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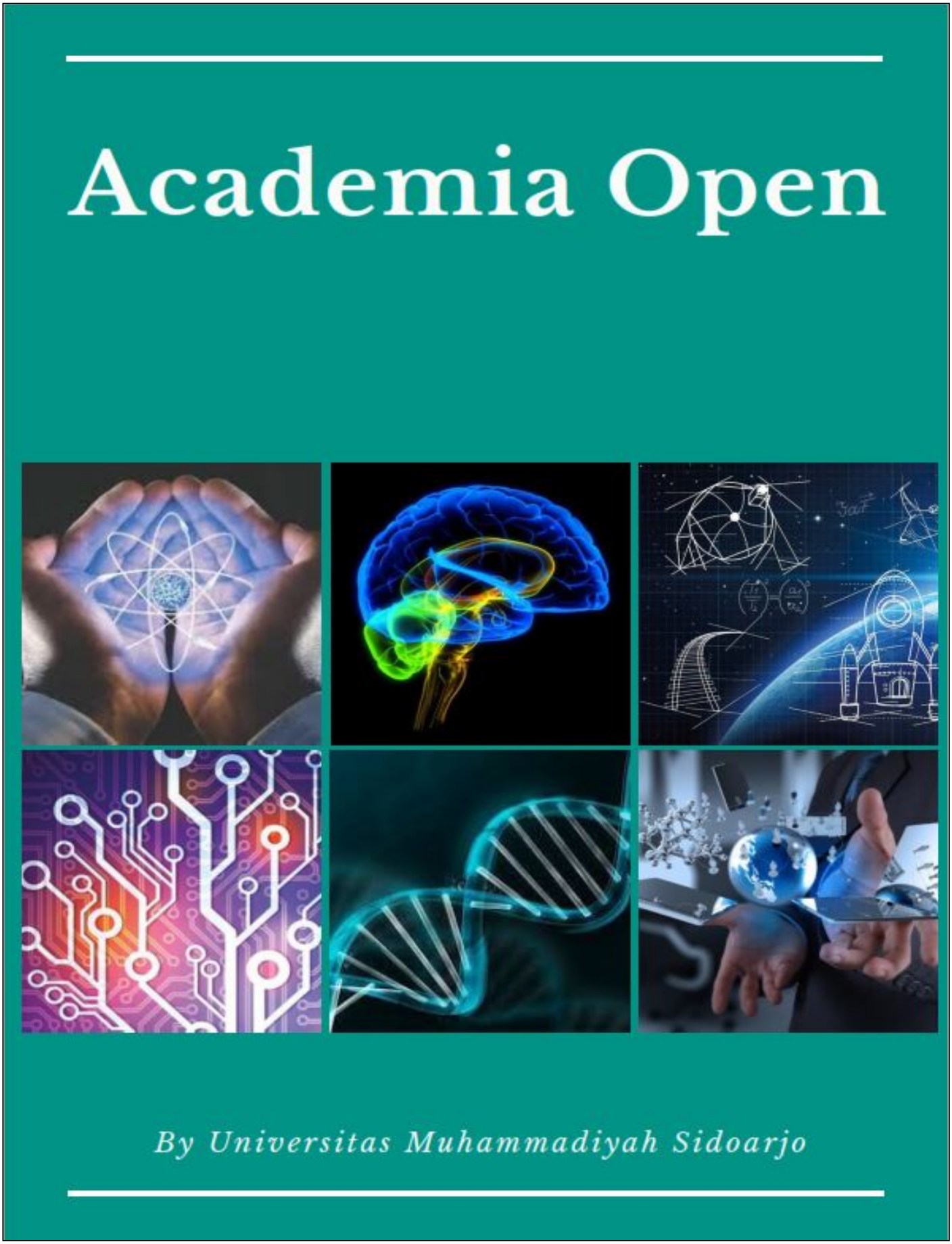


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# Study of Some Apoptotic Protein Expration in Human Mesenchymal Stem Cells During Toxoplasma Gondii Infection

## *Studi Ekspresi Beberapa Protein Apoptosis pada Sel Punca Mesenkim Manusia Selama Infeksi Toxoplasma Gondii*

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### Abstract

**Background:** *Toxoplasma gondii* (T. gondii) infection is a significant health concern, particularly during pregnancy, as it can lead to fetal harm and birth abnormalities.**Specific Background:** The role of apoptosis in managing *T. gondii* infection remains poorly understood, particularly regarding its molecular mechanisms. **Knowledge Gap:** The impact of T. gondii infection on apoptosis in mesenchymal stem cells (MSCs) derived from human umbilical cords has not been thoroughly studied in existing literature. **Aims:** This study aimed to investigate the activation of apoptosis and its regulatory mechanisms in human Wharton's Jelly mesenchymal stem cells (WJ-MSCs) during *T. gondii* infection. **Results:** Using non-enzymatic techniques, we isolated *T. gondii* from infected and aborted phase-specific placentas. Our findings demonstrated a significant increase in the expression of apoptosis-activating genes (CASP2, CASP3, Bak1) in WJ-MSCs following infection, with a marked decrease in cell viability observed within 2 to 4 hours of exposure to the parasite ($P \leq 0.05$). **Novelty:** This study provides novel insights into the relationship between *T. gondii* infection and apoptosis in WJ-MSCs, highlighting the specific gene expression changes that occur in response to infection. **Implications:** Research on T. gondii's apoptotic pathways is crucial for developing therapeutic strategies to mitigate pregnancy-related adverse effects and improve maternal and fetal health outcomes.

**Highlights:**

1. Increased Apoptosis: *T. gondii* enhances apoptosis in mesenchymal stem cells.
2. Cell Viability Impact: Significant decrease in WJ-MSC viability after infection.

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Clinical Relevance: Insights can inform strategies to reduce fetal infection risks.

**Keywords:** Toxoplasma gondii, apoptosis, Wharton's Jelly mesenchymal stem cells, pregnancy, gene expression

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# Introduction

In both warm-blooded animals and humans, toxoplasmosis is a highly prevalent infection worldwide. According to Weiss & Halonen (2013), 1/3 of the world's population has been infected with toxoplasmosis. Theoretically, such parasite can lead to infecting any warm-blooded animal, which include birds and mammals. The only animals which permit sexual reproduction resulting in oocyst excretion in feces are cats, or more broadly, members of the Felidae family. Ingestion of cysts found in oocysts found in vegetables contaminated through cat feces or cysts found in meat might cause infection. After formation of tissue cysts harboring bradyzoites, especially in brain, typically, the infection continues chronically form after an initial period the proliferation and dissemination of tachyzoite (Sullivan et al., 2013). Apoptosis is one of the processes that such parasites target, and most protozoan parasites could lead to the modulation of the host cell response. The mechanism through which cells trigger their destruction is referred to as the apoptosis, or programmed cell death. It was initially explained by Kerr etal. in 1972 and is a crucial path-way for the homeostasis as well as development of the tissues. Apoptosis, unlike necrosis, doesn’t entail an inflammatory response, and the integrity of the membrane is maintained. This event is strictly controlled, and any imbalance might result in diseases. A deficiency in apoptosis could contribute to carcinogenesis or cause autoimmune diseases, whereas excessive apoptosis could result in degenerative diseases. Numerous stimuli, including deprivation of growth factor, exposure to UV rays, and exogenous causes like cancer, could cause apoptosis. We might discriminate between death receptor and mitochondrial pathway signaling based on such signals. Also, a study conducted by Ran and associates reveals that throughout toxoplasmic encephalitis (TE), T. gondii could stimulate apoptosis through endoplasmic reticulum stress (ER) stress. The majority of pro-apoptotic stimuli are linked to an outer mitochondrial membrane permeabilization. Cytochrome C is released to cytosol as a result of this mechanism, which is either indirectly or directly controlled through BH3-only molecules. The proteases that are responsible for executing apoptosis, caspases, are activated by cytochrome c (Hammacher-Brady and Brady, 2015). It is majorly accepted that mesenchymal stem cell in an embryo is a pluripotent progenitor cell which multiplies multiple times and eventually gives rise to skeletal tissues such as tendon, cartilage, ligament, bone, and connective tissue. Those stem cells are not constrained to a certain number of mitotic divisions or regulated by them by definition. Yet, a variety of factors influence their progeny as they follow extremely specific developmental pathways wherein both extrinsic and intrinsic factors work together to control the molecular as well as cellular pattern of expression, leading to the development of particular tissues which carry out particular functions depending on the molecular repertoire (Jiao et al., 2012). For controlling T. gondii infection, apoptosis and autophagy are crucial. Yet, molecular mechanisms of such process are understood rather poorly. T. gondii infection throughout pregnancy could harm the fetus and result in birth abnormalities. In host defense mechanism against invading pathogens, apoptosis and autophagy are both crucial processes. In the activation of the host cells to defend from the intra-cellular pathogens, autophagy serves an anti-microbial and anti-parasitic role. Nevertheless, such pathogens may use host cell's autophagy to facilitate their proliferation. In eukaryotes, apoptosis is necessary for cell growth and to modulate tissue homeostasis in a range of diseases (Sinha et al., 2013). According to reports, T. gondii causes autophagy of the host cells in primary fibroblasts and HeLa cells, and this process aids in the growth of the parasite (Subauste, 2019). Moreover, a recent investigation revealed that T. gondii causes neural stem cells to undergo apoptosis through the ER stress pathway (Zhou etal., 2015). It is unknown, nevertheless, the way that T. gondii causes apoptosis or autophagy in hUC-MSCs, nor what molecular pathways underlie these processes.

The aims of the study

1.Mesenchymal stem cell isolation from the umbilical cords of both non-infected and infected placentas using Wharton's jelly.

2.Examine impacts of T. gondii on mesenchymal stem cells in vitro.

3.Evaluate a few apoptotic genes expression with using Quantitative real-time polymerase chain reaction (qRT-PCR).

# Methods

Study Sample

The research comprised 20 infected samples and 50 healthy samples from the umbilical cord and placenta of T. gondii-infected and uninfected individuals. To determine whether any samples had been infected with the parasite, IgM and IgG Toxoplasma antibodies have been used to detect them. Following receiving formal authorization, samples—both infected and uninfected—were taken from maternity and C-sections at the Maternity and Children Hospital in Al-Ramadi city in coordination with the obstetricians and gynecologists in the hospital and outpatient clinics. The samples of umbilical cord as well as placenta are kept at (20oC) ´ for a maximum of 1-12 hrs in sterile containers that are filled with antibiotics and roughly one liter of normal saline.

WJ of umbilical cord Isolation

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Note: Our research (ISOLATION OF MESENCHYMAL STEM CELLS FROM WHARTON'S JELLY OF HUMAN UMBILICAL CORD. https://connectjournals.com/03896.2021.21.4271) contains a comprehensive account of everything pertaining to stem cell isolation from the human umbilical cord.

Cell culture and T. gondii

Note: Our researches, which are entitled as (Non-Enzymatic Method to Isolation Toxoplasma Gondii from Placental Tissue and Effect on Lipid Peroxidation in Pregnant Women in Al-Ramadi City) (Ridhab et al., 2021) and our research (Isolation Toxoplasma gondii from Placental Tissue and its Effect on malondialdehyde levels in pregnant women in Al-Anbar Province) (Jassim et al., 2023) contain all the information regarding the isolation of the parasite Toxoplasma gondii. The cells have been extracted from the cell culture flask using 0.25% trypsin. The passage surgery has been repeated every two to three days. In MSC cells, T. gondii tachyzoites have been maintained alive. The culture for the MSCs cells was switched to DMEM medium containing 2% lethal bovine serum prior to a 12-hour T. gondii tachyzoite infection. A total of 4 hrs following infection, T. gondii tachyzoites have been introduced to culture medium, which was after that replaced with new fresh DMEM medium (which contain 2% fetal bovine serum). Centrifugation was utilized for harvesting MSCs cells and tachyzoites.

Real-time PCR

A total of 24 hours following transfection, cells were collected. ThermoFisher Scientific RevertAid First Strand cDNA Synthesis Kit (lot No. K-1621, Thermo Fisher Scientific, San Diego, CA, US) was used to generate cDNA after total RNAs were extracted. With the use of ABI7500 equipment (Applied Biosystems, Carlsbad, CA, US) and the SYBR-Green kit (Takara, Tokyo, Japan), quantitative real-time PCR has been performed. The synthetic primers shown in Table1 have been produced by Shenggong Biotechnology, located in Shanghai, China. The 2-ΔΔCt method was employed to examine the data, with the levels of gene expression being standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

| Product size (bp) | Annealing | Sequence of primer | Name of primer |
|---|---|---|---|
| 150 | 59 | GGCCTAGCACTGGTGTTGACTGTGCAGTCTGGTCACATAGA | CASP2 F CASP2 R |
| 158 | 58 | AGTGCTCGCAGCTCATACCT TGAGAATGGGGGAAGAGG | CASP3 F CASP3 R |
| 176 | 57 | AGTCTGGGAATCGATCTGGA CAGCATGATCCTCTGTCAAGTT | BCL2 F BCL2 R |
| 165 | 58 | GTGGCCACAGAGCAACTTCAGCCCAGTTTCCAGGAATG | BAK1 F BAK1 R |

**Table 1.** ***The name of genes and sequence of primers***

Statistical Analyses:

The Statistical Analysis System- SAS (2012) program has been used for the purpose of determining the impact of various components in research parameters. The least significant difference (LSD) test (ANOVA) was utilized in this investigation for significantly comparing the mean values.

# Result and Discussion

Gene Expression

RNA Extraction:

From every sample, total RNA was effectively extracted. RNA concentration varied between 186 and 488 ng/µl. The extraction conditions determine a good yield with a high concentration of total RNA; hence, strict aseptic procedures should be followed.

Gene Expression of Apoptotic genes by RT-PCR

BCL2 group (BCL2, BAK1) as well as the casspase group (cassp3, cassp2) were the two groups of programmed cell death proteins that were examined to quantify level of gene expression in study samples. The study samples have been split into 3 groups: naturally occurring (stem cells infected with T. gondii), in vitro infected (in vitro-infected stem cells), and control (uninfected mesenchymal stem cells). To find expression of Apoptotic gene, RT-PCR was used; samples have been evaluated and compared to the housekeeping β-actin gene expression. The comparative

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threshold cycle (CT) approach (2-ΔΔCt) was utilized to ascertain the relative alterations in the expression levels. SYBR Green qRT-PCR was used for amplification and apoptotic gene identification (Fig 1).

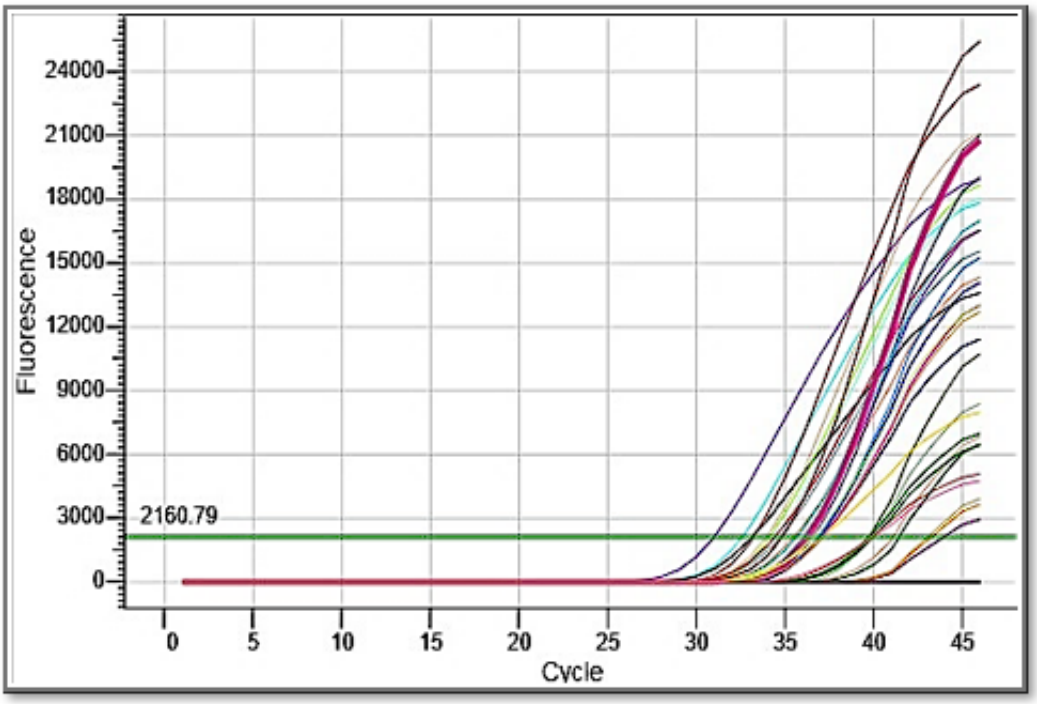


**Figure 1.** *Normalized Real-time PCR amplification curve for housekeeping and apoptotic genes*

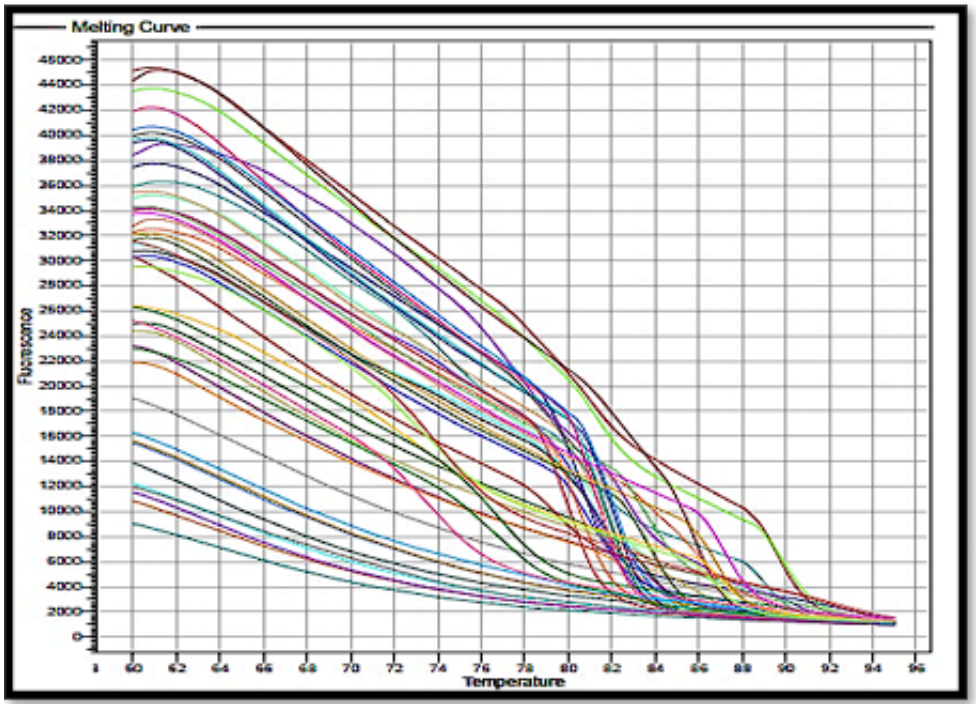


**Figure 2.** *Curve of melting temperature of housekeeping gene*

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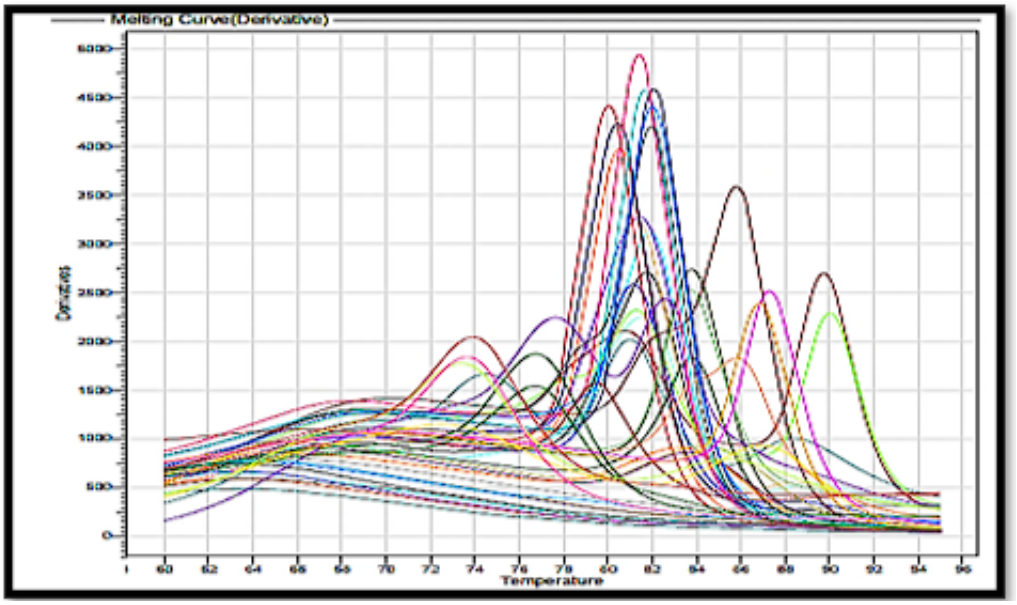


**Figure 3.** *Curve of melting temperature of housekeeping and apoptosis genes*

The present research's findings demonstrated that the sample groups under investigation differed greatly in terms of gene expression levels. These differences are described in more depth below:

BCL-2 family protein

The results of the gene expression of the BCL2 family showed a decrease in the lack of gene expression of bcl-2 in 98% of the study samples, where this gene is considered an anti-apoptosis gene in living cells and the results of the expression of 2% of samples (1.084-2.88) were low compared to the rest of the programmed death genes in study. While 100% of the current study samples showed an increase in the gene expression of Bak1 (78.3- 39.4) of the genes that Pro-apoptotic gene.

Table 2: data of BCL-2 gene expression:

| Sample type | Sample No. | 2(-ΔΔct) | Average |
|---|---|---|---|
| Naturally infected | 1 | 2.082 | 1.084 |
| In vitro infected | 6 | 0.34 | 2.88 |
| | | | |

**Table 2.**

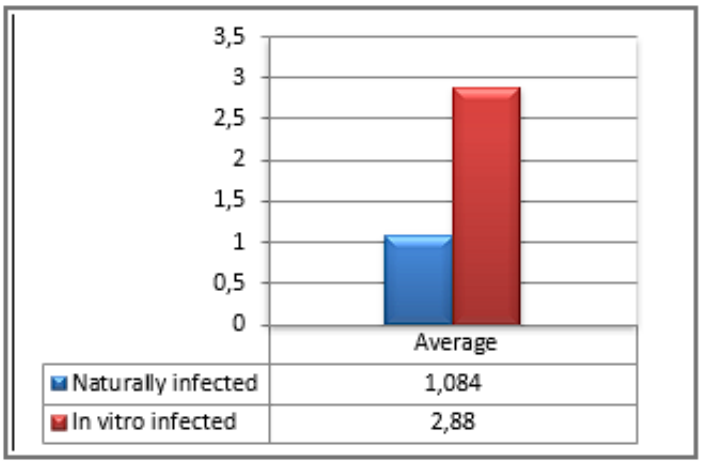


**Figure 4.** *Average of BCL-2gene expression*

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Table 3: data of BAK1 expression:

| Sample type | Sample No. | 2(-ΔΔct) | Fold of expression | Average |
|---|---|---|---|---|
| Naturally infected | 1 | 3.41 | 2.41 | 78.3 |
| | 2 | 0.86 | 1.16 | |
| | 3 | 232.32 | 231.32 | |
| In vitro infected | 4 | 100.43 | 99.43 | 39.4 |
| | 5 | 3.86 | 2.86 | |
| | 6 | 16.91 | 15.91 | |

**Table 3.**

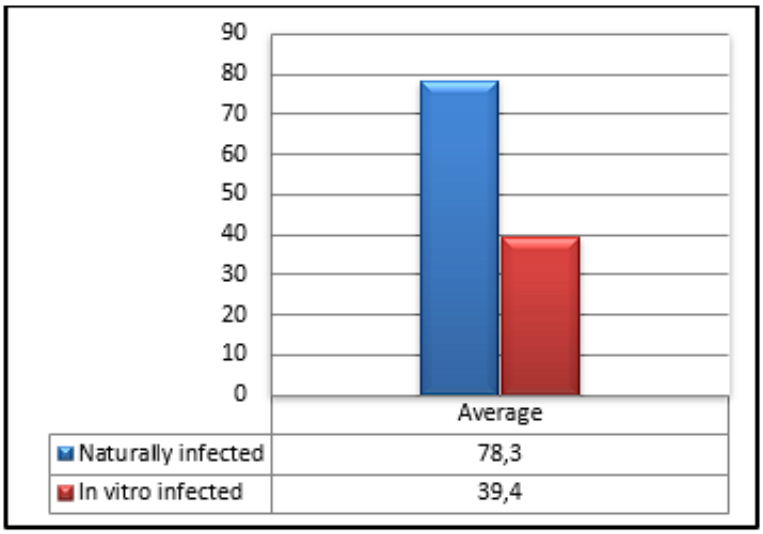


**Figure 5.** *Average of BAK1 gene expression*

Caspases (caspase 2, caspase3)

The results of this research showed increased of gene expression of caspase genes in the occurrence of programmed death in living cells, The gene expression rate for caspase 2 was (1.14, 0.57) in actual infected samples and in vitro infected samples respectively, while the gene expression rate for caspase 3 was (7.12, 7.73) in the study samples respectively as shown in the tables and charts below.

| Sample type | Sample No. | 2(-ΔΔct) | Fold of expression | Average |
|---|---|---|---|---|
| Naturally infected | 1 | 0.88 | 1.14 | 1.14 |
| In vitro infected | 2 | 1.39 | 0.39 | 0.57 |
| | 3 | 0.44 | 2.25 | |

**Table 4.** *data of caspase 2 expression*

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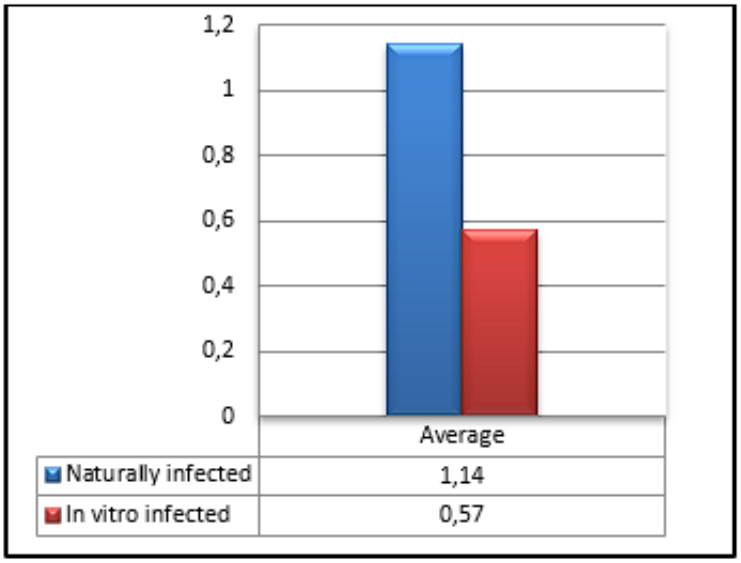


**Figure 6.** *Average of caspase2 gene expression*

| Sample type | Sample No. | 2(-ΔΔct) | Fold of expression | Average |
|---|---|---|---|---|
| Naturally infected | 1 | 6.02 | 5.02 | 7.12 |
| | 2 | 0.14 | 7.36 | |
| | 3 | 9.97 | 8.97 | 7.73 |
| In vitro infected | 4 | 8.88 | 7.88 | |
| | 5 | 0.13 | 7.57 | |

**Table 5.** *data of caspase 3 expression*

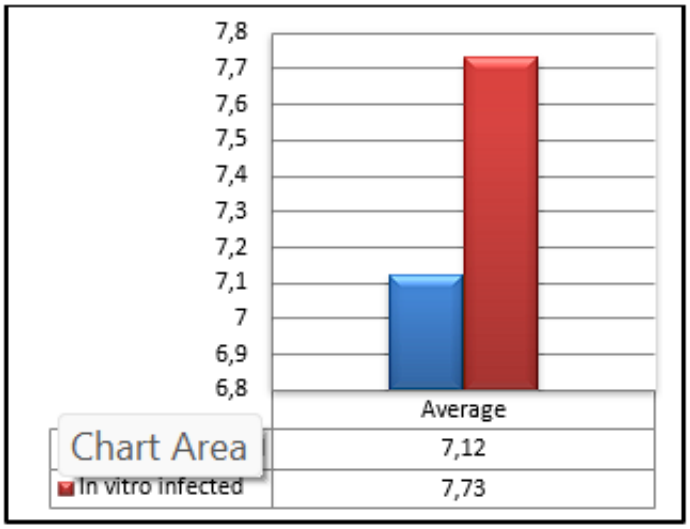


**Figure 7.** *Average of caspase3 gene expression*

DISCUSSION

Pro-apoptotic proteins BCL2-associated X protein (BAX) as well as BCL2 antagonist/killer-1 (BAK), which have a direct role in releasing Cytochrome C from mitochondria, are BCL2 family members (Strasser and Youle, 2008). Some members of BCL2 family, like BCL-XL and BCL2, operate as anti-apoptotic agents by inhibiting such pore-forming proteins, whereas other members, such as BID, BAD, and BIM, take part in the indirect or direct BAK or

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BAX activation. (Strasser and Youle, 2008). (Sinai and Carmen, 2011) have recently examined levels of BCL2 family proteins at different multiplicities of infection (MOI) and in presence of inhibitors for different protease classes in order to further identify requirements for T. gondii dependent degradation of BCL2 family proteins. The authors have been able to identify trends that demonstrate the intricacy of impact of T. gondii infection on stability of BCL2 family members, despite the fact that such trials fundamentally exhibit a considerable deal of variability. Pro-apoptotic proteins BAD, BAX, and BID as well as anti-apoptotic BCL2 were the main subjects of their investigation. whereas after infection, BAK1's transcript level rises. The present study's findings are in line with a recent investigation by Carmen and Sini (2011) on the function of microorganisms, including parasites, particularly T. gondii, which demonstrated the significant impact of the toxoplasmosis parasite on stability of BCL2 family proteins. Nuclear factor- B (NF-B) activation of phosphoinositide 3- (PI3-K), direct activation of apoptotic activity, and activation of Cytochrome C release from mitochondrion are all anti-apoptotic activities linked to T. gondii infection (Carruthers and Laliberte, 2008). The present investigation is incongruent with an earlier study that established a correlation between T. gondii-infected cells and elevated BCL2 transcript levels, inhibition regarding mitochondrial targeting as well as activation of BAX, and selective degradation of proapoptotic proteins BID, BAD, and BAX (Hippe etal., 2009). Between significantly infected and uninfected cells in a morning infaction, there is no variation in the amounts of BCL-2 protein. However, it is possible that T. gondii is amplifying the transcription of BCL-2 to counteract an increase in turnover of the protein in response to infection. Conversely, following a prolonged period of infection, T. gondii infection increases the transcript levels of the pro-apoptotic genes BAD and BID (Molestinna etal., 2003). Parasite-dependent protein degradation is noted for BAX at this time. The authors discover that NF-κB is not dependent on any impacts on the BCL2 proteins' stability through repeating such tests in mutant human cells.

Chromatin condensation, dNA fragmentation, and plasma membrane blabbing are among the classic morphological features of apoptosis that occur in the case where the caspases are activated in cells. They also cleave a broad range of substrates of particular proteins. Since caspase-2 represents the most evolutionarily conserved amongst all caspases, its function in apoptosis is still unknown despite a plethora of literature on the subject (Thompson, 1995). This is because there is considerable disagreement over where exactly caspase-2 fits into the apoptotic cascade, which makes it the orphan of caspase family. Our findings are in line with those of Keller etal. (2006), who verified the high level of caspase through Toxoplasma gondii-induced cytochromec-induced caspase activation in vitro, revealing new methods of interference with the apoptosis of host cells. The activation regarding the caspase cascade as well as cytochrome c release from mitochondria into cytosol might follow (Goebel etal., 2001). Dincel et al. (2015) have recently shown that such virulent strain causes severe neurodegeneration in mice with toxoplasmic encephalitis. At the level of Mesenchymal Toxoplasmosis, there were numerous studies that have demonstrated the role of T. gundii in activating apoptosis. The most recent of such studies is that of (Chu et al., 2017) in China, in which the stimulation regarding the infection of apoptosis in Mesenchymal Toxoplasmosis isolated from Wharton's Jelly, and their findings were fully consistent with our study through the decrease Those findings imply that apoptosis may be caused solely by T. gondii-mediated Mcl-1 down-regulation. Our research aligns with that of Mordue et al. (2001), which verified The high level of apoptosis found in the group study may have been caused by the high virulence of RH strain T. gondii, which is thought to be caused by its great capacity for replication and dissemination as well as its correlation with the induction of extraordinarily high levels of pro-inflammatory cytokines like IL12, IFNγ, TNFα, and IL-18 (Nishikawa et al., 2007). The splenic sections with positive caspase 3 immunostain partially illustrated the apoptotic pathway associated with T. gandii infection. It is important to remember that chromatin breakage and mitochondrial changes occur after caspase activation, leading to cytoplasmic acidification and cell death (Lawen, 2003). According to Alderson et al. (1995), the high levels of proinflammatory cytokines linked to RH strain T. gondii infection were found to induce the expression of death effector molecules like Fas ligand (FasL) and TNF receptor1 (TNFR1). Additionally, evidence of Casp-9 activation throughout acute infection was presented, pointing to the potential involvement of a mitochondrial-induced apoptosis pathway (Liles, 1997).

# Conclusion

Based on results that have been obtained from the present study, it is concluded that:

1.T. gondii affects the viability of cells and increase the apoptosis.

2.Pro-apoptotic and apoptosis genes were found to be more expressed after in vitro infection with T. gondii, while anti-apoptotic gene expression was found to be lower in the infected samples than in the control samples.

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