peroxide is produced in excess, and this induces oxidative stress. $H_2O_2$ reacts directly with cellular macro-molecules damaging the mitochondria and causing apoptosis of the cells. Due to the ability of hydrogen peroxide to induce oxidation resulting in programmed cell death, $H_2O_2$ has been widely used in experimental cases to produce antioxidant drugs that can aid in preventing neurodegenerative disorders (Zhang et al. 2007). $H_2O_2$ is commonly used to induce apoptosis since it is known to cause cytotoxicity in almost all cells, including SH-SY5Y (Akki et al. 2018; Xiang et al. 2016). Although the toxicity is

obtained at a variety of doses, the variance in dosing has not been apparent. The impacts of oxidative stress on cell growth inhibition and cell death will be examined in this chapter using $H_2O_2$ treatment on both undifferentiated and differentiated SH-SY5Y cells. In this scenario, both the differentiated and undifferentiated SH-SY5Y cells will be subjected to a range of $H_2O_2$ concentrations, and monitoring of the cell death process will be determined by MTT as well as checking the morphology changes. This will bring a further understanding of the behavior of differentiated and undifferentiated SH-SY5Y cells during oxidative stress.

## 5.2 Objectives.

The aim of this chapter is an investigate the effects of oxidant on neuronal cell viability and morphology. There are two main objectives:

- Investigate the effects of oxidative stress on undifferentiated SH-SY5Y cells.
- Investigate the effects of oxidative stress on differentiated SH-SY5Y cells.

## 5.3 Methods.

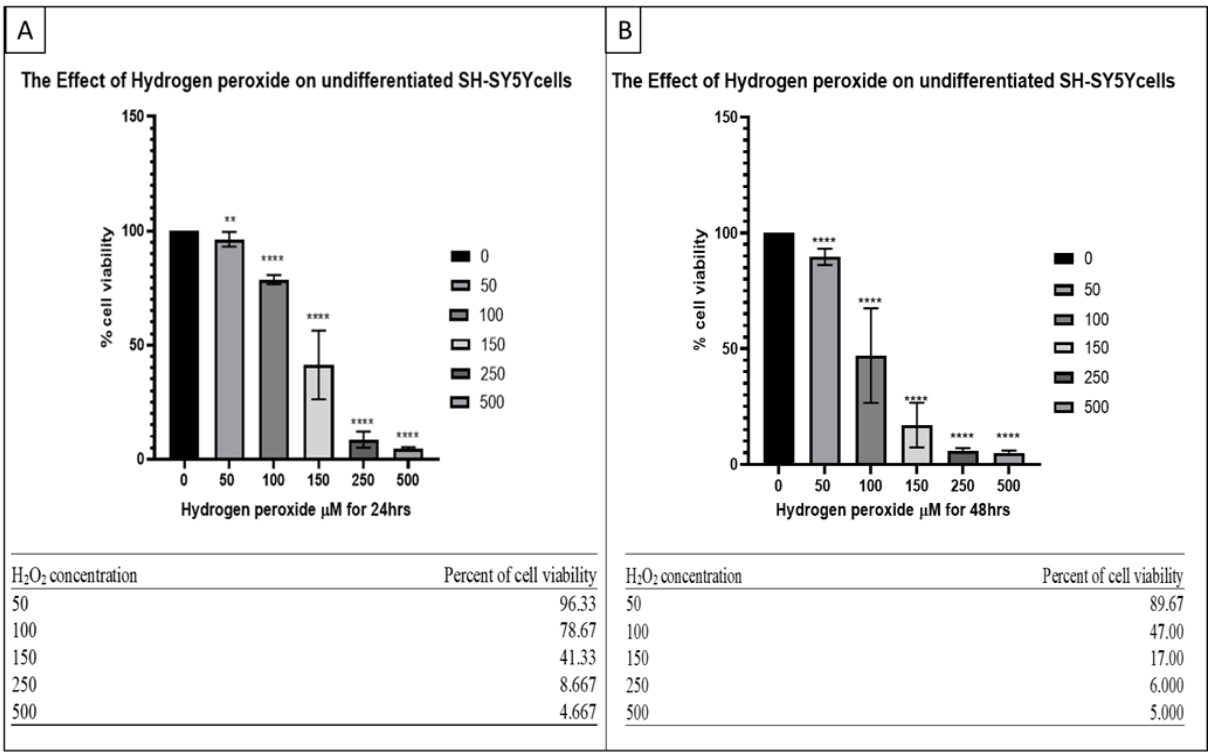
The detailed methods used in this chapter are described in chapter 2 Materials and Methods. Briefly the SHSY5Y cell line was used to study the effect of oxidative stress on the neural cells during *T. gondii* infection. To study the effect of oxidative stress on undifferentiated and differentiated SH-SY5Y cells, different concentrations of H2O2 (50µM, 100µM, 150 µM, 250µM, 500µM) were used to treat differentiated and undifferentiated SH-SY5Y cells in DMEM. Then cell death was determined by MTT assay, DAPI staining and morphology change.

## 5.4 Results.

### 5.4.1 Investigation of the effect of oxidative stress on undifferentiated SH-SY5Y cells.

#### 5.4.1.1 Effects of hydrogen peroxide on MTT reductive activity.

The main aim of this objective was to examine the effects of different concentrations of hydrogen peroxide ($H_2O_2$) on undifferentiated SH-SY5Y cells. Hydrogen peroxide is known for its oxidising activity and its ability to trigger cell oxidative stress. The effect of hydrogen peroxide-triggered cell death in undifferentiated SH-SY5Y cells was investigated, using the MTT assay, by exposing the cells to different concentrations of $H_2O_2$ (50µM, 100µM, 150µM, 250µM, 500µM) for either 24 or 48 hours. As demonstrated in figure 5. 1 A, B, treatment using $H_2O_2$ at concentrations of 50µM for 24 hours showed no effect on the viability of the cells when compared to the control samples. However, as shown in figure 5. 1 A and B, treatment using 50µM $H_2O_2$ for 48 hours and 100µM, 150µM, 250µM or 500µM $H_2O_2$ for either 24 or 48 hours caused a significant decline in cell viability compared to the cells which were not treated ($P < 0.00001$, Figure 5. 1). This shows a concentration- and time-dependent cytotoxic effect. Based on these findings, treatment with $H_2O_2$ at low concentrations of 50µM for 24 hours as shown in Figure 5. 1 A and, B), did not elicit any cell death while exposure to 50µM for 48 hours and 100µM, 150Mm, 250µM or 500µM $H_2O_2$ for either 24 or 48 hours induced cell damage. In order to explore this further, the viability of the cells was visualised using a nuclear stain.




| $H_2O_2$ concentration | Percent of cell viability |
|---|---|
| 50 | 96.33 |
| 100 | 78.67 |
| 150 | 41.33 |
| 250 | 8.667 |
| 500 | 4.667 |

| $H_2O_2$ concentration | Percent of cell viability |
|---|---|
| 50 | 89.67 |
| 100 | 47.00 |
| 150 | 17.00 |
| 250 | 6.000 |
| 500 | 5.000 |

**Figure 5-1** The Effect of Hydrogen peroxide on undifferentiated SH-SY5Y cell viability using an MTT assay. SH-SY5Y cells were cultured in 24-well plates ($1\times10^5$ cells/1000 µL/mL)) and incubated for 24 hours at 37°C, 5% $CO_2$. The cells treated with different concentrations of $H_2O_2$ (50,100,150,250 and 500$\mu$M) for 24 (A) or 48 (B) hours at 37°C, 5% $CO_2$. Control cells were kept with a medium for 24 (A) or 48 (B) hours at 37°C, 5% $CO_2$. Viable cell numbers were analyzed using thiazolyl blue tetrazole (MTT). Data are expressed as the percentage of viable cells in relation to the control (mean ± S.D.) and are representative of three independent experiments in triplicate. *P< 0.05, **P< 0.005, ***P< 0.0005 and ***P< 0.00005.

#### 5.4.1.2 Effects of hydrogen peroxide on nuclear condensation and cellular viability by using DAPI staining.

Differential staining can be used to distinguish between viable cells and dead cells. DAPI is a precise nuclear stain; all live cells take up the dye while dead cells do not. As described in earlier results, the hydrogen peroxide triggered cell death in undifferentiated SH-SY5Y cells as shown by an MTT assay. In this study, investigations were carried out on the effect of $H_2O_2$ induced cell death in undifferentiated SH-SY5Y cells and the cell viability was determined using DAPI. Treatment of cells was done using different concentrations of $H_2O_2$ (50µM, 100µM, 150µM, 250µM, 500µM) for 24hours. Cells were stained with DAPI, after which, the estimation of the nuclear uptake of the stain was determined as the percentage of living cells having stained nuclei (Figure 5. 2).

The results show that the addition of an increasing amount of $H_2O_2$ resulted in variations in SH-SY5Y density: in the field of view, the number of live cells was lost due to $H_2O_2$ treatment. The strong effect of $H_2O_2$ concentration increased from 250 and 500 µM of $H_2O_2$ (Figure 5. 2 E, F) which showed the existence of only a few living cells. Having established that $H_2O_2$ affects cell viability, it raises the question as to whether it has an impact on the cell morphology.

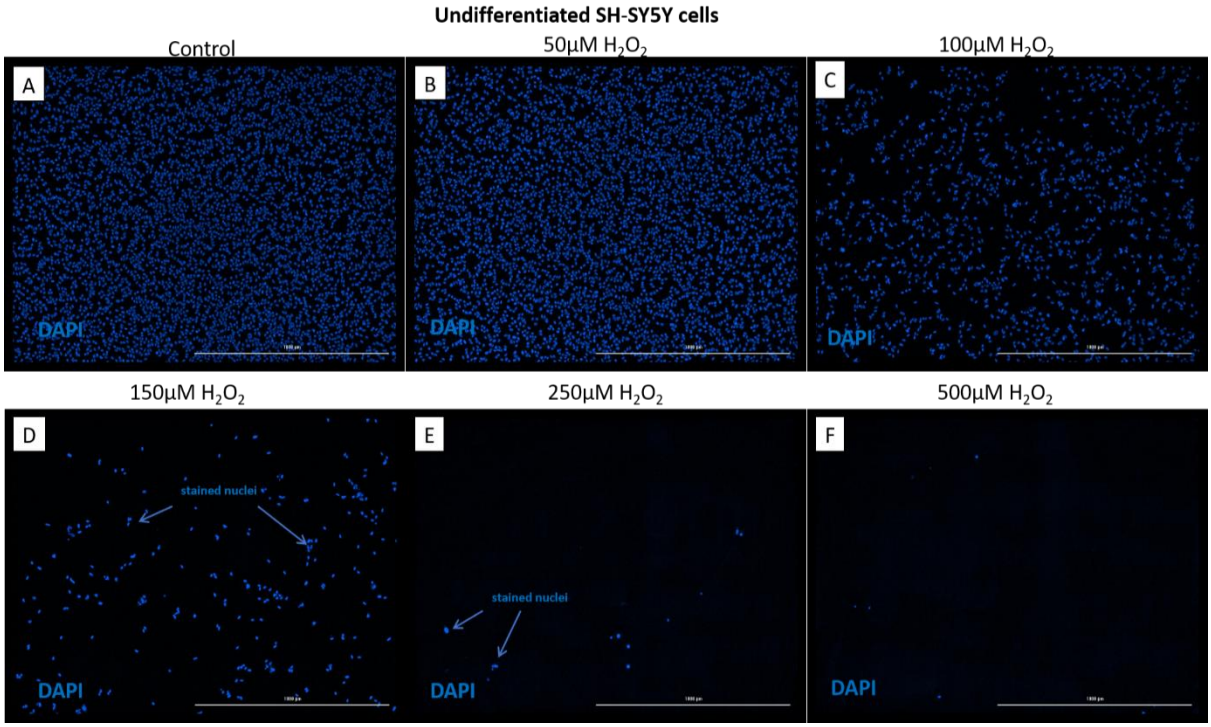



**Figure 5-2** Photographs of DAPI staining of undifferentiated SH-SY5Ycells after treatment with different concentrations of $H_2O_2$for 24h. Undifferentiated SH-SY5Y cells were cultured in 6-well plates ($5\times10^4$ cells/1000 µL/mL) and incubated for 24 hours at 37°C, 5% $CO_2$. The cells were treated with different concentrations of $H_2O_2$ (50, 100, 150, 250 and 500$\mu$M) for 24 hours at 37°C, 5% $CO_2$. Control cells were kept with a medium for 24 hours at 37°C, 5% $CO_2$. Vectashield medium containing DAPI was used for staining. Pictures were attained through the Cytation 3 imaging reader. Scale bar, 1000 µm.

#### 5.4.1.3 Effects of hydrogen peroxide on cell morphology.

As shown in prior results, as the $H_2O_2$ concentration increased, it caused a diminished possibility of survival in the undifferentiated SH-SY5Y cells. It was essential to confirm these results on the cell morphology of undifferentiated SH-SY5Y cells. Here, the morphology of cells undergoing treatment with $H_2O_2$ was investigated using microscopy, as was described above. Different concentrations of $H_2O_2$ (50µM, 100µM, 150µM, 250µM, 500µM) were used for the treatment of these cells (Figure 5. 3).

Images that are shown in figure 5. 3 confirmed that the addition of an increasing amount of $H_2O_2$ caused changes in SH-SY5Y morphology. Within the field of view, there was a loss of the adherent neuroblasts among the densely covered areas due to $H_2O_2$ treatment. These cells were noted to be rounding up and clumping, which appeared to be condensed. Overall, it was clear that the presence of $H_2O_2$ in ascending concentration caused the death of undifferentiated SH-SY5Y cells, especially in high concentrations of $H_2O_2$. These overall conclusions were consistent when examined by MTT, nuclear staining and cell morphology. This raises the question as to whether the same observations apply to $H_2O_2$ treated differentiated SH-SY5Y cells.

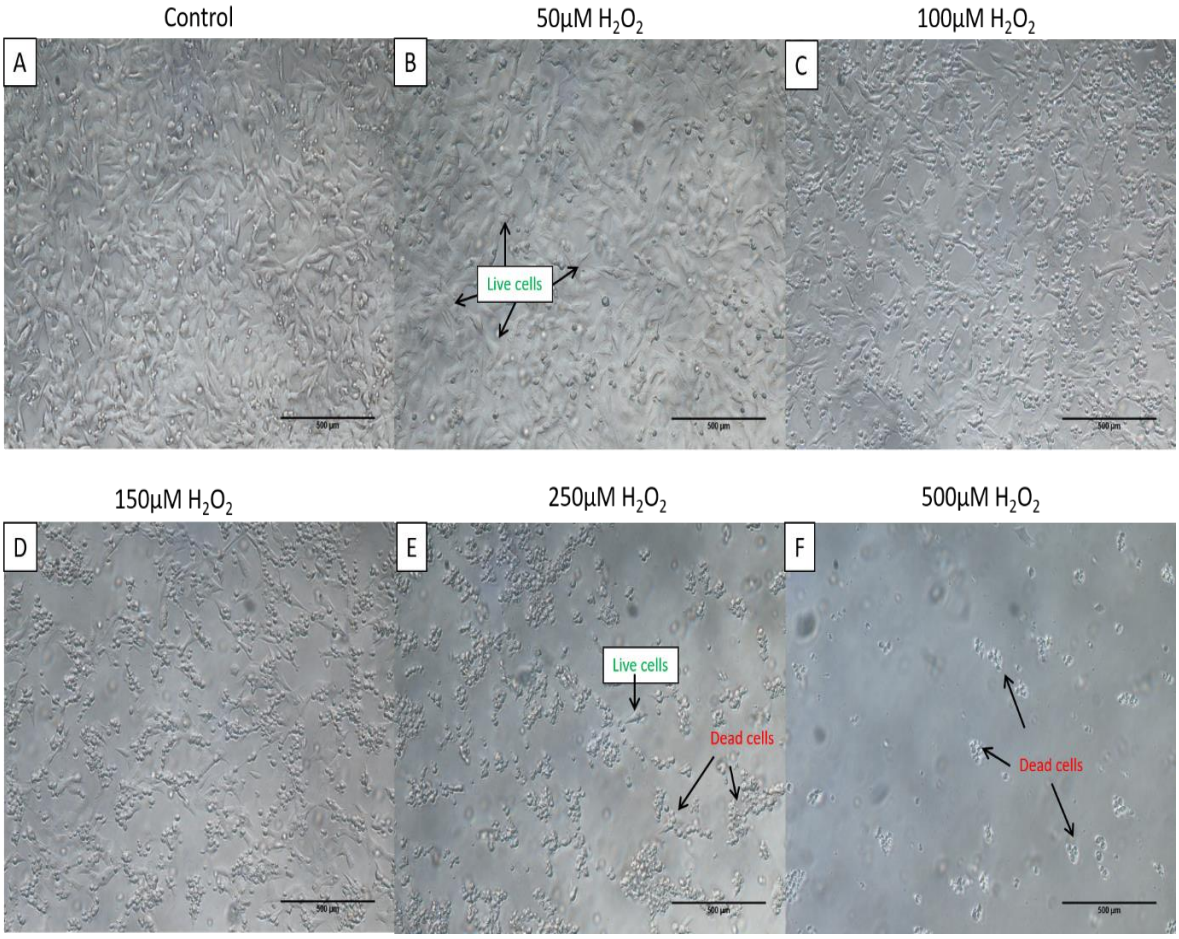


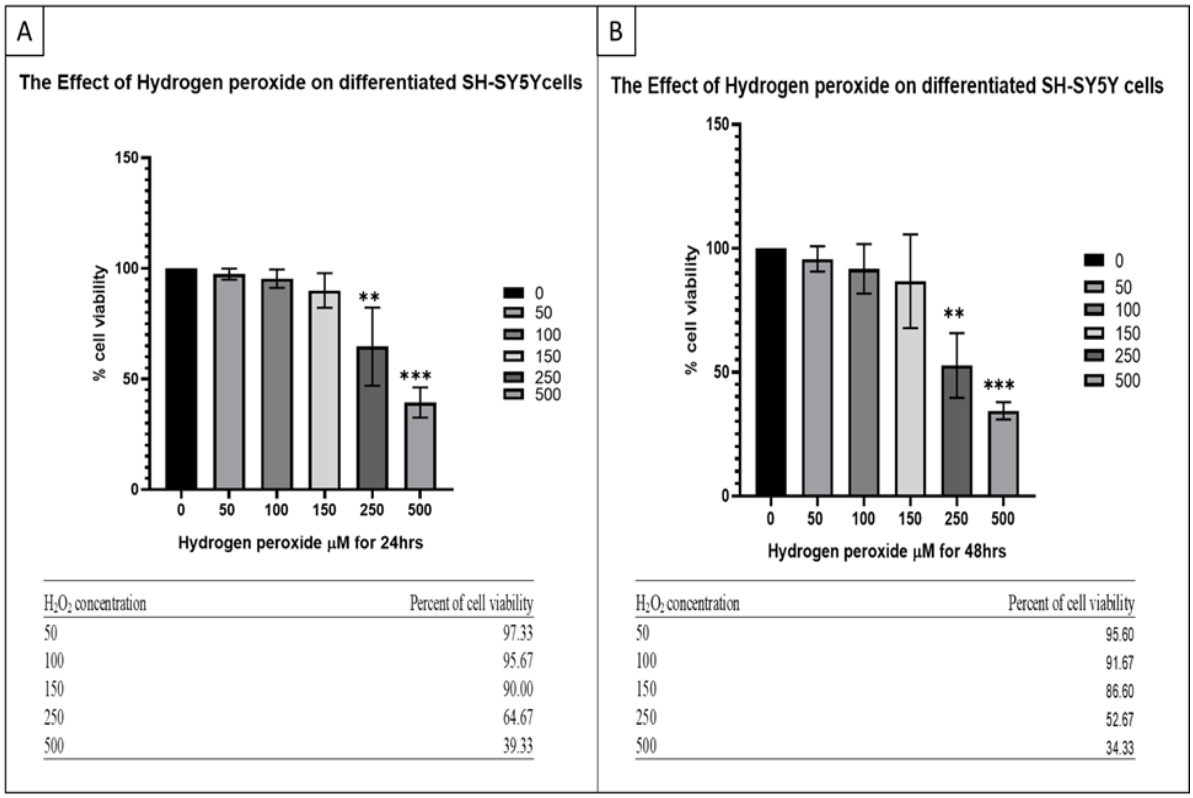

**Figure 5-3** Morphological changes in undifferentiated SH-SY5Ycells after treatment with different concentrations of $H_2O_2$ for 24h. Undifferentiated SH-SY5Y cells were cultured in 6 well plates ($5\times10^4$ cells/1000 µL/mL) and incubated for 24 hours at 37°C, 5% $CO_2$. The cells were treated with different concentrations of $H_2O_2$ (50,100,150,250 and 500$\mu$M) for 24 hours at 37°C, 5% $CO_2$. Cells were kept with medium (control) for 24 hours at 37°C, 5% $CO_2$. Images were taken by light microscopy. Scale bar, 500 µm.

## 5.4.2 Investigation of the effect of oxidative stress on differentiated SH-SY5Ycells.

### 5.4.2.1 Effects of hydrogen peroxide on MTT reductive activity.

The main aim was to examine the effect of different concentrations of hydrogen peroxide ($H_2O_2$) on differentiated SH-SY5Y cells. The earlier results demonstrated the negative growth impacts of $H_2O_2$ on undifferentiated SH-SY5Y cells. This raises the question as to whether the same effects occur in differentiated SH-SY5Y cells. To determine this, an MTT assay was done through the treatment of the cells with a range of concentrations of $H_2O_2$ (50µM, 100µM, 150µM, 250µM, 500µM) for either 24 or 48 hours. The SH-SY5Y cells were first taken through the process of differentiation for 21 days. After differentiation, the differentiated SH-SY5Y cells were subjected to the different $H_2O_2$ concentrations (Figure 5. 4 A, B).

As portrayed in figure 5. 4 A, B, treatment of the differentiated cells with $H_2O_2$ at all concentrations (up to 150µM) for either 24 or 48 hours showed no significant effect on the cell viability as compared to controls. However, as shown in figure 5. 4 A, B, treatment with either 250µM or 500µM $H_2O_2$ for either 24 or 48 hours induced a significant decline in cell viability when compared to the cells, which were not treated ($P < 0.001$ and $P < 0.0001$; respectively, Figure 5. 4 A, B). This effect is dependent entirely on the concentration levels and not in the time taken in incubation to achieve toxicity. Interestingly, the differentiated cells seem to be able to tolerate a higher concentration of $H_2O_2$ than the undifferentiated cells. Having established the effects of $H_2O_2$ on cell viability, does this affect cell morphology?




| $H_2O_2$ concentration | Percent of cell viability |
|---|---|
| 50 | 97.33 |
| 100 | 95.67 |
| 150 | 90.00 |
| 250 | 64.67 |
| 500 | 39.33 |

| $H_2O_2$ concentration | Percent of cell viability |
|---|---|
| 50 | 95.60 |
| 100 | 91.67 |
| 150 | 86.60 |
| 250 | 52.67 |
| 500 | 34.33 |

**Figure 5-4** The Effect of Hydrogen peroxide on differentiated SH-SY5Y cell viability using an MTT assay. SH-SY5Y cells were cultured in 24-well plates ($2\times10^4$ cells/1000 µL/mL) and incubated for 48 hours at 37°C, 5% $CO_2$, then treatment by RA 1 µM for 21 days to differentiated the cells, after 21 days, the cells were treated with different concentrations of $H_2O_2$ ( 50,100,150,250 and 500$\mu$M) for 24 (A) or 48 (B) hours at 37°C, 5% $CO_2$. Control cells were kept with a medium for 24 (A) or 48 (B) hours at 37°C, 5% $CO_2$. Viable cell numbers were analyzed using thiazolyl blue tetrazole (MTT). Data are expressed as the percentage of viable cells in relation to the control (mean ± S.D.) and are representative of three independent experiments in triplicate. **P< 0.005 and ***P< 0.0005. There was some variability in the percentage of cell viability between experiments. For example, in Figure 5. 4 the cell viability at 150$\mu$M for 24 and 48 hours are 90 % 86.6 %, respectively, and in Figure 6.3 the cell viability at 150$\mu$M for 24 hours is 77.5 %.

#### 5.4.2.2 Effects of hydrogen peroxide on cell morphology

The previous results showed that the high concentration of $H_2O_2$ resulted in diminished cell viability in differentiated SH-SY5Y cells. Confirmation of this outcome by examining cell morphology, which demonstrates the effect of accumulating the $H_2O_2$ concentration on the phenotype of the differentiated SH-SY5Y cells, is essential. In this case, the morphology of differentiated SH-SY5Y cells undergoing treatment with $H_2O_2$ was examined under the microscope, as it was defined above. SH-SY5Y cells underwent differentiation for 21 days, after which the differentiated SH-SY5Y Cells were treated using diverse $H_2O_2$ concentrations (50µM, 100µM, 150µM, 250µM, 500µM) for 24 hours.

As portrayed in figure 5. 5 B, C and D treatment of the differentiated SH-SY5Y cells with $H_2O_2$ at all concentrations (up to 150µM) for 24 hours showed no significant reduction in densely covered areas of adherent differentiated SH-SY5Y cells when compared with untreated cells. However, as shown in figure 5. 5 E, F, treatment with either 250µM or 500µM $H_2O_2$ 24 hours resulted in a significant reduction in compactly covered areas of adherent cells when matched with untreated cells.

Generally, all the outcomes clearly showed that the increased concentration of $H_2O_2$ tends to induce cell oxidative stress, which resulted in the death of cells among the differentiated SH-SY5Y cells at higher $H_2O_2$ concentration. The above experiments allow a comparison between the effect of $H_2O_2$ on differentiated and undifferentiated SH-SY5Y cells, which clearly shows that the differentiated cells are more resistant to $H_2O_2$ toxicity. This raises the question as to how these cells behave in response to oxidative stress when infected with *T. gondii* type I and type II strains in differentiated and undifferentiated SH-SY5Y cells.

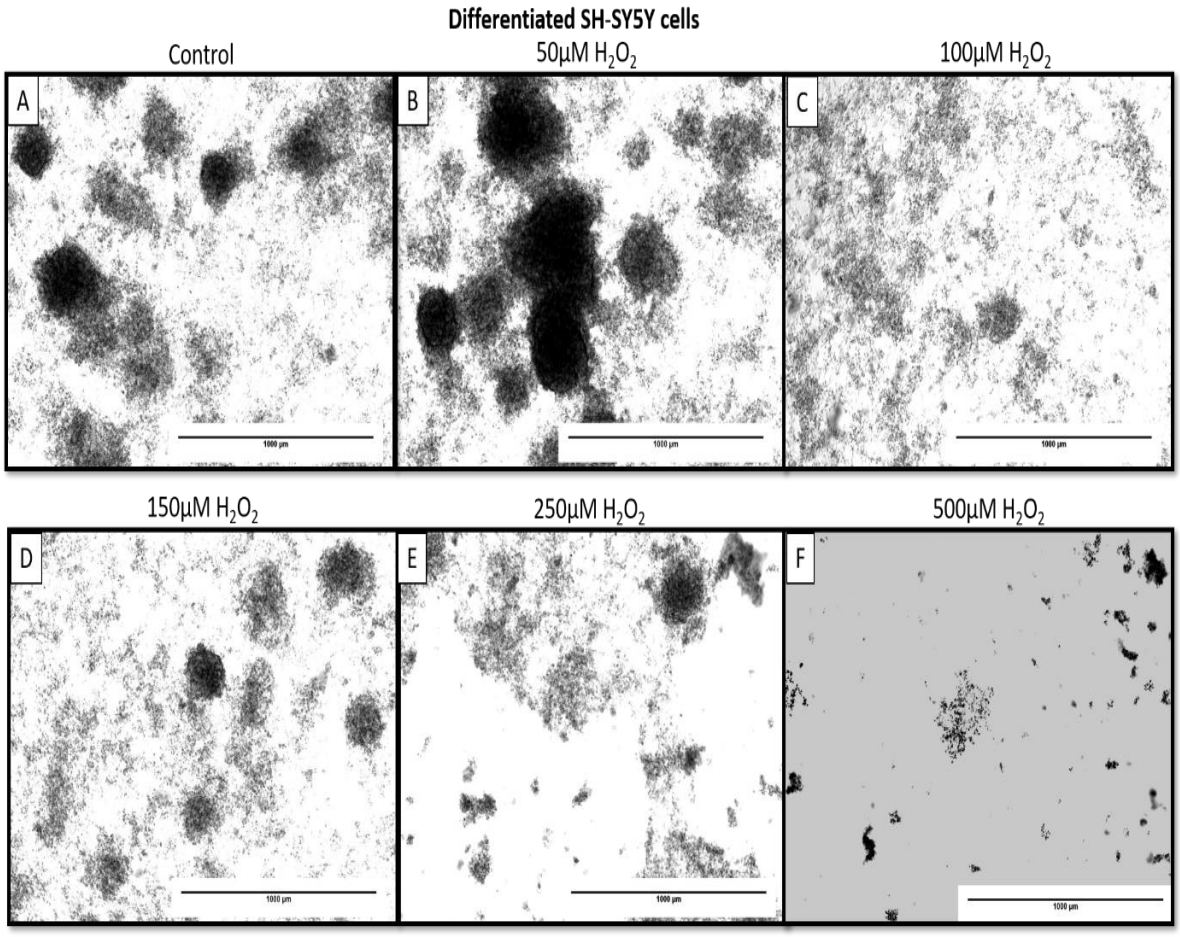



**Figure 5-5** Morphological changes in differentiated SH-SY5Ycells after treatment with different concentrations of $H_2O_2$ for 24h. Differentiated SH-SY5Y cells were cultured in 6-well plates ( $2\times10^4$ cells/1000 µL/mL)) and incubated for 48 hours at 37°C, 5% $CO_2$, then treated with retinoic acid (RA) 1 µM for 21 days to differentiate the cells. After 21 days the cells were treated with different concentrations of $H_2O_2$ (50,100,150,250 and 500$\mu$M) for 24 hours at 37°C, 5% $CO_2$. Control cells were kept with a medium for 24 hours at 37°C, 5% $CO_2$. Cytation 3 imaging was used to capture the pictures. Scale bar, 1000 µm.

## 5.5 Discussion.

As mentioned in the literature review (Chapter 1), oxidative stress has a significant role in the pathological progression of many neurodegenerative disorders such as Alzheimer's disease (AD). In this study, exogenous $H_2O_2$ was used as a source of generation of oxidative stress in the SH-SY5Y cells. The study gave focus on the influence that oxidative stress has on cell growth inhibition and cell death in the differentiated and undifferentiated SH-SY5Y cells, which have been treated with hydrogen peroxide. The outcomes indicated that there was compatibility between the results obtained with the MTT assays, DAPI staining, and examination of morphology.

These results showed that with increased $H_2O_2$ doses for either 24 hours or 48hours (150, 250-500µM), the living cells of undifferentiated SH-SY5Y cells as indicated by MTT assay and images that showed the cell viability and density. With the increasing $H_2O_2$ doses, the cell viability and density diminished. According to previously published work (Akki et al. 2018), cells that were treated with hydrogen peroxide experienced oxidative stress generating ROS that induced neural cell death. Again, the results showed that exposure of the differentiated SH-SY5Y cells to high $H_2O_2$ concentrations (250- 500µM) caused a decrease in the number of live cells, as indicated by MTT assay and images. This further demonstrated that cell viability and density decreased when under increased $H_2O_2$ doses. These findings showed that the differentiated cells were considerably more resistant to the effects of $H_2O_2$ (Figure 5. 4), as was illustrated in the results section. When differentiated cells were treated with $H_2O_2$, which are at a concentration between 250µM to 500µM for 24 hours, there was a decline in the cell viability to percentage levels of 64.67% and 39.33% respectively. When comparing these results with those of the undifferentiated cells, they showed a much higher reduction of cell viability (8.66% and 4.66%) respectively. These results indicate that the differentiated SH-SY5Y cells are more resilient to the oxidative stress triggered cell death as compared to the

undifferentiated SH-SY5Y cells (Figure 5. 1 and Figure 5. 4). These results are in agreement with the work published by (Schneider et al. 2012), who demonstrated that cell differentiation induced by retinoic acid changes the mitochondrial function in SHSY5Y cells. It has been shown that RA increases mitochondrial membrane potential, levels of cytochrome c oxidase and MnSOD and bioenergetic reserve capacity.

# 6 Chapter 6: Investigation of the effects of *Toxoplasma* infection on oxidative stress in a model brain cell culture system.

## 6.1 Introduction

*T. gondii* is an obligate intracellular pathogenic parasite that reproduces inside a eukaryotic cell and infects warm-blooded vertebrates including humans (Halonen and Weiss 2013). Toxoplasma's ability to survive in its hosts as dormant tissue cells (bradyzoites) that tend to transmit the infection to new hosts via predation is one feature that encourages the parasite's pervasiveness in the human species (Jeffers et al. 2018). The protozoan parasite has evolved the ability to balance parasite metabolism, multiplication, and cyst formation to enable rapid propagation and encystment across host tissues while avoiding destroying the host necessitates the ability to effectively counter host cell defenses (White, Radke, and Radke 2014). *T. gondii* infection, for example, is thought to inhibit parasite reproduction and spread due to oxidative stress. However, according to (Augusto et al. 2021), the mechanisms through which *T. gondii* decreases oxidative stress are still unclear. Also, these strategies can have an impact on the host cells as well. As a measure, it's vital to examine the influence of *T. gondii* cells on infected cells amid oxidative stress. This chapter will explore the effects of *T. gondii* on hydrogen peroxide-induced cell damage in SH-SY5Y neuroblastoma cells. *T. gondii* would be used to infect undifferentiated SH-SY5Y cells in this situation, and then the undifferentiated SH-SY5Y cells will be treated with $H_2O_2$. MTT assay and morphology will be used to determine their viability. Besides, the variations between *T. gondii* type I and type II strains will be determined to investigate whether they can protect SH-SY5Y cells from hydrogen peroxide toxicity.

## 6.2 Objectives

The aim of this chapter is an investigate the effects of *Toxoplasma* infection on oxidative stress in a model brain cell culture system. There are two main objectives:

- Investigate the effects of *T. gondii* type I and II infection on oxidative stress in undifferentiated SH-SY5Y cells.
- Investigate the effects of *T. gondii* type I and II infection on oxidative stress in differentiated SH-SY5Y cells.

## 6.3 Methods

The detailed methods used in this chapter are described in chapter 2 Materials and Methods. Briefly the SHSY5Y cell line was used to study the effect of *Toxoplasma* infection on the expression of molecules associated with oxidative stress and Alzheimer's in a model brain cell culture system. In this chapter, the effect of infection of *T. gondii* strain type I and II on $H_2O_2$ toxicity in undifferentiated and differentiated SH-SY5Y cells was examined. Here, the I and II strains of the *T. gondii* were used to infect the differentiated and undifferentiated SH-SY5Y cells. They were then incubated for 24 hours at 37ºC. Treatment was then done using 150µM of $H_2O_2$ for 24 hours at 37°C. After 24 hours, then the cell's death was determined by MTT assay and Giemsa stain.

## 6.4 Results.

### 6.4.1 Investigation into the effect of *T. gondii* type I and II infection on oxidative stress in undifferentiated SH-SY5Y cells.

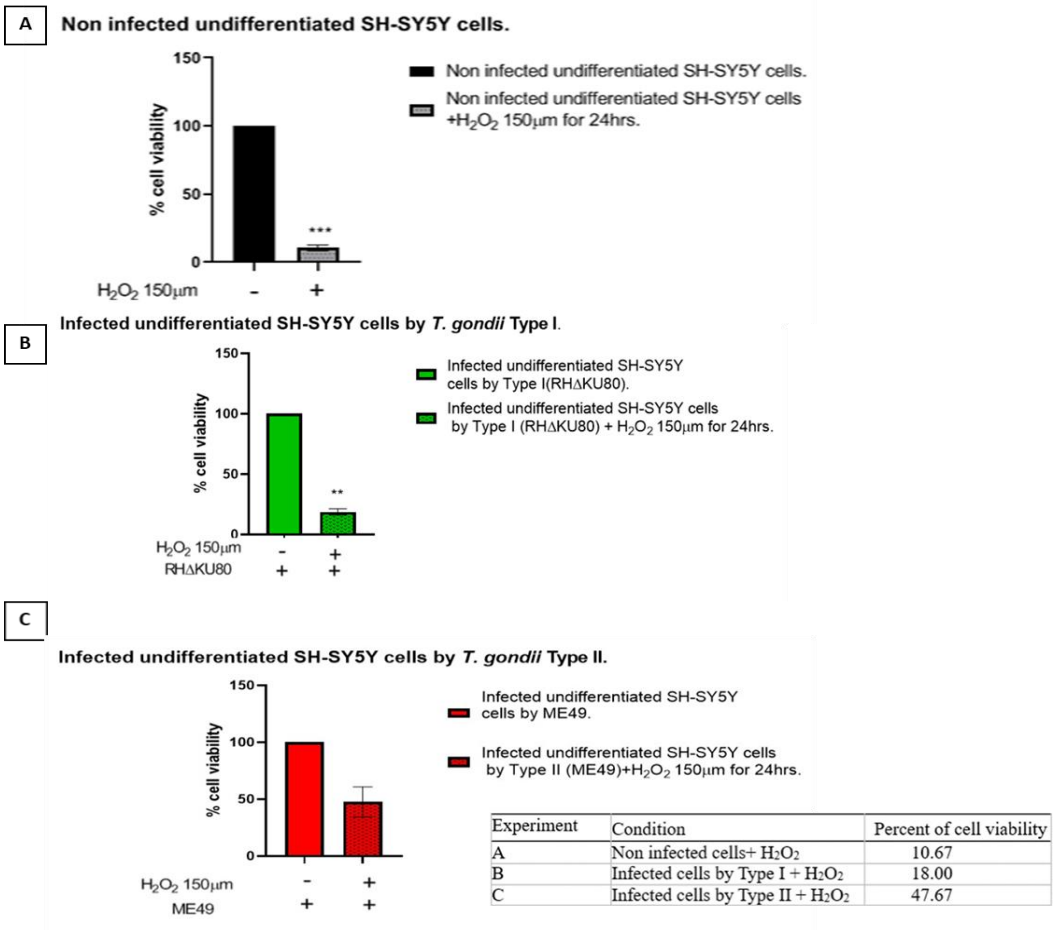
#### 6.4.1.1 Effects of hydrogen peroxide on MTT reductive activity.

The main aim was to examine whether the infection of type I and type II strains of *T. gondii* can counter the $H_2O_2$ triggered cell death in undifferentiated SH-SY5Y cells. The previously described experiments demonstrated that the exposure of the SH-SY5Y cells to different $H_2O_2$ concentrations and time periods culminated in the death of these cells. These toxicity effects, which were correlated with the concentration of $H_2O_2$, tested various times of exposure. In the current study, the aim was to have the undifferentiated SH-SY5Y cells exposed to 150$\mu$M $H_2O_2$ for 24hours. This concentration value is known to initiate cell death.

The undifferentiated SH-SY5Y cells were cultured for 24h then for some cells the *T. gondii* strains were used to infect them, with another set of cells uninfected as controls, then both sets were incubated for 24 hours. Both the infected and uninfected cells were then exposed to 150 $\mu$M $H_2O_2$ for 24hours. The outcome showed that the cells that were treated with 150 $\mu$M $H_2O_2$ for 24 hours appeared to have a marked decrease in their cell viability (10.67%) when they were compared to the control samples ($P < 0.0002$) (Figure 6. 1 A). On the other hand, the undifferentiated SH-SY5Y cells infected by *T. gondii* strains type I and II followed by treatment of 150$\mu$M $H_2O_2$ for 24 hours improved the cell viability to 18.67% ($P < 0. 001$) and 47.67 % respectively when compared with the control samples (Figure 6.1 B-C).

Generally, results showed that infection with both strains of the *T. gondii* provided a better protective effect against the $H_2O_2$ induced toxicity when compared to uninfected cells treated with $H_2O_2$. Having established the protective effect of infection of *T. gondii* strains

against $H_2O_2$ toxicity on cell viability of undifferentiated SH-SY5Y cell, does this affect cell morphology?




| Experiment | Condition | Percent of cell viability |
|---|---|---|
| A | Non infected cells+ $H_2O_2$ | 10.67 |
| B | Infected cells by Type I + $H_2O_2$ | 18.00 |
| C | Infected cells by Type II + $H_2O_2$ | 47.67 |

**Figure 6-1** The Effect of Hydrogen peroxide on the infected undifferentiated SH-SY5Y cell viability using the MTT assay with infection by Type I (RH***Δ***KU80) or Type II (ME49) *T. gondii* strains. SH-SY5Y cells were cultured in 24-well plates ($1\times10^5$ cells/1000 µL/mL)) and incubated for 24 hours at 37°C, 5% $CO_2$. (A) non-infected undifferentiated SH-SY5Y cells were treated with 150 $\mu$M of $H_2O_2$ or cells kept with medium (controls) for 24 hours at 37°C, 5% $CO_2$. Undifferentiation SH-SY5Y cells were infected by $1\times10^5$ of *T. gondii* strain I (RH***Δ***KU80) (B) or II(ME49) (C) before incubating for 24h at 37°C, 5% $CO_2$. Infected undifferentiated SH-SY5Y cells were treated with 150 $\mu$M of $H_2O_2$ or cells kept with medium (control) for 24 hours at 37°C, 5% $CO_2$. Viable cell numbers were analyzed using thiazolyl blue tetrazole (MTT). Data are expressed as the percentage of viable cells in relation to the control (mean ± S.D.) and are representative of three independent experiments in triplicate. **P< 0.005 and ***P< 0.0005.

#### 6.4.1.2 Effects of hydrogen peroxide on cell morphology

The other outcomes, as discussed above, show that infection with the *Toxoplasma* parasite protected against the effect of the $H_2O_2$ triggered toxicity in the undifferentiated SH-SY5Y cells as compared to the exposure to the $H_2O_2$ performed by the MTT assay. However, it is vital to confirm these outcomes through the use of a morphology method, which clearly shows the effect of the infection of *Toxoplasma* on oxidative stress in undifferentiated SH-SY5Y cells. In this case, a differential staining such as Giemsa is used to distinguish between the viable cells and dead cells. Giemsa stain is a primary stain where all living cells will take up the dye. As demonstrated above, undifferentiated SH-SY5Y cells were cultured on coverslips for 24hours then followed by infecting some of the cells by two strains of *T. gondii* for 24hours. After this, some infected and non-infected cells were treated with $H_2O_2$ for 24hours, eventually staining by the Giemsa stain was done for all the cells.

The results showed most of the cells that were not infected by the *T. gondii* were lost due to the exposure to $H_2O_2$ 150$\mu$M treatment (Figure 6. 2 D). Where the cells were infected by both the strains and treated with the 150$\mu$M $H_2O_2$ for 24 hours, a high number of viable cells were elicited when compared to the non-infected cells treated with the $H_2O_2$ (Figure 6. 2 E-F). Generally, these outcomes gave similar results to the MTT assay indicating that the two strains of the *T. gondii* infection offered better protection against the $H_2O_2$ triggered undifferentiated SH-SY5Y cell toxicity when compared to the $H_2O_2$ treated cases. This raises the question as to how differentiated SH-SY5Y cells behave in response to oxidative stress when infected with *T. gondii* type I and II strains.

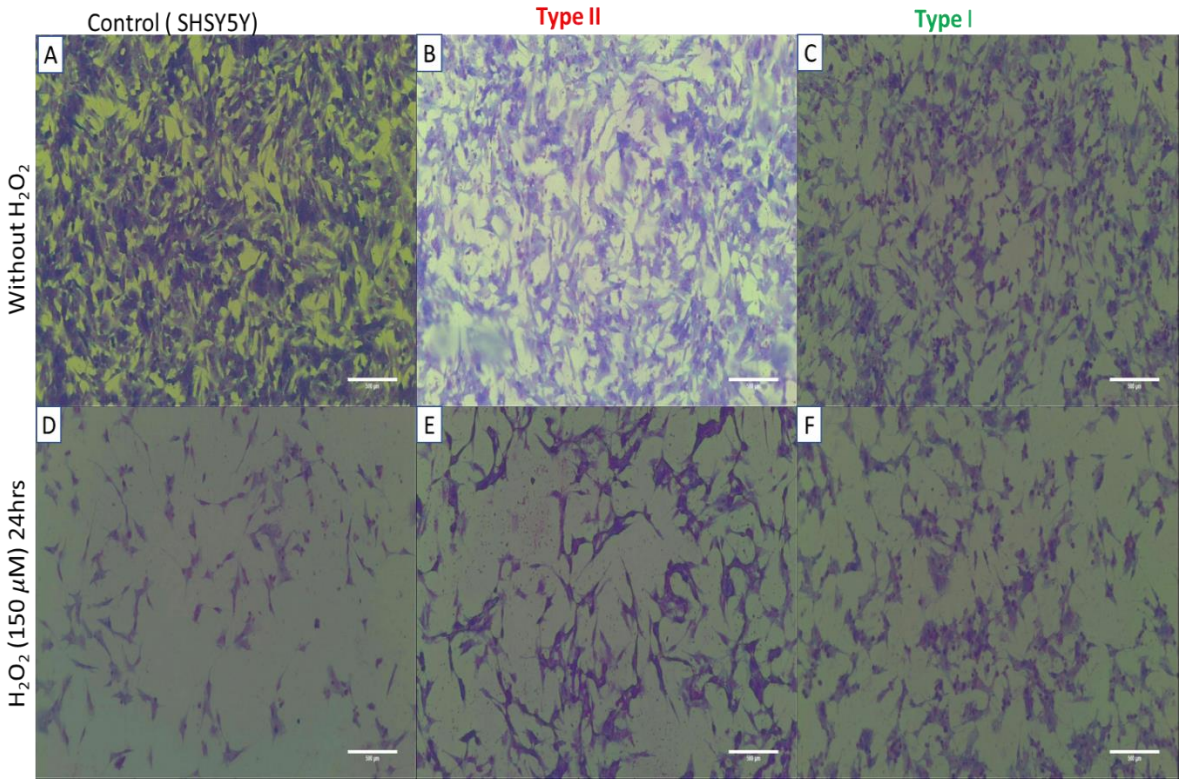



**Figure 6-2** Photographs of Giemsa staining of infected undifferentiated SH-SY5Ycells exposed to $H_2O_2$ for 24h. Undifferentiated SH-SY5Y cells were cultured on coverslips in 6-well plates ( $5\times10^4$ cells/1000 µL/mL) and incubated for 24 hours and then infected by *T. gondii* strains type I and II at a ratio of 1 cell: 5 parasites for 24 hours then treated with 150 $\mu$M of $H_2O_2$ for 24h. (A) Undifferentiated SH-SY5Y cells after 48h of culture. (B) Undifferentiated SH-SY5Y cells after 48h of culture and after 24 h of infection with *T. gondii* strain II ( ME49). (C) Undifferentiated SH-SY5Y cells after 48h of culture and after 24 h of infection with *T. gondii* strain I ( RH***Δ***KU80) (D) Undifferentiated SH-SY5Y cells after 48h of culture then treated with 150 $\mu$M of $H_2O_2$ for 24h. (E) Undifferentiated SH-SY5Y cells after 48h of culture and after 24 h of infection with *T. gondii* strain II ( ME49) were then treated with 150 $\mu$M of $H_2O_2$ for 24h. (F) Undifferentiated SH-SY5Y cells after 48h of culture and after 24 h of infection with *T. gondii* strain I (RH***Δ***KU80) then treated with 150 $\mu$M of $H_2O_2$ for 24h. Then the cells were washed, fixed, stained with Giemsa stain and analysed under a light microscope. Scale bar, 500 µm.

## 6.4.2 Investigation into the effects of *T. gondii* type I and II infection on oxidative stress in differentiated SH-SY5Y cells.

### 6.4.2.1 Effects of hydrogen peroxide on MTT reductive activity.

The main aim was to examine whether the infection of *T. gondii* strains I and II can counter the $H_2O_2$ triggered cell deaths in differentiated SH-SY5Y. The earlier experiments demonstrated that the *Toxoplasma* infection offers protection against the $H_2O_2$ triggered cell death among the undifferentiated cells as compared to the cells treated using $H_2O_2$ as performed by the MTT assay and cell morphology methods. This experiment will address whether *T. gondii* infections offer protection against $H_2O_2$ triggered cell toxicity in the differentiated SH-SY5Y cells. At this point, the undifferentiated SH-SY5Y cells were taken through differentiation for 21 days. After that, the *T. gondii* strains were used to infect some of the cells for 24 hours. Both the non-infected and infected cells were treated with 150$\mu$M $H_2O_2$ for 24hours.

Results showed that treatment with 150$\mu$M $H_2O_2$ for 24hours caused a significant decline in the cell viability (77.5%) as compared to the control samples ($P < 0.004$) (Figure 6. 3A). However, differentiated SH-SY5Y cells infected by *T. gondii* type I and II followed by treatment of 150$\mu$M $H_2O_2$ for 24 hours improved the cell viability to 98% and 84.5%, respectively compared with the control samples (Figure 6. 3 B-C). These results showed that infection with the type I and II strains of *T. gondii* demonstrated a better protective effect against $H_2O_2$ -induced cell toxicity when compared to treatment with $H_2O_2$.

Also, the experiment aimed to determine which strain provides maximum protection against the effects of hydrogen peroxide ($H_2O_2$). A comparison between the protective effect of infection with the type I and II strains of *T. gondii* against $H_2O_2$ was carried out. The investigation of induced cell toxicity was carried out in differentiated and undifferentiated SH-

SY5Y cells to determine the toxic effect of $H_2O_2$. The results of previous experiments showed that when undifferentiated cells were infected by *T. gondii* strains type I and II followed by treatment of 150$\mu$M $H_2O_2$ for 24 hours, cell protection which restored cell viability was 18.67% and 47.67%, respectively, compared to untreated cells (Figure 6.1 B-C). This indicated that the *T. gondii* strain type II provides a greater protective effect against $H_2O_2$. When a comparison was made with infected differentiated SH-SY5Y cells by *T. gondii* strain types I and II when treated by150$\mu$M $H_2O_2$ for 24 hours, the cell viability improved to 98% and 84.5%, respectively, from 77.5 % ($P < 0.004$) (Figure 6. 3). It indicates that *T. gondii* strain types provide a greater provides a greater protective effect against $H_2O_2$ in differentiated cells but that the Type I strain provided a greater protective effect. This raises the question as to how these cells behave in response to oxidative stress when treated with conditioned media (CM) from *T. gondii* type I and II strains grown in undifferentiated SH-SY5Y cells. CM was used to investigate whether it is a soluble component in the conditioned medium that is involved in the effect.

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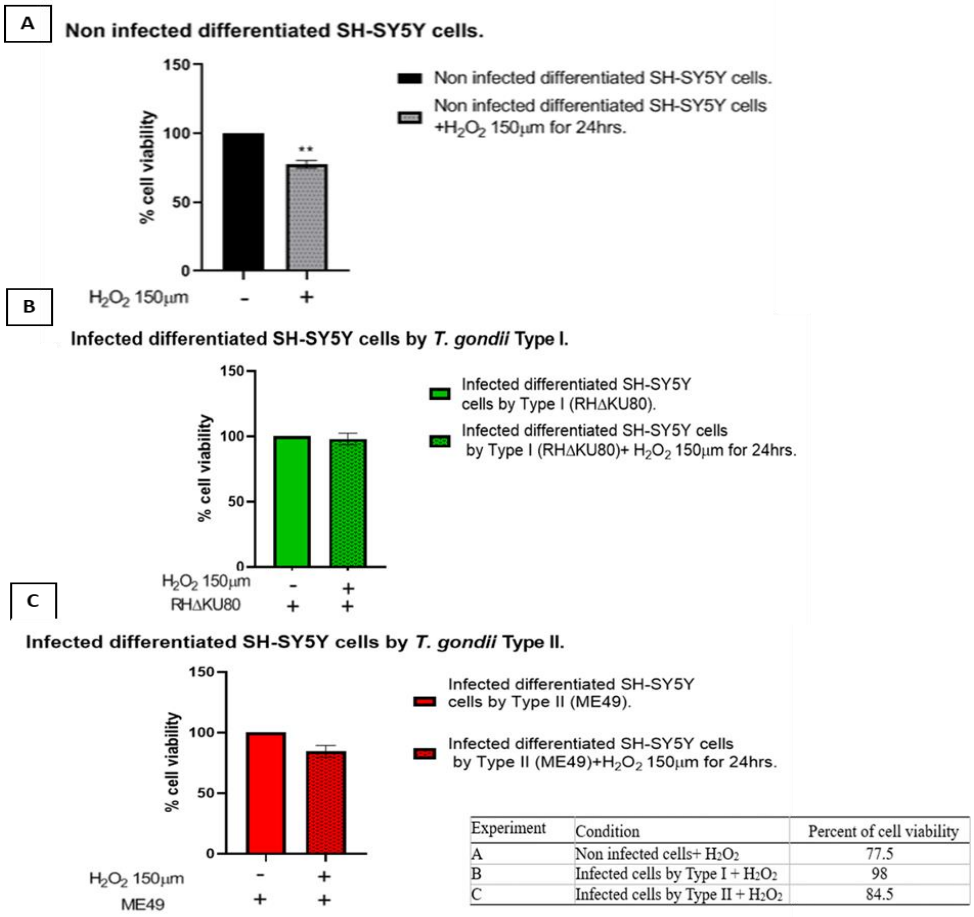



| Experiment | Condition | Percent of cell viability |
|---|---|---|
| A | Non infected cells+ $H_2O_2$ | 77.5 |
| B | Infected cells by Type I + $H_2O_2$ | 98 |
| C | Infected cells by Type II + $H_2O_2$ | 84.5 |

**Figure 6-3** The Effect of Hydrogen peroxide on the infected differentiated SH-SY5Y cell viability using MTT assay. SH-SY5Y cells were cultured in 24-well plates ($2\times10^4$ cells/1000 µL/mL) and incubated for 48 hours at 37°C, 5% $CO_2$, then the SH-SY5Y cells were taken through the process of differentiation for 21 days, (A) non-infected differentiated SH-SY5Y cells were treated with 150 $\mu$M of $H_2O_2$ or cells kept with medium (control) for 24 hours at 37°C, 5% $CO_2$. Differentiation SH-SY5Y cells were infected by $1\times10^5$ of *T. gondii* strain I (RH$\Delta$KU80) (B) or II (ME49) (C) before incubating for 24h at 37°C, 5% $CO_2$. Infected differentiated SH-SY5Y cells were treated with 150 $\mu$M of $H_2O_2$ or cells kept with medium (control) for 24 hours at 37°C, 5% $CO_2$. Viable cell numbers were analyzed using thiazolyl blue tetrazole (MTT. Data are expressed as the percentage of viable cells in relation to the control (mean ± S.D.) and are representative of three independent experiments in triplicate. **P < 0.005 (RM one way ANOVA and Paired t-test). There was some variability in the percentage of cell viability between experiments. For example, in Figure 5 4 the cell viability at 150$\mu$M for 24 and 48 hours are 90 % 86.6 %, respectively, and in Figure 6.3 the cell viability at 150$\mu$M for 24 hours is 77.5 %.

## 6.5 Discussion

As mentioned in the literature review, *Toxoplasma* can infect brain cells includes neuronal cells. *In-vitro*, SH-SY5Y cell line was used as a model of neuronal cell. In this study, the effect of T. gondii infection against cell damage caused by hydrogen peroxide in SH-SY5Y neuroblastoma cells was investigated. Two strains of *T. gondii* were used to infect differentiated and undifferentiated SH-SY5Y cells followed by treatment to $H_2O_2$. The results of this study showed that infection with the *T. gondii* type I and II strains play a significant role in protecting undifferentiated and differentiated SH-SY5Y cells from $H_2O_2$ induced cell death. To our knowledge, this is a novel finding in human brain cell lines and has not been reported before. Also, the study examined the difference in the preventive effect of the infection *T. gondii* strains type I and II. Differentiated and undifferentiated SH-SY5Y cells were infected with *T. gondii* strains type I and II. The cells were then treated with $H_2O_2$ to determine which strains provide the best protection for differentiated and undifferentiated SH-SY5Y cells against oxidative stress. The results show that the *T. gondii* strain type II provides a greater protective effect against $H_2O_2$-induced cell toxicity in undifferentiated SH-SY5Y cells. In contrast, the infection of differentiated SH-SY5Y cells with *T. gondii* strain type I conferred better protection compared with Type II. This finding supports previous research in mouse brain with AD which suggest that *T. gondii* strain type II offered maximum protection compared with Type I (Cabral et al. 2017). Previous studies indicated that the protection conferred by *T. gondii* was induced by anti-inflammatory cytokines, such as TGF-β and IL-10 (Cabral et al. 2017; Jung et al. 2012). Also, it was found that there was a reduction in Aβ plaque formation in Alzheimer's disease-*Toxoplasma*-infected mice compared with uninfected mice (Möhle, Israel, Paarmann, Krohn, Pietkiewicz, Müller, Inna N Lavrik, et al. 2016). Indeed, the difference between *T. gondii* strains requires more research to determine the exact point of variation and how it relates to my hypothesis of the relationship between *T. gondii* and Alzheimer's disease.

# 7 Chapter 7: Investigation of the effects of *Toxoplasma* conditioned media on cell growth associated with oxidative stress in a model brain cell culture system.

## 7.1 Introduction

Toxoplasmosis is caused by *Toxoplasma gondii*, a parasite that is known to infect the brain by forming cysts in the brain cells. Previous studies have shown that among immunocompromised patients, the parasite damages brain regions such as the cortex and hippocampus, which can also play a role in AD (Blanchard, Dunay, and Schlüter 2015; Haroon et al. 2012; Munoz, Liesenfeld, and Heimesaat 2011). Conversely, other studies show that *T. gondii* infects the brain, causing protection from neuronal degeneration. In this particular infection, interferon (IFN)-γ is produced as an immunological response against the parasite. The IFN-γ activates the microglia and simulates the production of transforming growth beta 1 (TGF-β), which prevents expression of inducible nitric oxide synthase (iNOS), which in turn has a significant influence on host cell apoptosis (Parlog et al. 2015; Rozenfeld et al. 2005). Even though some studies have been conducted on the neuroprotective effects of anti-inflammatory and antioxidants against $H_2O_2$ apoptosis in systems such as *(M)*-bicelaphonol A (Wang et al. 2013); however, no study has yet shown that *T. gondii* infection can protect SH-SY5Y cells from cytotoxicity.

In this chapter, the effect of conditioned media (CM) of *Toxoplasma* against cell damage caused by hydrogen peroxide in SH-SY5Y neuroblastoma cells will be further analyzed. In this case, conditioned media (CM) of *Toxoplasma* obtained from *T. gondii* growth medium will be used to treat them prior to exposing the undifferentiated SH-SY5Y to $H_2O_2$. Apoptosis in the

cells and their viability will then be determined using MTT assay and flow cytometry. Additionally, we will determine the differences between the type I and II strains of *T. gondii* (non-cyst forming and cyst-forming strains) to investigate their ability to protect the SH-SY5Y cells from hydrogen peroxide toxicity.

## 7.2 Objectives

The aim of this chapter is an investigate the effects of *Toxoplasma* conditioned media on cell growth associated with oxidative stress in a model brain cell culture system. There are two main objectives:

- Investigate the effects of *T. gondii* type I and II conditioned media on oxidative stress in undifferentiated SH-SY5Y Cells by MTT reductive activity.
- Investigate the effects of *T. gondii* type I and II conditioned media on oxidative stress in undifferentiated SH-SY5Y Cells by FACS.

## 7.3 Methods

The detailed methods used in this chapter are described in chapter 2 Materials and Methods. Briefly the SHSY5Y cell line was used to study the effect of *Toxoplasma* conditioned media on the expression of molecules associated with oxidative stress and Alzheimer's in a model brain cell culture system. In this study, the effect of CM of *T. gondii* strain I and II on $H_2O_2$ toxicity in undifferentiated and SH-SY5Y cells was determined. Here, diverse conditions (CM of *T. gondii* strain I and II and 150µM of $H_2O_2$) were used to treat the undifferentiated SH-SY5Y cells, and MTT and FACS assays carried out to determine the effects on cell growth.

## 7.4 Results

### 7.4.1 Investigation into the effect of *T. gondii* type I and II conditioned media on oxidative stress in undifferentiated SH-SY5Y Cells by MTT reductive activity.

The main objective was to investigate whether conditioned medium (CM) from *T. gondii* strain type I and II co-treatment, post-treatment, and pre-treatment were capable of neutralizing $H_2O_2$ triggered undifferentiated SH-SY5Y cell death. This would show whether the conditioned medium contains some components that may play an important role in protecting cells from the toxic effect of $H_2O_2$. Previous experiments indicated that infection with *Toxoplasma* in the SH-SY5Y cells gave protection against $H_2O_2$ cell damage. The following experiments will investigate the conditioned media (CM) from the *Toxoplasma* strains that were extracted from the culture medium of the *T. gondii* infected undifferentiated SH-SY5Y cells. In this study, the SH-SY5Y cells were challenged by treatment with 150 $\mu$M $H_2O_2$ (a concentration value known to induce cell death) for either 24hours or 48hours. As described in the methods section, undifferentiated SH-SY5Y cells were grown for 24hours. The cells were then either treated with CM (for either 24h and 48h), $H_2O_2$ (for either 24h and 48h), co-treatment ($H_2O_2$ and CM for either 24h and 48h), post-treatment ($H_2O_2$ for 24h then CM for 24h) or pre-treatment (CM for 24h then $H_2O_2$ for 24h). After this, the viability of the cells was determined using the MTT assay.

Firstly, when *T. gondii* strain I CM was used, the results showed that undifferentiated SH-SY5Y cells, subjected to 150 $\mu$M of $H_2O_2$ for either 24 or 48 hours, caused a decrease in cell viability (36% and 25.25%; respectively as compared to the control samples ($p < 0.00001$, Figure 7. 1 A- Figure 7. 2 A). The results also indicated that co-treatment ($H_2O_2$ and CM for either 24h and 48h) and post-treatment ($H_2O_2$ for 24h then CM for 24h) with *T. gondii* CM

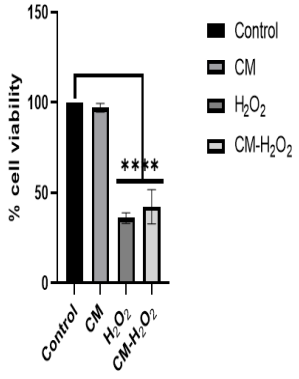
from strain type I showed no restoration in cell viability, respectively (42.25%, 32.50%, and 28.00%) (Figure 7. 1 A- Figure 7. 2 A). Nevertheless, the pre-treatment with CM and $H_2O_2$ for 24h did show the major protection by restoring the cell viability to 85.75% compared with the $H_2O_2$ group ($p < 0.00001$, (Figure 7. 2 A).

For the second case, when CM was used from the *T. gondii* strain II CM, the outcomes showed that the undifferentiated SH-SY5Y cells which results were subjected to 150 $\mu$M of $H_2O_2$ for either 24 hours or 48hours caused a significant decrease in the cell viability (38.25% and 25.25%; respectively) as compared with control samples ($p < 0.00001$, Figure 7. 1 B- Figure 7. 2 B). The co-treatment ($H_2O_2$ and CM for either 24h and 48h) and post-treatment ($H_2O_2$ for 24h then CM for 24h) with *T. gondii* CM strain type II failed to restore cell viability as follows 41%, 31.75%, and 25.00% respectively (Figure 7. 1 B- Figure 7. 2 B). However, pre-treatment with CM and $H_2O_2$ for 24hours led to protection restoration of cell viability of up to 89.25% as compared to the $H_2O_2$ group ($p < 0.00001$, Figure7. 2 B).

In the use of the CM of *T. gondii* strain, I & II for both cases gave similar results this showed that treatment with CM revealed good protection effect against $H_2O_2$ - triggered cell toxicity in the undifferentiated SH-SY5Y cells when compared to those that were exposed to the $H_2O_2$. Thus, it is clear that the presence of *T. gondii* CM has a significant influence on SH-SY5Y cell protection. This raises a question about the effects of *T. gondii* CM on the protection of SH-SY5Y cells against hydrogen peroxide triggered apoptosis.

A

Effect of *T. gondii* type I C.M. on undifferentiated SH-SY5Y cells with $H_2O_2$ (150μM) 24hrs

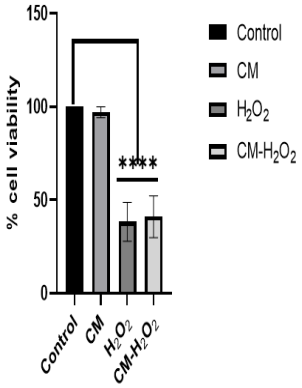



| Condition | Percent of cell viability |
|---|---|
| C.M | 97.3 |
| $H_2O_2$ | 36.00 |
| C.M.+$H_2O_2$ | 42.25 |

C.M.= Condition Media

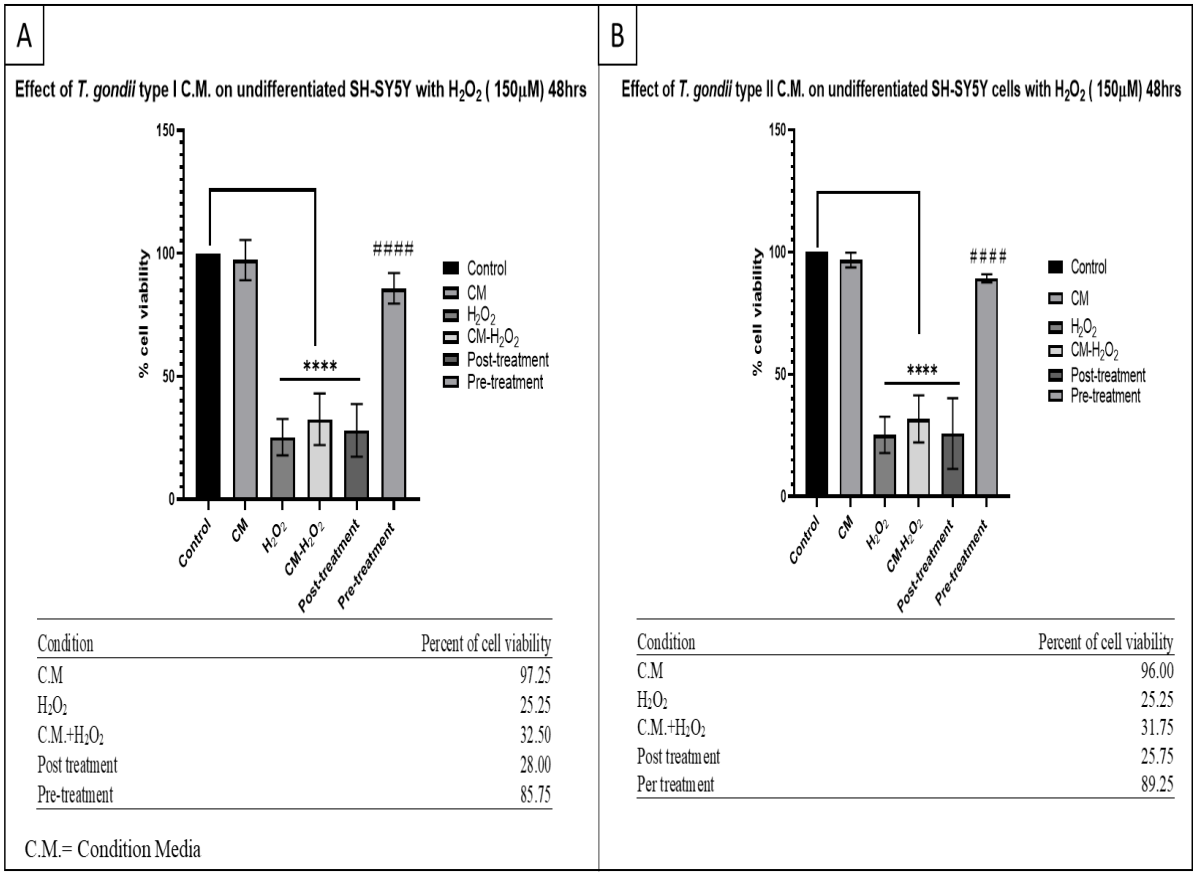
B

Effect of *T. gondii* type II C.M. on undifferentiated SH-SY5Y cells with $H_2O_2$ (150μM) 24hrs




| Condition | Percent of cell viability |
|---|---|
| C.M | 97 |
| $H_2O_2$ | 38.25 |
| C.M.+$H_2O_2$ | 41.00 |

**Figure 7-1** Inhibitory effect of *T. gondii* strain CM (Condition Medium) on $H_2O_2$ induced in undifferentiated SH-SY5Y cells injury for 24h. Undifferentiated SH-SY5Y cells were cultured in 24-well plates ($1\times10^5$ cells/1000 μL/mL) and incubated for 24 hours at 37◦C, 5% $CO_2$. In the first group, cells were kept with medium (control) for 24 hours at 37◦C, 5% $CO_2$. For the second group, cells treated with CM of *T. gondii* strain I (RH***Δ***KU80) (A) or strain II (ME49) (B) for 24 hours at 37◦C, 5% $CO_2$. The third group of cells was treated with $H_2O_2$ (150 $\mu$M) for 24 hours at 37◦C, 5% $CO_2$. The fourth group of cells was co-treated with CM of *T. gondii* strain I (RH***Δ***KU80) (A) or strain II (ME49) (B) and $H_2O_2$ (150 $\mu$M) for 24 hours at 37◦C, 5% $CO_2$. Viable cell numbers were analyzed using thiazolyl blue tetrazole (MTT) after 24 h treatment. Data were presented as the percentage of survival relative to control cells from three independent experiments and as mean ± SD. ****$p < 0.00005$ vs. control.




| Condition | Percent of cell viability |
|---|---|
| C.M | 97.25 |
| $H_2O_2$ | 25.25 |
| C.M.+$H_2O_2$ | 32.50 |
| Post treatment | 28.00 |
| Pre-treatment | 85.75 |

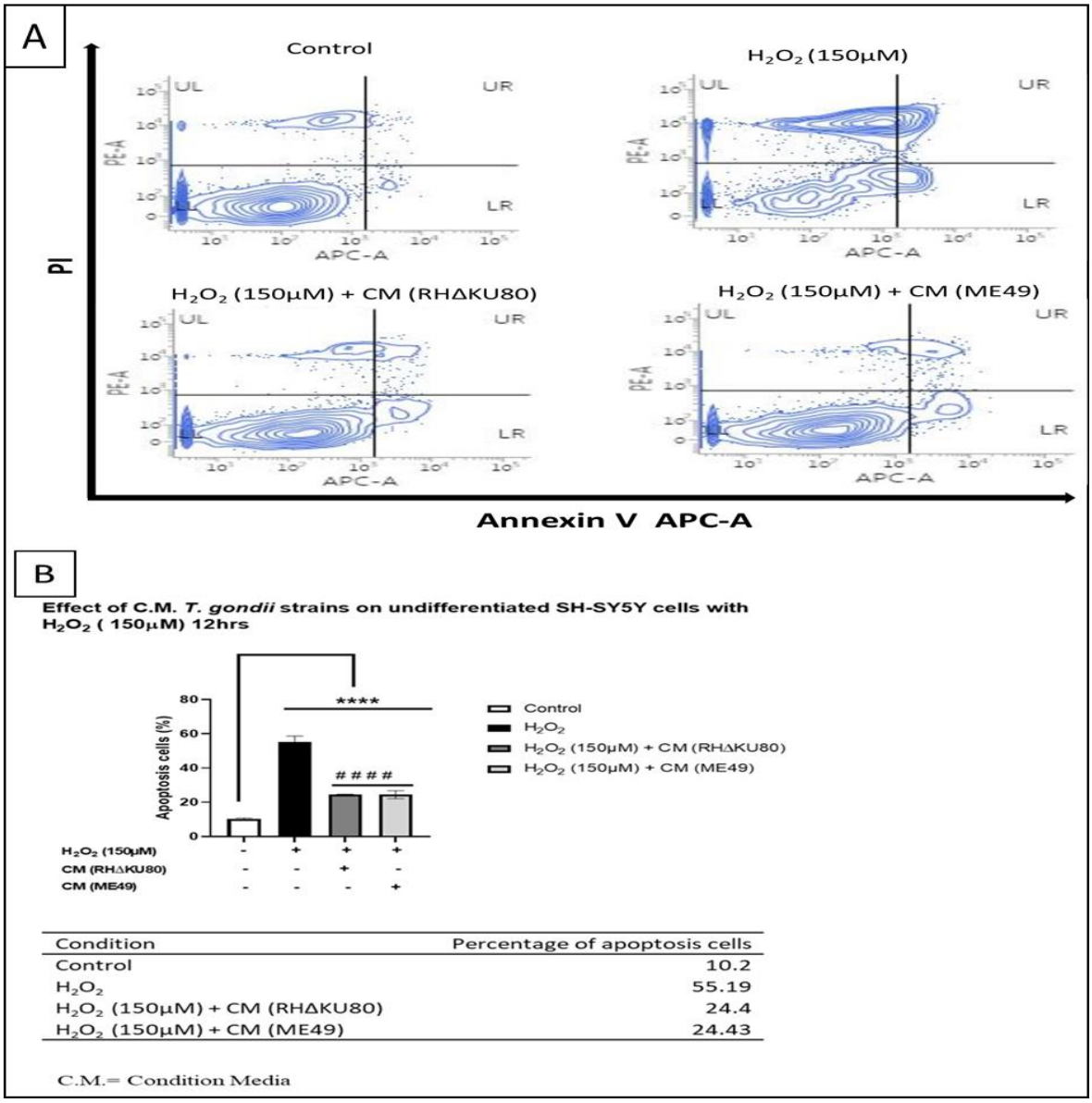
C.M.= Condition Media

| Condition | Percent of cell viability |
|---|---|
| C.M | 96.00 |
| $H_2O_2$ | 25.25 |
| C.M.+$H_2O_2$ | 31.75 |
| Post treatment | 25.75 |
| Per treatment | 89.25 |

**Figure 7-2** Inhibitory effect of *T. gondii* strain CM (Condition Medium) on $H_2O_2$ induced in undifferentiated SH-SY5Y cells injury for 48h. Undifferentiated SH-SY5Y cells were cultured in 24-well plates ($1\times10^5$ cells/1000 µL/mL) and incubated for 24 hours at 37◦C, 5% $CO_2$. In the first group, cells were kept with medium (control) for 24 hours at 37◦C, 5% $CO_2$. For the second group, cells treated with CM of *T. gondii* strain I (RH***Δ***KU80) (A) or strain II (ME49) (B) for 48 hours at 37◦C, 5% $CO_2$. The third group of cells was treated with $H_2O_2$ (150 $\mu$M) for 48 hours at 37◦C, 5% $CO_2$. The fourth group of cells were post-treated with CM of *T. gondii* strain I (A) or strain II (B) and $H_2O_2$ (150 $\mu$M) for 48 hours at 37◦C, 5% $CO_2$. The fifth group of cells was pre-treated with CM of *T. gondii* strain I (A) or strain II (B) for 24 hours, before incubating with 150 $\mu$M of $H_2O_2$ for 24 hours at 37◦C, 5% $CO_2$. Viable cell numbers were analyzed using thiazolyl blue tetrazole (MTT) after 24 h treatment. Data were presented as the percentage of survival relative to control cells from three independent experiments and as mean ± SD. ****$p < 0.00005$ vs. control; $^{\#\#\#\#}p < 0.00005$ vs. $H_2O_2$ group.

### 7.4.2 Investigation into the effect of *T. gondii* type I and II conditioned media on oxidative stress in undifferentiated SH-SY5Y cells by FACS.

The main objective of this set of experiments was to examine if the conditioned medium (CM) of *T. gondii* strain type I and II pre-treatment were capable of neutralizing $H_2O_2$ triggered undifferentiated SH-SY5Y cell apoptosis. The previously obtained results show that the use of the *Toxoplasma* strain CM offered protection to the SH-SY5Y cells against $H_2O_2$ triggered cell damage. The following experiments were aimed at investigating whether the CM of the *Toxoplasma* strains gives protection to the SH-SY5Y cells against $H_2O_2$ induced apoptosis. Annexin V-APC binding analysis and PI staining (Figure 7. 3 A) was performed to detect cells undergoing early and late apoptosis, while intact cells cannot be stained using the PI and Annexin V-APC. In the control samples (Figure 7. 3 A), there was only a minimal cell number (10.2 %) were positive to PI and Annexin V-APC staining. Then they were exposed to 150 µM $H_2O_2$ for 24 hours, and there was a significant increase in the apoptosis percentage (55.19%; $p < 0.00001$ compared to control) as shown in (Figure 7. 3 B). In cases where pre-incubation with *T. gondii* CM strain type I and II for 24 hours was done, there was a marked decline in the number of apoptotic cells. The dose-dependent apoptosis rate was also reduced to the following percentages 24.4% and 24.43%. (Figure 7. 3 B; $p < 0.00001$ vs. $H_2O_2$ group). Results showed that the exposure of undifferentiated SH-SY5Y cells to $H_2O_2$ triggered an increase in the rate at which cell apoptosis occurred. However, the pre-treatment with *T. gondii* CM resulted in a significant reduction of apoptotic cells. Thus, the CM of *T. gondii* played an essential role in offering protection to the SH-SY5Y cells against hydrogen peroxide toxicity.




| Condition | Percentage of apoptosis cells |
|---|---|
| Control | 10.2 |
| $H_2O_2$ | 55.19 |
| $H_2O_2$ (150µM) + CM (RHΔKU80) | 24.4 |
| $H_2O_2$ (150µM) + CM (ME49) | 24.43 |

C.M.= Condition Media

**Figure 7-3** Inhibitory effect of *T. gondii* strains CM (Conditioned Medium) on $H_2O_2$ induced apoptosis in undifferentiated SH-SY5Y cells. SH-SY5Y cells were cultured in 24-well plates ($1\times10^5$ cells/1000 µL/mL) and incubated for 24 hours at 37◦C, 5% $CO_2$. The first group of cells was control cells. The second group of cells was treated with $H_2O_2$ (150 $\mu$M) for 24 hours at 37◦C, 5% $CO_2$. The third group of cells was pre-treated with *T. gondii* strain I CM for 24 hours, before incubation with 150 $\mu$M of $H_2O_2$ for 24 hours at 37◦C, 5% $CO_2$. The fourth group of cells was per-treated with *T. gondii* strain II CM for 24 hours, before incubation with 150 $\mu$M of $H_2O_2$ for 24 hours at 37◦C, 5% $CO_2$. (A) Flow cytometric analysis shows the percentage of apoptotic cells: the sum of the UR and LR quadrants. (B) The histogram of the apoptosis percentage (n = 3). Data were presented as mean ± SD. ****$p < 0.00005$ vs. control; $^{\#\#\#\#}p < 0.00005$ vs. $H_2O_2$ group.

## 7.5 Discussion

It has been noted that infection with the *T. gondii* type I and II strains play a significant role in protecting SH-SY5Y cells from $H_2O_2$ induced cell death. In this study, the effect of conditioned media (CM) of *Toxoplasma* against cell damage caused by hydrogen peroxide in SH-SY5Y neuroblastoma cells was investigated. The exposure of the cultured cells to $H_2O_2$ results in various changes in the cells, including dysfunction of the mitochondrial, misfolding of the proteins, genetic mutation, and eventual cell death (Klamt et al. 2009). In this study, it was established that undifferentiated SH-SY5Y cell pre-treatment using different conditioned media (CM) from *T. gondii* strains reduced the effects induced by the $H_2O_2$. This was achieved through a dose-dependent approach where the results were confirmed through the viability of the SH-SY5Y cells assay (Figure 7. 1 and Figure 7.2). These findings indicated that the infection of *T. gondii* could offer protection to the SH-SY5Y cells from $H_2O_2$ triggered neurotoxicity. Also, the quantification of the protective properties of the CM of *T. gondii* against $H_2O_2$ triggered cell apoptosis was done through flow cytometry. It is established that $H_2O_2$ can cause damage to the cells and induce apoptosis (Chen et al. 2010; Klamt et al. 2009). Consistently, these previous reports showed that, when double staining was done using PI and Annexin V-APC, treatment with $H_2O_2$ led to a noticeable upsurge in the dead cells (Figure 7. 3). Comparable to the earlier mentioned cell survival test findings, CM pre-treatment significantly weakened the effect of oxidation, demonstrating that the neuroprotective effect of CM pre-treatment may be partly linked to its ability to prevent cell apoptosis.

Generally, this study showed that cells pre-infected by *T. gondii* or pre-treated with CM of *T. gondii* reduced cell viability decline and apoptosis induced by $H_2O_2$. Thus, this study shows that the infection with *T. gondii* is associated with its protective impact against OS effects, which can be partially accredited to the immunological changes that occur during the

infection with *T. gondii*. In the course of infection with *T. gondii*, anti-inflammatory cytokines levels that include the IL-10 and TGF-β rises in the brain tissues and cultivated neurons, whereas nitric oxide (NO) is produced in decreased levels (Blanchard et al. 2015; Jung et al. 2012). These anti-inflammatory cytokine levels can be can be determined by using ELISA kits (Jung et al. 2012). Additionally, there was a study that was conducted using Alzheimer's disease murine model, and it showed that β-Aβ plaque deposition was considerably lower in *T. gondii*-infected animals (Jung et al. 2012). A probable explanation was offered in a study done by (Rozenfeld et al. 2005). Here the outcomes showed that microglia activated by interferon IFN-γ has a significant influence in the protection of neurons by stimulating the production of TGF-β, which blocks NO which plays a role in decreasing reactive oxygen species (ROS), which in turn leads to a reduction of the effect of oxidative stress and prevents cell death (Martindale and Holbrook 2002; Valko et al. 2007).

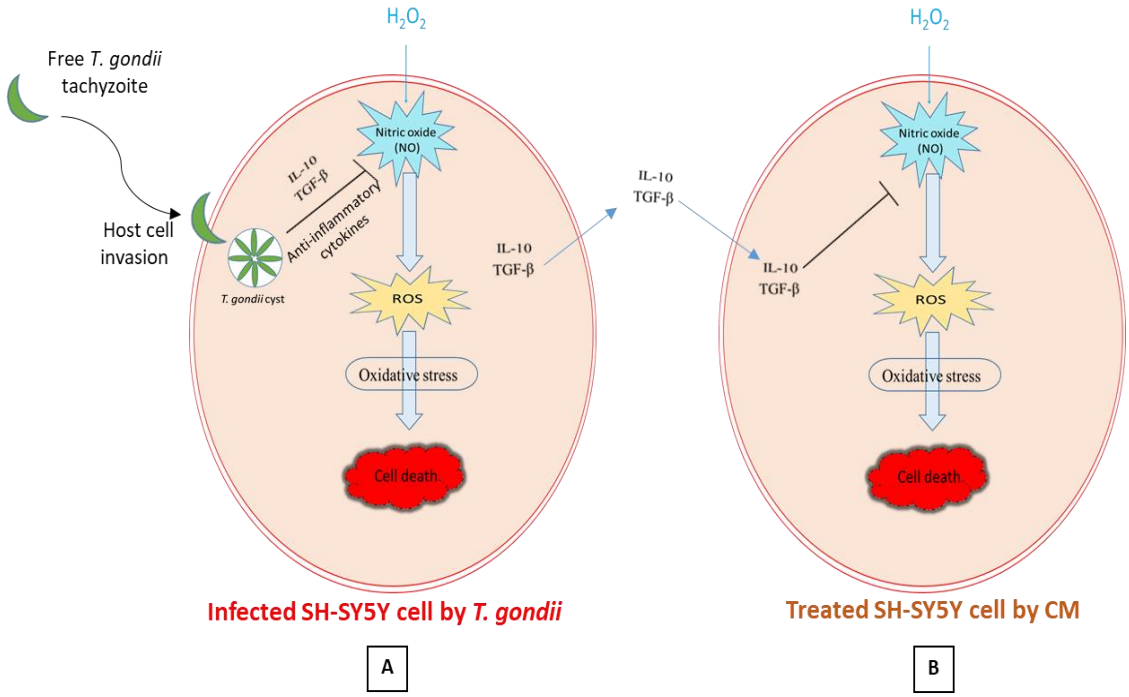



**Figure 7-4** A schematic representation of the cellular Oxidative stress pathway during infection with (A) *Toxoplasma gondii* or (B) treated with conditioned media (CM) of *T. gondii*. Anti-inflammatory cytokines, such as transforming growth beta (TGF-β) and interleukin-10 (IL-10), are secreted during this infection which prevent the formation of reactive oxygen species (ROS) and protect the cell from death. The infection of *T. gondii* particularly affects the nitric oxide (NO) pathway and the production of ROS.

Therefore, these results show that infection with *T. gondii* is associated with its protective impact on oxidative stress. During the course of *T. gondii* infection, the levels of anti-inflammatory cytokines rise which play a role as an antioxidative effect against oxidative stress. This is in harmony with the work published by (Alvariño et al. 2017; Md et al. 2018; Wang et al. 2013), which showed that many antioxidative molecules could scavenge ROS and protect against oxidative stress. It has been shown that these neuroprotective properties are associated with a decline in ROS. Thus, neuroprotective abilities could be used as a potential property to base the development of drugs for the management of neurodegenerative diseases

associated with oxidative stress. Overall, this study proposes that the anti-oxidative effects of *T. gondii* on neuronal cell damage and its unique potential mechanisms provide significant support for the development of drugs that are intended to assist in diagnosing and treating clients against AD. Nevertheless, firm evidence on the efficacy of the pre-treatment effect of *T. gondii* CM in primary cultured neurons, animal models, and the specific mechanisms of the valuable aspects of this compound needs to be established. Hence, future research on this aspect is recommended.

## 7.6 Recommendations and future work:

The research presented in this thesis has addressed several questions regarding the relationship between the infection of *T. gondii* and Alzheimer's disease; however, there are still questions that need to be addressed. Future work should investigate the effect of *T. gondii* type I and II conditioned media on oxidative stress in differentiated SH-SY5Y cells by MTT assay and FACS. The same question for differentiated cells was not addressed in the study here due to the timing and interruption due to the Covid-19 pandemic and an inability to produce sufficient conditioned media in the time frame. The answer to this question allows the researcher to establish the difference in the effect of the conditioned media of *T. gondii* strains type I and II between undifferentiated and differentiated SH-SY5Y cells.

Future investigations might use different conditioned media obtained from infected differentiated SH-SY5Y by *T. gondii* strains and repeat all previous experiments and study the difference between these conditioned media. Likewise, it is important to measure the level of anti-inflammatory cytokines, such as TGF-β and IL-10, in both conditioned media obtained from infected differentiated and undifferentiated SH-SY5Y cells by *T. gondii* strains, using the ELISA technique. Previous studies have proven that the *T. gondii* infection induces TGF-β and IL-10, which play an important role in preventing Alzheimer's disease. Studies confirm a decrease in β -Aβ plaque formation in Alzheimer's disease-*Toxoplasma*-infected mice (Jung et al. 2012; Möhle, Israel, Paarmann, Krohn, Pietkiewicz, Müller, Inna N Lavrik, et al. 2016). Therefore, it leads us to the importance of estimating the levels of inflammatory cytokines secreted during infection with different *T. gondii* strains.

# 8 Chapter 8: General discussion.

The parasite *Toxoplasma gondii* is widespread in both animals and humans. The feline family act as a definitive host. This is very important in the parasite's lifecycle and the ultimate release of the infective oocysts into the environment occurs through the feline faeces (Hutchison et al. 1969, 1971). However, in the intermediate host, the infection persists due to the cystic nature of the parasite. Whereby the parasites lodge into tissues and muscles in the form of cysts, which contain bradyzoites, and typically the brain tissues and muscles are commonly affected (Dubey, J.P., Lindsay, D.S. & Speer 1998). Infection of this parasite in the brain is known to cause changes to the immunity and pathology of the host, as described in the early attempts to culture the parasite in brain cells (Fischer et al. 1997; Jones et al. 1986; Parlog et al. 2015). Later, investigations into associations between AD and *Toxoplasma* infection and the use of mouse models led to suggestions that an association may exist (Jung et al. 2012; Kusbeci et al. 2011). Studies on chronic *T. gondii* infections have been associated with AD (Bayani et al. 2019) since the parasite is prevalent globally although this is not universally accepted (Mahami-Oskouei et al. 2016; Perry et al. 2016). There is a general consensus that *T. gondii* infections can be associated with some other neurological conditions such as schizophrenia (Kezai et al. 2020) but not others such as pregnancy and post-partum related depression (Gao et al. 2019). In cases of chronic infections of the brain, this infection can cause alterations to the neural tissue. Thus, parts of the brain that are infected by the parasite for a long time have also been reported to be affected by AD (Berenreiterová et al. 2011). Limited information is available about the relationship between the *T. gondii* infection and AD. This project was, therefore aimed at investigating this broader question using various approaches.

*T. gondii* infects the human body and the common tissues involved include the brain tissues. There is very little data available on the extensive spread of the *T. gondii* parasite in throughout the brain (Di Cristina et al. 2008). In chapter 3, the prevalence of *T. gondii* in the

brains of Alzheimer's disease patients was investigated. Changes in the morphology can be observed using the IHC technique as a tool in the examination of Alzheimer's disease patients and the aim was to see if there was any association with the presence of *T. gondii* cysts. In order to make a case for applying to the Manchester Brain Bank (MBB) for a wider study to investigate this association, and gaining consequent ethical approval, a pilot study was carried out on an existing collection of slides. In this case, sections prepared from the hippocampus and the frontal lobe of human brains collected from Alzheimer's disease patients.

The examination of these samples was done under the light microscope to check for the *T. gondii* cysts. A total of 89 human brain frontal lobe samples and 35 hippocampal samples were used in this study to examine the distribution and prevalence of the *Toxoplasma* parasite in the samples. The investigation found that *T. gondii* cysts were undetectable in the brain samples were negative for *T. gondii* cysts. Sines previous studies have shown that *T. gondii* present in 10% of the UK people, the findings suggest that the volumes for these small samples were too small to be able to be confident that they were representative of the total brain tissue of these patients. Each sample represented 0.063$cm^3$ of brain tissue and, since the total volume of the human brain is approximately 1130-1260 $cm^3$, these samples only constituted 0.0049% of the volume of the human brain. Also, since the tissues were also not prepared specifically for this project, specific *Toxoplasma* staining was not used and, although the slides were very carefully analysed, had this been possible it may have improved detection of small or nascent cysts. The overall conclusions of this work were that convincing pilot data could not be obtained to justify the development of a project involving the MBB. A study which was published concurrently with the start of this study (Burrells et al. 2016) used 3g of brain tissue and sensitive tests like PCR to determine whether patients were infected or not. In order to progress a project like the one that we envisaged – investigation of the association between AD and *T. gondii* infection alongside the spatial investigation of cysts with AD related tissue lesions – the required tissue

amounts would have been unjustifiable with MBB. If this pilot study was repeated, it is proposed that more sensitive techniques like immunohistochemistry and PCR with specific biomarkers be used for the detection of *T. gondii* in these samples. The biomarkers that could be included are anti-BAG1 and anti-SAG1 antibodies (Passeri et al. 2016; Tomita et al. 2017) which detect cysts and tachyzoites. This would need to be conducted alongside a sampling strategy that improved the effective sample size of brain tissue used. As a result of the outcome of this chapter, the direction of the project changed towards using *in vitro* cell culture systems to investigate the effects of *T. gondii* on brain tissue.

In chapter 4, an investigation was carried out on the use of cell culture as a model to describe the effects of infections by *T. gondii* in the brain cells. Three types of cell lines were examined in this particular study. The HFF cell line was used to improve the tissue culture technique and the MDBK cell line was used for the production of *T. gondii* in *vitro*. The SH-SY5Y cell line was included as an *in vitro* model in the study of Alzheimer's disease, as these cells have the ability to differentiate into neuronal cells by treating the SH-SY5Y cells with retinoic acid (RA) (Schneider et al. 2012). Differentiated and undifferentiated SH-SY5Ycells have been widely used in many of neuroscience studies. Thus the use of this specific cell line in AD research is very crucial (Das et al. 2012; Lopes et al. 2010; Schneider et al. 2012; Shipley, Mangold, and Szpara 2017). As discussed in the previous results, the differentiation of this cell line was successfully achieved after implementing two differentiation protocols which included 10 day and 21day protocols. Morphologically, extensive projections of neurites were observed as a characteristic of differentiated SH-SY5Y cells that is comparable to other research (Vesanen et al. 1994; Xun et al. 2012). Many other studies have shown that, during differentiation, there is the expression of key neuronal markers such as MAP2, NF and NES (Cheung et al. 2009; Clelland et al. 2009; Higashi et al. 2015; Schneider et al. 2012; Teppola et al. 2016). In this study, the differentiation of SH-SY5Y cells was confirmed through the use

of immunofluorescent methods and demonstration of the detection of these key biomarkers (NES, MAP2, and NF).

Also in chapter 4, a comparison between two strains of *T. gondii* was also made. The strains used were: Type I strain, RHΔKU8, and type II strain, ME49, respectively. The differences in the growth rate of the *T. gondii* strains in the undifferentiated SH-SY5Y cells have only been determined for these strains in one other previous study with this cell line, to our knowledge (Mammari et al. 2014). The primary purpose of this research was to establish the growth and infection rate for the identification of the differences among the two strains of *T. gondii* and to establish a robust assay system. The growth rate among the undifferentiated SH-SY5Y cells showed that for the type I strain; $2x10^6$ tachyzoites per ml were harvested while the type II strain showed a lower recovery of $1.2x\ 10^6$ tachyzoites per ml at the same time point. It was shown that the type I strain had a faster growth rate compared to type II. The same process was attempted using differentiated cells however there was no outcome obtained. These experiments could not be completed due to technical issues with the cell differentiation on this occasion, lengthy interruption due to the dedicated pathogen incubator failure and finally followed by a closure of the labs due to Covid-19. The infection rate of *T. gondii* strains in undifferentiated SH-SY5Y cells, as shown by the results, illustrated that the type I strain parasites (RHΔKU8) caused infections in 29% of the host cells while the type II strain (ME49) had caused only 7.3% of the host cells to be infected. This shows the faster infection rate in type I compared to type II. This faster growth rate in Type I strains concurred with other previous studies, carried out in different cell lines, that showed that the type I strains multiplied three times faster compared to strain type II and type III (Asis and Grigg 2016; Boothroyd and Grigg 2002; Switaj et al. 2005). Future studies are required to determine the growth rate of *T. gondii* strains in differentiated SH-SY5Y cells. Additionally, future work could be aimed at measurement of infection rate using specific staining, such as immunofluorescence staining,

which is able to detect the presence of *T. gondii* in host cells. The sensitivity of immunofluorescence staining in detecting the *T. gondii* tachyzoites and bradyzoites in host cells and the use of special biomarkers such as surface antigen (SAG) and bradyzoite specific antigen (BAG) that are associated with *T. gondii* has been shown to be very useful in some previous studies (Buchholz et al. 2011; Chang et al. 2015; Rozenfeld et al. 2005; Weiss and Kim 2000; Zhang et al. 2001). Finally, chapter 4 described the development of a method of culturing *T. gondii* in SH-SY5Y cells. There are only three studies, to our knowledge, that describe the interactions between *T. gondii* and SH-SY5Y cells, one of these investigates growth rates (Mammari et al. 2014) and two studies that use *T. gondii* proteins to investigate their specific effect on these cells (Chang et al. 2015; Fan et al. 2016).

In chapter 5, the effect of oxidative stress in SH-SY5Y cells was investigated. The initial part of this chapter shows how the differentiated and undifferentiated SH-SY5Y cells are affected by an oxidative stress reaction using exogenous $H_2O_2$ to generate oxidative stress in the SH-SY5Y cell line. The results that were obtained indicated that there is an increase in the death of undifferentiated SH-SY5Y cells when there is an increase in the $H_2O_2$ doses (150, 250-500µM) within 24-48 hours. This result was demonstrated by the MTT assay and pictures were taken to show cell density and viability. As the $H_2O_2$ doses were increased, it further led to the diminishing of the cell viability and density that has been widely reported in other cell types. The results, described in this study, were in accordance with other recent studies, reported since this study was started, that showed that treatment of SH-SY5Y cells with $H_2O_2$ caused oxidative stress which led to the production of ROS that induced the death of these neural cells by apoptosis (Akki et al. 2018). In our study, the examination of the $H_2O_2$ effects on the differentiated SH-SY5Y cells showed that when higher doses of $H_2O_2$ (250- 500 µM) were used, it resulted in apoptosis of these cells as determined by morphology and the MTT assays. These outcomes also demonstrated that the differentiated cells showed a greater degree

of resistance to the effects of $H_2O_2$ than the undifferentiated cells. These results are in agreement with the work published by (Schneider et al. 2012). In the latter study, it was shown that cell differentiation induced by retinoic acid changes the mitochondrial function in SHSY5Y cells. It has been shown that RA increases mitochondrial membrane potential, levels of cytochrome c oxidase and MnSOD and bioenergetic reserve capacity.

An investigation of the effects of *T. gondii* infections on oxidative stress in SH-SY5Y cells was reported in chapter 6 and 7. This study showed that both *T. gondii* type I and II strains infections gave protection to both differentiated and undifferentiated SH-SY5Y cells from $H_2O_2$ related cell-induced apoptosis. It was also demonstrated that undifferentiated SH-SY5Y cells which were exposed to pre-treatment with conditioned media (CM) of *T. gondii* strains demonstrated a decrease in the effects caused by $H_2O_2$ on the morphology of these cells. Through the findings that were obtained, it was shown that *T. gondii* infections gave protection to the SH-SY5Y cells against $H_2O_2$ induced cell death. Flow cytometry (Chen et al. 2010; Klamt et al. 2009) was used to quantify protection capabilities by the *T. gondii* CM against $H_2O_2$ induced cell death. Treatment with the type I and II strains of *T. gondii* CM showed a decrease in the number of cells that ultimately got fatally affected by the $H_2O_2$ treatment. This study shows that cells that were either exposed to pre-treatment with *T. gondii* or others that were pre-treated with *T. gondii* CM gave outcomes that led to a slow decline of the cell viability and cell death caused by cytotoxicity to hydrogen peroxide. Moreover, the results of this study showed that the *T. gondii* strain type II provides a greater protective effect against $H_2O_2$-induced cell toxicity in undifferentiated SH-SY5Y cells. In contrast, the infection of differentiated SH-SY5Y cells with *T. gondii* strain type I conferred better protection compared with Type II. Therefore, this study indicates that *T. gondii* infections were closely related to the protective effects on the cells against apoptosis, and can be partly attributed to the

immunological alterations that happen during *T. gondii* infection. During the *T. gondii* infections, there is a rapid increase in the number of anti-inflammatory cytokines released, such as IL-10 and TGF-β by the brain tissues. In addition, inflammatory mediators that include nitric oxide (NO) are released in decreased amounts (Blanchard et al. 2015; Jung et al. 2012). Consequently, reactions by anti-inflammatories are shown to prevent tissue damage and mostly assist in the creation of chronic host-parasite balance relations (Jung et al. 2012; Rozenfeld et al. 2005). In addition, there was a study that was performed utilising an Alzheimer's disease murine model indicating that infections caused by *T. gondii* resulted in the prevention of neuronal degeneration. A possible explanation was given in a study performed by (Rozenfeld et al. 2005). The results illustrated that the activation of microglia by interferon IFN-γ impacted the protection of neurons significantly. This happens through alteration of the TGF-β levels, which blocks NO pathway, which led to a decrease in ROS production. As per the study, damage caused by oxidative stress is mostly due to the generating of excess reactive oxygen species (ROS) (Martindale and Holbrook 2002; Valko et al. 2007). Results from these studies show a close association between *T. gondii* infection and the protective influence against oxidative stress. In the process of the infection, induced by *T. gondii*, there is an increase in anti-inflammatory cytokines that act against oxidative stress.

This study is in agreement with previous work (Alvariño et al. 2017; Md et al. 2018; Wang et al. 2013) showing that antioxidants have the ability to scavenge ROS, thus protecting the cells from oxidative stress. This is further elaborated by the neuroprotective abilities resulting in a decrease in the production of ROS. Therefore, an understanding of neuronal cell protection properties can be potentially applied to the development of drugs that can be used for the treatment of diseases related to neurodegeneration as a result of oxidative stress like AD. Generally, this study has suggested that the anti-oxidative effects acquired from infection with the *T. gondii* parasite assisted in the protection of neuronal cells from cellular damage. Also,

these distinct mechanisms may offer important knowledge to assist with a breakthrough in developing drugs that could be specifically aimed at assisting the treatment of patients suffering from Alzheimer's disease.

The protective nature of *T. gondii* infection against oxidative stress in brain tissues also raises the question as to whether *T. gondii* infection offers a selective advantage to infected hosts in an evolutionary context. The opening statements in this thesis (and most *T. gondii* papers) refer to the ubiquitous nature of this parasite – it infects all warm-blooded animals and infects 30% of the global human population. It has many adaptations to transmission, the spread of infections and adaptation to intracellular life in its hosts. Perhaps a further weapon in its armoury involves providing the host with a selective advantage based on protection against oxidative damage?

## Recommendations:

This study has elaborated on the vital part that *T. gondii* infection plays in the protection of neuronal cells against oxidative stress and the resultant cell damage. Therefore, it is recommended that forthcoming studies should focus on the exploration of anti-inflammatory cytokines like TGF-β and IL-0 that might be released from undifferentiated as well as differentiated SH-SY5Y cells infected by the *T. gondii* strains I and II. This could be realized through the application of techniques such as western blot and Immunofluorescence as well as enzyme-linked immunosorbent assay (ELISA). Furthermore, growth experiments using fractionated conditioned media could reveal other factors that might be involved in the protection of oxidative stress in neuronal tissue. SH-SY5Y cell culture systems could be a useful tool for investigating these molecules and for testing new drugs that could improve protection against oxidative stress. Perhaps, ideas for such new drugs could be inspired by a greater understanding of how *T. gondii* manages to mediate protection against oxidative damage.

# 9 Appendix

**Table 9-1** The results of the examination of 124 slides of samples from the parts of the brain tissues, from Frontal lobe which stained by immunohistochemistry staining with antibodies AT8 and Beclin-1, and other samples from hippocampus which stained by immunohistochemistry staining with antibodies AT8.

| **Case No** | **Immunohistochemistry Staining with the previous antibody** | **Brain region** | **Cyst of *Toxoplasma gondii*** |
|---|---|---|---|
| **10/31** | AT8 | frontal lobe | × |
| **09/13** | AT8 | frontal lobe | × |
| **09/37** | AT8 | frontal lobe | × |
| **12/03** | AT8 | frontal lobe | × |
| **11/25** | AT8 | frontal lobe | × |
| **10/04** | AT8 | frontal lobe | × |
| **09/05** | AT8 | frontal lobe | × |
| **09/13** | AT8 | frontal lobe | × |
| **10/10** | AT8 | frontal lobe | × |
| **10/20** | AT8 | frontal lobe | × |
| **10/14** | AT8 | frontal lobe | × |
| **09/27** | AT8 | frontal lobe | × |
| **10/10** | AT8 | frontal lobe | × |
| **10/31** | AT8 | frontal lobe | × |
| **09/24** | AT8 | frontal lobe | × |

| | | | |
|---|---|---|---|
| **09/24** | AT8 | frontal lobe | × |
| **09/05** | AT8 | frontal lobe | × |
| **09/31** | AT8 | frontal lobe | × |
| **10/18** | AT8 | frontal lobe | × |
| **09/22** | AT8 | frontal lobe | × |
| **09/37** | AT8 | frontal lobe | × |
| **11/02** | AT8 | frontal lobe | × |
| **11/06** | AT8 | frontal lobe | × |
| **12/05** | AT8 | frontal lobe | × |
| **12/17** | AT8 | frontal lobe | × |
| **11/22** | AT8 | frontal lobe | × |
| **09/22** | AT8 | frontal lobe | × |
| **10/01** | AT8 | frontal lobe | × |
| **11/28** | AT8 | frontal lobe | × |
| **09/31** | AT8 | frontal lobe | × |
| **11/29** | AT8 | frontal lobe | × |
| **11/09** | AT8 | frontal lobe | × |
| **10/40** | AT8 | frontal lobe | × |
| **11/15** | AT8 | frontal lobe | × |
| **09/22** | AT8 | frontal lobe | × |
| **09/26** | AT8 | frontal lobe | × |
| **11/28** | AT8 | frontal lobe | × |
| **09/31** | AT8 | frontal lobe | × |
| **11/09** | AT8 | frontal lobe | × |

| | | | |
|---|---|---|---|
| **10/31** | AT8 | frontal lobe | × |
| **12/20** | AT8 | frontal lobe | × |
| **09/27** | AT8 | frontal lobe | × |
| **09/26** | AT8 | frontal lobe | × |
| | | | |
| **12/33** | Beclin-1 | frontal lobe | × |
| **09/26** | Beclin-1 | frontal lobe | × |
| **09/13** | Beclin-1 | frontal lobe | × |
| **12/17** | Beclin-1 | frontal lobe | × |
| **10/18** | Beclin-1 | frontal lobe | × |
| **11/15** | Beclin-1 | frontal lobe | × |
| **10/10** | Beclin-1 | frontal lobe | × |
| **09/05** | Beclin-1 | frontal lobe | × |
| **11/28** | Beclin-1 | frontal lobe | × |
| **10/14** | Beclin-1 | frontal lobe | × |
| **14/16** | Beclin-1 | frontal lobe | × |
| **14/09** | Beclin-1 | frontal lobe | × |
| **13/09** | Beclin-1 | frontal lobe | × |
| **11/29** | Beclin-1 | frontal lobe | × |
| **12/03** | Beclin-1 | frontal lobe | × |
| **14/08** | Beclin-1 | frontal lobe | × |
| **13/44** | Beclin-1 | frontal lobe | × |
| **13/39** | Beclin-1 | frontal lobe | × |
| **12/29** | Beclin-1 | frontal lobe | × |

| | | | |
|---|---|---|---|
| **09/37** | Beclin-1 | frontal lobe | × |
| **09/24** | Beclin-1 | frontal lobe | × |
| **12/33** | Beclin-1 | frontal lobe | × |
| **10/20** | Beclin-1 | frontal lobe | × |
| **13/45** | Beclin-1 | frontal lobe | × |
| **13/04** | Beclin-1 | frontal lobe | × |
| **10/04** | Beclin-1 | frontal lobe | × |
| **14/27** | Beclin-1 | frontal lobe | × |
| **10/20** | Beclin-1 | frontal lobe | × |
| **11/22** | Beclin-1 | frontal lobe | × |
| **13/12** | Beclin-1 | frontal lobe | × |
| **14/07** | Beclin-1 | frontal lobe | × |
| **11/06** | Beclin-1 | frontal lobe | × |
| **11/06** | Beclin-1 | frontal lobe | × |
| **14/14** | Beclin-1 | frontal lobe | × |
| **11/28** | Beclin-1 | frontal lobe | × |
| **11/02** | Beclin-1 | frontal lobe | × |
| **14/04** | Beclin-1 | frontal lobe | × |
| **11/25** | Beclin-1 | frontal lobe | × |
| **09/27** | Beclin-1 | frontal lobe | × |
| **14/20** | Beclin-1 | frontal lobe | × |
| **12/05** | Beclin-1 | frontal lobe | × |
| **10/40** | Beclin-1 | frontal lobe | × |
| **09/22** | Beclin-1 | frontal lobe | × |

| | | | |
|---|---|---|---|
| **09/24** | Beclin-1 | frontal lobe | × |
| **11/25** | Beclin-1 | frontal lobe | × |
| **12/34** | Beclin-1 | frontal lobe | × |
| | | | |
| **11/28** | AT8 | hippocampus | × |
| **13/40** | AT8 | hippocampus | × |
| **14/16** | AT8 | hippocampus | × |
| **12/20** | AT8 | hippocampus | × |
| **13/45** | AT8 | hippocampus | × |
| **14/14** | AT8 | hippocampus | × |
| **11/15** | AT8 | hippocampus | × |
| **12/29** | AT8 | hippocampus | × |
| **12/05** | AT8 | hippocampus | × |
| **12/17** | AT8 | hippocampus | × |
| **12/03** | AT8 | hippocampus | × |
| **14/07** | AT8 | hippocampus | × |
| **11/25** | AT8 | hippocampus | × |
| **14/16** | AT8 | hippocampus | × |
| **14/09** | AT8 | hippocampus | × |
| **11/29** | AT8 | hippocampus | × |
| **11/15** | AT8 | hippocampus | × |
| **11/28** | AT8 | hippocampus | × |
| **12/33** | AT8 | hippocampus | × |
| **13/09** | AT8 | hippocampus | × |

| | | | |
|---|---|---|---|
| **13/04** | AT8 | hippocampus | × |
| **14/08** | AT8 | hippocampus | × |
| **14/20** | AT8 | hippocampus | × |
| **09/24** | AT8 | hippocampus | × |
| **14/27** | AT8 | hippocampus | × |
| **13/39** | AT8 | hippocampus | × |
| **10/40** | AT8 | hippocampus | × |
| **10/14** | AT8 | hippocampus | × |
| **10/18** | AT8 | hippocampus | × |
| **14/04** | AT8 | hippocampus | × |
| **09/37** | AT8 | hippocampus | × |
| **09/13** | AT8 | hippocampus | × |
| **10/20** | AT8 | hippocampus | × |
| **14/16** | AT8 | hippocampus | × |
| **12/20** | AT8 | hippocampus | × |

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