Influence of Nitrofurantoin on Toll-Like Receptor Pathway in Human Trophoblastic Cells (Bewo) Infected with Toxoplasma Gondii

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Abstract

Toxoplasmosis is a common parasitic disease caused by the obligatory intracellular *Toxoplasma gondii*. To investigate the effect of nitrofurantoin (NF) on the signaling pathway of TLR (Toll-Like Receptor) in BeWo, a human trophoblastic cell line, infected with *T. gondii*. Primarily, to determine the concentration of NF, giemsa staining method, trypan blue staining method and MTS (3-(4,5-dimetiltiyazol-2-yl)-5-(3-karboksimetoksifenil)-2-(4-sülfofenil)-2H-tetrazolyum) test were used on negative-positive controls and experimental groups. After MTS measurement, IC50 (half maximal inhibitory concentration) and EC50 (half maximal effective concentration) values and the appropriate concentration as 1.25 µM were calculated using GraphPad Prism 8.0. In order to determine the effect of NF on the TLR pathway, samples were collected at three different times: 24, 48 and 72 hours. After RNA isolation, cDNA was synthesized and Real-Time PCR (RT-qPCR) analyzes were performed. Proportional changes in gene expression were calculated using reference genes. According to this, the most effective time period of the drug was found to be 48 hour period based on the decrease in expression levels in some apoptosis-related genes and, TLR6 (Toll-like receptor 6) and TLR8 (Toll-like receptor 8). There were significant differences after 48 ($p = 0.015$) and 72 ($p = 0.049$) hours in the parasitic load at experimental groups. As a result, it was shown that NF inhibited *T. gondii* infection by affecting genes in the TLR pathway. Furthermore, to know that these knowledges has shown the possibility that new preparations with less side effects can be developed as an alternative method in the treatment of the disease.

Introduction

Toxoplasmosis is a common disease caused by the obligate intracellular protozoan parasite *Toxoplasma gondii (T. gondii). T. gondii* is an apicomplexant parasite that can infect all warm-blooded vertebrates (Blume and Seeber 2018; Montazeri et al 2018a). It has been reported that about a third of the people in the world are chronically infected with *T. gondii* (Kato 2018). The seroprevalence of toxoplasmosis varies from 1% in some countries in the Far East and up to 90% in some parts of Europe and South America (Fallahi et al 2018). Toxoplasmosis is transmitted to humans by the intake of tissue cysts from raw or undercooked meat and / or by consumption of food and drink contaminated with oocysts (Montazeri et al 2018a) and also infection is caused by congenital transmission, organ transplantation, blood transfusion (Blume and Seeber 2018; Montazeri et al 2018a).

Congenital toxoplasmosis causes serious clinical symptoms in the prenatal and postnatal process (Pittman et al 2014; Wujcicka et al 2014; Lau et al 2016). In the postpartum period, *T. gondii* infection can occur with a wide variety of clinical symptoms such as neonatal malformations and may cause blindness, chorioretinitis, mental retardation, permanent neurological damage, and tissue and organ disorders (Chaudhry et al 2014). It carries out the clinical consequences it causes by immune mechanisms such as signaling mechanisms that depend on activated Toll-like receptors (TLR). Cytokines and chemokines are secreted by immune system cells such as macrophages, dendritic cells and neutrophils through TLR-dependent signaling mechanisms in the chronic and acute phase of the infection. TLR are protein structures that play a key role in the first encounter of the innate immune

system with pathogens. Through these receptors, innate immunity recognizes the structure of pathogens and initiates the inflammatory response. These receptors also act as a bridge between the innate and acquired immune system (Gazzinelli et al 1993; Kundakcı & Pirat 2012).

Currently, there is not much information about these adapter molecules and their pathways in congenital contamination, but especially drugs to be used in the treatment of toxoplasmosis are desired to have high efficacy and low toxicity. Therefore, it is necessary to determine the toxicity of the drugs to be used and to investigate the signalling pathways that are vital during the formation of the immune response.

It will be useful to know the molecules in the TLR pathway stimulated by the effective dosage of Nitrofurantoin (NF), which can be used in pregnancy, to combat toxoplasmosis, so it was hypothesized that new preparations acting through these molecules can be developed as an alternative method in the treatment of the disease with less side effects and wide areas of use. Within the scope of this hypothesis, it was aimed to investigate the effect of NF on the TLR signaling pathway in *T. gondii* infected BeWo cell line, which is a human trophoblastic choriocarcinoma cell line.

Materials And Methods

Infection of cell line and preparation of drug solutions

In the study, the effect of NF (Sigma Aldrich, USA), a commercial preparation, was investigated by infecting BeWo Cell Line (ATTC, USA) with *T. gondii* tachyzoids obtained from Ankara Public Health Laboratory. The tachyzoites and cells were kept at -80 ° C until the test period. 1 M stock solution was prepared using NF dissolved in DMSO (BioShop, Burlington, USA), and kept at room temperature for use in the experiment. Cell culture was carried out in DMEM-HAM's / F12 medium (Biochrom, Germany) medium containing 10% inactivated FBS (Capricorn Sciencetific, South America), 1% penicillin-streptomycin (Capricorn Sciencetific, South America) and 1% L-glutamine (Biochrom, Germany) solution. BeWo cells were transferred into a 25 cm^2 flask with 4 ml of medium and incubated in an incubator with 5% CO_2 at 37 ^{0}C. After the cells covered 80–100% of the flask surface, they were treated with Trypsin-EDTA (Sigma, USA) and passaged. Cells were examined every other day under an inverted microscope. Proliferated cells were inoculated into 6-well plates for use in morphology assay with giemsa staining and cell viability test with Trypan blue method, and inoculated into 96-well plates for MTS test. The tachyzoites were counted on the thoma slide and made ready for the infection step of the experiment, and the cells covering the flask surface were infected at the ratio of 1 tachyzoite per 1 cell (1: 1). Drug solutions were prepared from the stock solution at concentrations of 320 µM, 160 µM, 80 µM, 40 µM, 20 µM, 10 µM, 5 µM, 2.5 µM and 1.25 µM. These solutions were added to the infected cells into individual wells and morphology assays with giemsa staining, cell viability tests using MTS and trypan blue methods were performed.

Morphology analysis with giemsa staining

BeWo cells planted in six-well culture plates were infected after 24–48 hours after reaching the confluency, and NF at the specified concentrations was added to the wells and incubated for 24 hours at 37 ^{0}C, 5% CO_2 in an incubator. After the incubation, the cells were fixed with methanol and then treated with giemsa dye for 45 minutes. After staining, the cells were examined under an inverted microscope.

Cell viability test with MTS

BeWo cells were inoculated into 96-well culture plates for MTS testing, after 24–48 hours, all wells were infected with tachyzoites except those belonging to the control group containing only BeWo cells. 20 µl of MTS (CellTiter 96® AQueous One Solution Reagent) reagent was added to each well of the culture plate containing drug groups and control groups, and incubated for 1–4 hours at 37 ^{0}C, 5% CO_2 in an incubator. The absorbance of the samples was measured at 490 nm in an ELISA reader. IC_{50}, EC_{50} and R^2 values were determined using the GraphPad Prism 8.0 statistical program.

Cell Viability Test With Trypan Blue Staining

BeWo cells grown in 25 cm^2 flasks were planted in 6-well culture plates. After 24–48 hours, NF in determined concentrations and tachyzoites (1:1 cell / tachyzoite ratio) were added to the cells covering the surface and incubated for 24 hours at 37^{0}C in a 5% CO_2 incubator. To determine the number of BeWo cells and tachyzoites, 100 µl trypan blue dye was added to 100 µl cell-medium mixture and counted with thoma slide under microscope. The numbers of dead or live cells and tachyzoites were determined.

Parasite load in cell culture, RNA isolation and cDNA synthesis

Samples were collected at 24, 48 and 72 hours from infected BeWo cells (at a rate of 1 tachyzoite / 1 cell) incubated until the confluency in an incubator with 5% CO_2 in six-well culture plates at 37 ℃ to measure the efficacy of the drug concentration determined after morphology and cell viability tests. 500 µl of TRI reagent was added to the collected samples and transferred to tubes for RNA isolation.

The TRI reagent protocol developed by Chomczynski (2006) was modified and RNA isolation was performed. The amount of RNA was measured in the nanodrop device and stored at -80 ℃ until cDNA (complementary DNA) synthesis. A commercial kit (Roche-Transcriptor High Fidelity cDNA Syntesis Kit, USA) was used for cDNA synthesis. Total RNA in the amount of 1 pg-1 µg at final concentration, RNase, DNase free dH2O, 60 µM Random Hexamer and OligodT primers, 1x buffer, RNase inhibitor, dNTP mix (0.1 nmol), DTT and reverse transcriptase (1 U / µl) enzyme containing RT-qPCR mix was prepared and incubated on ice for 5 minutes. 2 µl of enzyme was added to the mixture and after the total volume was completed to 20 µl, it was incubated in the PCR device for 15 minutes at 42 ℃, 5 minutes at 85 ℃ and 15 minutes at 65 ℃. The cDNAs obtained were stored at -20 ℃ for use in RT-qPCR reaction.

Calculation Of Parasite Loads And Tlr Gene Expressions By Rt-qpcr

'House keeping' genes, was obtained commercially and PCR Array analysis was performed (Table 1). For RT-qPCR, 1100 µl mixture was prepared with 550 µl Syber Green mix, 440 µl dH2O and 20 µl cDNA. The mixture was dispensed into a 96-well plate, in 9 µl in each well. A total reaction of 10 µl was prepared by taking 1 µl of primer from each well of the TLR panel and adding it to the wells respectively. RT-qPCR was performed using B1 gene specific primers (F: 3'-CCCACCACGCAGAATCAT-5 'and R: 3'-CCCACCACGCAGAATCAT-5') using cDNAs obtained from samples collected at 24, 48 and 72 hours to calculate parasite loads and plasmid DNA standards were used as positive controls. RT-qPCR program was applied using Roche LightCycler 480II device, Cq (Quantification cycle) values for genes were calculated and relative quantification was made.

Table 1
List of TLR pathway genes whose expression levels were calculated by RT-QPCR

Genes	Definition of abbreviation
AKT3	V-akt murine thymona viral oncogene homolog3
CXCL12	Chemokine(C-X-Cmotif)ligand12
PIK3CB	Phosphatidylinositol-4,5-bisphosphate3-kinase, cataytic subunit beta
PIK3CD	Phosphatidylinositol-4,5-bisphosphate3-kinase, cataytic subunit delta
PIK3R5	Phosphoinositide-3-kinase, regulatory subnit 5
STAT1	Signal transducer and activator of transcription 1
CD40	CD40 molecule, TNF receptor superfamily member 5
IKBKE	Inhibitor of kappa light polypeptide gene e0nhancer in B-cells, kinase epsilon
IL1β	Interleukin 1, beta
MAPK2	Mitogen-avtivated protein kinase kinase 2
MAPK3	Mitogen-activated protein kinase 3
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells2(p49/p100)
TLR1	Toll-like receptor 1
TLR7	Toll-like receptor 7
AKT1	V-akt murine thymona viral oncogene homolog1
AKT2	V-akt murine thymona viral oncogene homolog2
CASP8	Caspase 8, apoptosis-related cysteine peptidase
CCL3	Chemokine(C-C motif) ligand 3
CCL5	Chemokine(C-C motif) ligand 5
CCL4	Chemokine(C-C motif) ligand 4
CD14	CD14 molecule
CD80	CD 80 molecule
CD86	CD86 molecule
CHUK	Conserved helix-loop-helix ubiquitous kinase
CXCL10	Chemokine(C-X-Cmotif)ligand10
CXCL11	Chemokine(C-X-Cmotif)ligand11
FADD	Fas (TNFRSF6)- asociated via death domain

Genes	Definition of abbreviation
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
IFNA7	Interferon, alpha7
IFNaR5	IFNAR 1 Interferon (alpha, beta and omega) receptor 5
IFNAR5	Interrferon (alpha, beta, and omega) receptor5
IFNB1	I Interferon, beta1, fibroblast
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma
IL12A	Interleukin 12A
IL12B	Interleukin 12B
IL6	Interleukin 6(ınterferon, beta2)
IL8	Interleukin 8
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK4	Interleukin-1 receptor-associated kinase4
IRF3	Interferon regulatory factor 3
IRF5	Interferon regulatory factor 5
IRF7	Interferon regulatory factor 7
JUN	Jun oncogene
LBP	Lipopolysaccharide binding protein
LY96	Lymphocyte antigen 96
MAP2K1	Mitogen-avtivated protein kinase kinase 1
MAPK3	Mitogen-avtivated protein kinase kinase 3
MAP2K4	Mitogen-avtivated protein kinase kinase 4
MAP2K5	Mitogen-avtivated protein kinase kinase 5
MAP2K7	Mitogen-avtivated protein kinase kinase 7
MAP3K7	Mitogen-avtivated protein kinase kinase kinase 7
MAP3K8	Mitogen-avtivated protein kinase kinase kinase 8
MAPK1	Mitogen-activated protein kinase 1
MAPK10	Mitogen-activated protein kinase 10

Genes	Definition of abbreviation
MAPK9	Mitogen-activated protein kinase 9
MAPK13	Mitogen-activated protein kinase 13
MAPK14	Mitogen-activated protein kinase 14
MYD88	Myeloid differentiation primary response gene (88)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NFKBIB	NFKBIA Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitör, beta
PIK3CA	Phosphatidylinositol-4,5-bisphosphate3-kinase, cataytic subunit alpha
PIK3CG	Phosphatidylinositol-4,5-bisphosphate3-kinase, cataytic subunit gamma
PIK3R1	Phosphoinositide-3-kinase, regulatory subnit 1(alpha)
PIK3R2	Phosphoinositide-3-kinase, regulatory subnit 2(beta)
PIK3R3	Phosphoinositide-3-kinase, regulatory subnit 3(gamma)
RAC1	RAS- related C3 botilinum substrate 1
RELA	V-rel reticuloendotheliosis viral oncogene homolog A(avain)
RIPK1	Receptör- interacting serine- threonine kinase1
SPP1	Secreted phosphoprotein 1
Table 1	TGF-beta activated kinase 1/MAP3K7 binding protein 1
Table 2 protein2	TGF-beta activated kinase 1/MAP3K7 binding protein 2
TBK1	TANK-binding kinase 1
TICAM1	Toll-like receptor adaptör molecule 1
TICAM2	Toll-like receptor adaptör molecule 2
TIRAP	Toll-interleukin 1 receptor(TIR) domain containing adaptör protein
TLR10	Toll-like receptor 10
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TLR6	Toll-like receptor 6

Genes	Definition of abbreviation
TLR8	Toll-like receptor 8
TLR9	Toll-like receptor 9
TNF	Tumor necrosis factor(TNF superfamıly, member 2)
TOLLIP	Toll interacting protein
TRAF4	TNF receptor-associated factor 4
TRAF1	TRAF6 TNF receptor- associatedfactor 1
ACTB	Actin, beta
B2M	Beta-2-microglobulin
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
GAPD	Glyceraldehyde-3-phosphate dehydrogenase
GUSB	Glucuronidase, beta
PGK	Phosphoglycerate kinase 1
PPIA	Peptidylproly isomerase A
RPL13A	Ribosomal protein L 13a

Statistical analysis

Relative quantification was made with reference to GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and B2M (Beta-2-microglobulin) genes from house keeping genes that constitute the in-experiment control groups, and the change in expression levels of genes related to TLR pathways was calculated. In addition, the parasite loads of samples collected at 24th, 48th and 72nd hours using plasmid DNA standards as positive controls were determined by the Kruskal Wallis H-test and the Mann Whitney U-test at $p < 0.05$ significance level using IBM SPSS Version 16.0 package program.

Results

Evaluation of morphology test results using giemsa staining method

In the morphology experiment performed using the Giemsa staining method, it was observed that 1.25 µM NF concentration was the best dosage as a result of the comparison of the negative control (only BeWo cells) and the positive control (infected BeWo cells with no drug) and the experimental groups (infected BeWo cells with added drug). While BeWo cells treated with a concentration of 1.25 µM

consisted of a large number of viable cells with normal morphology attached to the surface with similar characteristics to the control groups, it was observed that the structures of a small number of tachyzoites were morphologically larger (Fig. 1).

Evaluation of cell viability using trypan blue test

In the cell viability experiment performed using trypan blue staining method, it was observed that 1.25 µM NF concentration was the best dosage as a result of the comparison of the negative control and positive control groups and the experimental groups. While there were more living BeWo cells with normal morphology covering the whole surface at 1.25 µM final concentration, it was observed that there was a decreasing number of tachyzoites in the well to which 1.25 µM NF was added compared to the positive control group, but there were more tachyzoites compared to higher concentrations (Fig. 2).

Evaluation of Cell Viability Using MTS Test

With the MTS test, it was observed that the dose of 1.240 µM drug concentration, which is the toxic dose for tachyzoites with the highest cellular viability, gave similar results with the 1.25 µM dosage determined by both giemsa and trypan blue staining methods (Fig. 3).

Once the appropriate dosage was found, BeWo cells were planted in 6-well plates and a cell culture setting containing negative control, positive control and experimental groups was formed again. Later, cells were observed under an inverted microscope, and samples were collected from each group at three different times: at 24th hour, 48th hour and 72nd hour. In order to determine the effect of NF on the TLR pathway, RNA isolation was performed from the samples to perform Real-Time PCR (RT-qPCR) analysis, and then cDNA (complementary DNA) was synthesized.

Parasite Load in Infected Cell Culture

To be used as a standard, undiluted DNA (0.0175 µg / µl) and 7 serial dilutions were prepared by diluting 10 times from this DNA to be used with both primers and copy / ml ratios were determined (Table 2). The standard curve was constructed according to the copy numbers obtained from the serial dilutions (Fig. 4 and Fig. 5). The positivity rates of the experimental groups were determined in line with the data obtained.

Table 2
B1 gene serial dilution rates.

Dilution	B1 primer	
	Cq value	DNA (µg/µl)
1:1	12.90	$175x10^{-1}$
1:10	13.68	$175x10^{-2}$
1:100	14.85	$175x10^{-3}$
1:1000	15.60	$175x10^{-4}$
1:10000	16.32	$175x10^{-5}$
1:100000	17.19	$175x10^{-6}$
1:1000000	18.09	$175x10^{-7}$

In the experimental groups, the change in parasite loads at 24, 48 and 72 hours was examined (Fig. 6), and while no significant difference was observed after 24 hours, it was determined by RT-qPCR that the parasite load significantly decreased in samples taken from cell cultures after 48 and 72 hours (Table 3).

Table 3
Kruskal Wallis H-test result of parasite loads after 24,48 and 72 hours.

Experimental Groups	n*	average rank	sd*	X^2	p
24.saat	4	4.75	2	4.902	0.086
48.saat	4	7.25	2	8.375	0.015
72.saat	4	6.75	2	6.038	0.049
n: mean, sd: standard deviation, X^2: chi-square, p: significance					

Evaluation of TLR Gene Expression Levels

Genes with changes in expression level include immune modulator genes, oncogenes, and apoptosis genes, which also participate in the TLR pathway. The expression levels of these genes in the cells in the experimental group compared to the control groups were determined. Accordingly, changes were observed in CCL5, CD40, FADD, MAP2K2, MAP2K3, MAPK3, TLR1 and TOLLIP genes in cells in the experimental group compared to the positive control groups at 24 hours. At 48 hours, changes were observed in CCL3, CD14, CXCL11, FOS, IFNaR5, IKBKB, MAP2K4, MAPK13, MAPK14, PIK3CB, PIK3R1, RELA, SPP1, TBK1, TLR6 and TLR8 genes in cells in the experimental group compared to the positive

control groups. At 72 hours, changes were observed in CCL5, FOS, IFNa7, IKBKB, IL12b, JUN, MAP2K1, MAPK14, MAPK3, RAC1 and STAT1 genes in cells in the experimental group compared to the positive control groups. When compared with the positive and negative control groups, the genes with a change in the expression level at one of the 24th, 48th and 72nd hour time points in the experimental groups were CHUK, FADD, IKBKB, IL12b, IL6, MAP2K1, MAP2K2, MAPK1, MAPK9, MAPK3, MYD88, NFKb1, RAC1. SPP1, STAT1, TLR6, and TLR8. It was found that expression levels of the CHUK, FADD, IKBKB, IL12b, IL6, MAP2K1, MAP2K2, MAPK9, MAPK3, MYD88, NFKb1, RAC1 and STAT1 genes gradually decreased from the 24th to the 72nd hour (Figs. 7–9).

Discussion

Congenital toxoplasmosis causes serious clinical symptoms in the period from fetus to adulthood and poses a great problem for human health. Understanding the changes occurring through the *T. gondii* infection in the placenta-blood barrier is important for prevention of congenital toxoplasmosis. Previous studies indicated that placental trophoblasts directly play a role in the pathogenesis of toxoplasmosis (Franco et al 2011). The role of trophoblast cells in the infection with intracellular parasites, and thus pregnancy immunology, was explored using human choriocarcinoma cells such as BeWo cell line (Franco et al 2011).

It has been reported that the high sensitivity of BeWo cells to *T. gondii* is related with immunomodulation mechanisms and trophoblast cells facilitate infection in placental tissues (Barbosa et al 2014; Carvalho et al 2010). *Toxoplasma gondii* has developed various strategies to successfully circumvent and manipulate the immune system and to infect host cells (Lima and Lodoen 2019). In the current study, BeWo cell line was used to create a placental microenvironment and infected with *T. gondii* tachyzoids. The changes in the TLR pathway were investigated when the infection was suppressed with the addition of NF. With a large number of different recognition molecules, TLRs are involved in the detection of a variety of microbial molecules, including carbohydrates, lipids, nucleic acids, and proteins. (Yarovinsky et al 2006). Recent experimental studies have shown drug resistance in toxoplasmosis. Thus understanding the mechanisms of drug resistance in *T. gondii* plays an important role in controlling the disease, especially among patients with congenital toxoplasmosis or immunocompromised patients (Montazeri et al 2018b). In this context, identifying host or parasite targets, such as the TLR pathway, allows to choice of drug or drug combinations to be used against toxoplasmosis. In addition, commonly used preparations can cause side effects including neutropenia, leukopenia, thrombocytopenia and hypersensitivity reactions (Kaye 2011; Montoya and Remington 2008; Montazeri et al 2018b). How the mechanisms and receptor pathways related to drug-pathogen interaction cause these side effects and how they affect trophoblast cells need further investigation.

Yeo et al (2016), investigated the in vitro and in vivo anti-*T. gondii* effects of NF and pyrimethamineusing the concentrations of 5, 10, 20 and 40 µM. and calculated EC_{50} value of NF against *T. gondii* and HeLa cells as 14.7 µM and 33.1 µM, respectively. The researchers (Yeo et al 2016) highligted that NF is a potential anti-Toxoplasmosis preparation and can be used clinically. Our results results areparallel to the

findings of Yeo et al., 2016 and support to the conclusion that NF can be used as an anti-Toxoplasmosis preparation. We determined that NF dissolves better at lower concentrations in the medium and is effective on tachyzoites. Lee et al (2008) reported that, the production of IL-8 was induced by NF κβ - activation via TLR-2 and TLR-4 expressions after stimulation with *T.gondii* lysate or LPS, in HEK-293 cells immunosuppressed by transfecting anti-TLR2 and anti-TLR4. In another experimental toxoplasmosis model, the existence of a non-lymphoid IFN-γ source, which is evidence of TLR-mediated *T. gondii* recognition, has been demonstrated (Sturge et al 2013). In a study by Salazar Gonzalez et al (2014), a multiple sequence alignment phylogenetic analysis program was used to compare human and mouse species and it was stated that human TLR5 is an evolutionarily close member in the TLR gene family related to the mouse TLR11. In the same study, IL-6, IL-8 and IL-12p70 response of human TLR5 receptor to *T. gondii*-profilin-like ligand was also investigated. As a result, it has been observed that there is the production of cytokines both in the human cell line and in peripheral blood monocytes. It has also been indicated that peripheral blood monocytes carrying the R392X mutation on the TLR5 gene cannot produce cytokines in response to stimulation with the *T. gondii*-profilin (Salazar Gonzalez et al 2014). In another study (Del Rio et al 2004), *T. gondii* is thought to trigger the production of neutrophil IL-12 and chemokine ligand 2 (CCL2; monocyte chemoattractant protein 1) by tightly binding to functional MyD88. In addition, TLR2 is defined as a receptor that triggers CCL2 production, but it is stated that the parasite-dependent IL-12 production is not TLR dependent. It has been reported that the production of IL-12 and CCL2 occurs after neutrophil activation by IFN-γ. In addition, it has been stated that the synergistic effect of IFN- γ on IL-12 is not dependent on CCL2, but is dependent on STAT1 signal transduction (Del Rio et al 2004). Recent studies show that TRAF 6, an adapter molecule, plays a role in TLR signaling pathways in the immune response against *T. gondii* infection. It has been reported that in this pathway it binds to serine / threonine kinases involved in the activation of both NF-κβ and mitogen-activated protein kinase (MAPK).

In the current study, post-RT-qPCR analyzes revealed that the gene expression levels of (a) MYD88 and FADD molecules, which are mediator molecules in all apoptatic pathways; (b) MAP2K1, MAP2K2, MAPK9, MAPK3 molecules that are members of the MAP-kinase gene family and activated in apoptosis due to mitochondrial damage; (c) IL-12b, IL-6 molecules, which are cytokines that trigger phagocytosis by stimulating macrophages after inflammation; (d) STAT1 molecule, whose level increases especially after *T. gondii* infection and provides activation of pro-apoptotic genes by stimulation of intrinsic factors in the cell; (e) CHUK, RAC1, IKBKB molecules, which are in the intermediate steps of TNF-mediated cellular death, significantly changed in cells treated with NF. While a gradual decrease observed in the expression levels of these genes from the 24th to the 72nd hours, the most effective time zone of the drug was the 48th hour due to the decrease in the expression level observed in the MAPK1, SPP1, TLR6 and TLR8 genes. Therefore, it was determined that the apoptotic process initiated by *T. gondii* tachyzoids with cellular recognition by TLRs after infection was inhibited after treatment with NF. Thus, it has been shown that NF inhibits the infection by affecting the TLR pathway activated after infection with *T. gondii* and the affected genes in the pathway were determined. In addition, it has been statistically shown that NF has a significant lethal effect on tachyzoites in cell culture after 24 hours.

Consequently, unique data on TLRs and other pathway-related molecules that play a role in the specific immune response that develops after the interaction of NF with *T. gondii*-infected BeWo cells in in vitro culture medium has been obtained with our study. We also demonstrated that NF, known to have little toxic effect on the liver, can be used as an effective treatment option in the treatment of toxoplasmosis. The determination of the molecules stimulated by the effective dosage of NF might constitute a model for the studies to be carried out on the development of new preparations in the treatment of toxoplasmosis with less side effects.

Declarations

Author contribution ES determined the subject of the article together with AI. ES, MY worked together in all steps of the study and created all the tables and figures of the article. TA assisted in the preparation of drug concentrations in laboratory work. AY, AI assisted the in organizing the entire article, especially the material method, in form and content and analysed the data. All authors reviewed the manuscript.

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Data availability All data presented here are available upon request.

Ethics approval This study does not require an ethics committee decision.

Consent to participate All authors give their consent to participate in this article. Consent for publication All authors give their consent for the publication of this article.

Conflict of interest The authors declare no competing interests.

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Figures

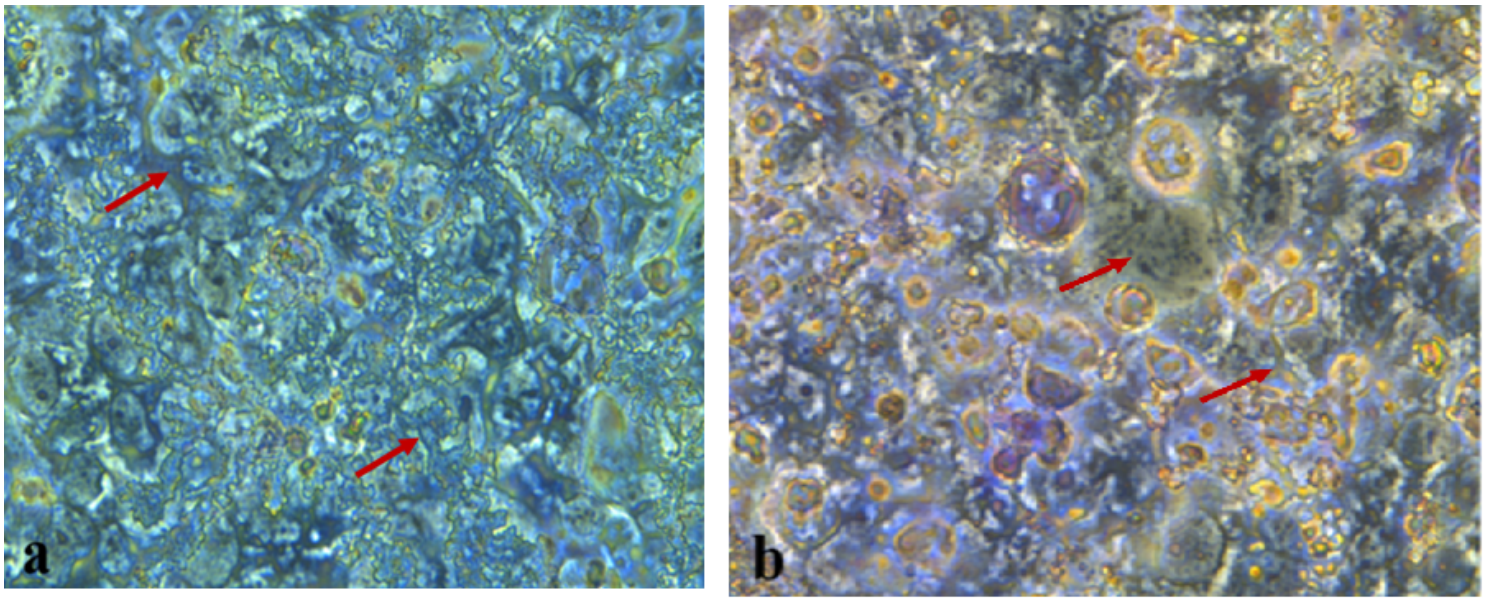

Figure 1

Inverted microscope images of the morphology experiment performed with the giemsa staining method (x40); **a)** Negative control group **b)** Pozitive control group **c)**1,25 µM NF.

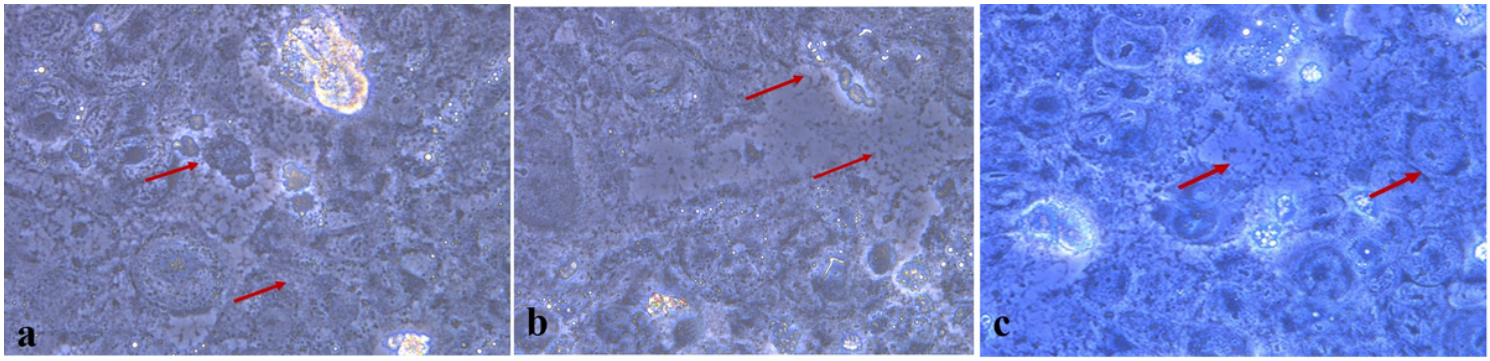

Figure 2

Inverted microscope images of the celluler viability experiment performed with the trypan blue staining method (x40); **a)** Negative control group **b)** Pozitive control group **c)**1,25 µM NF.

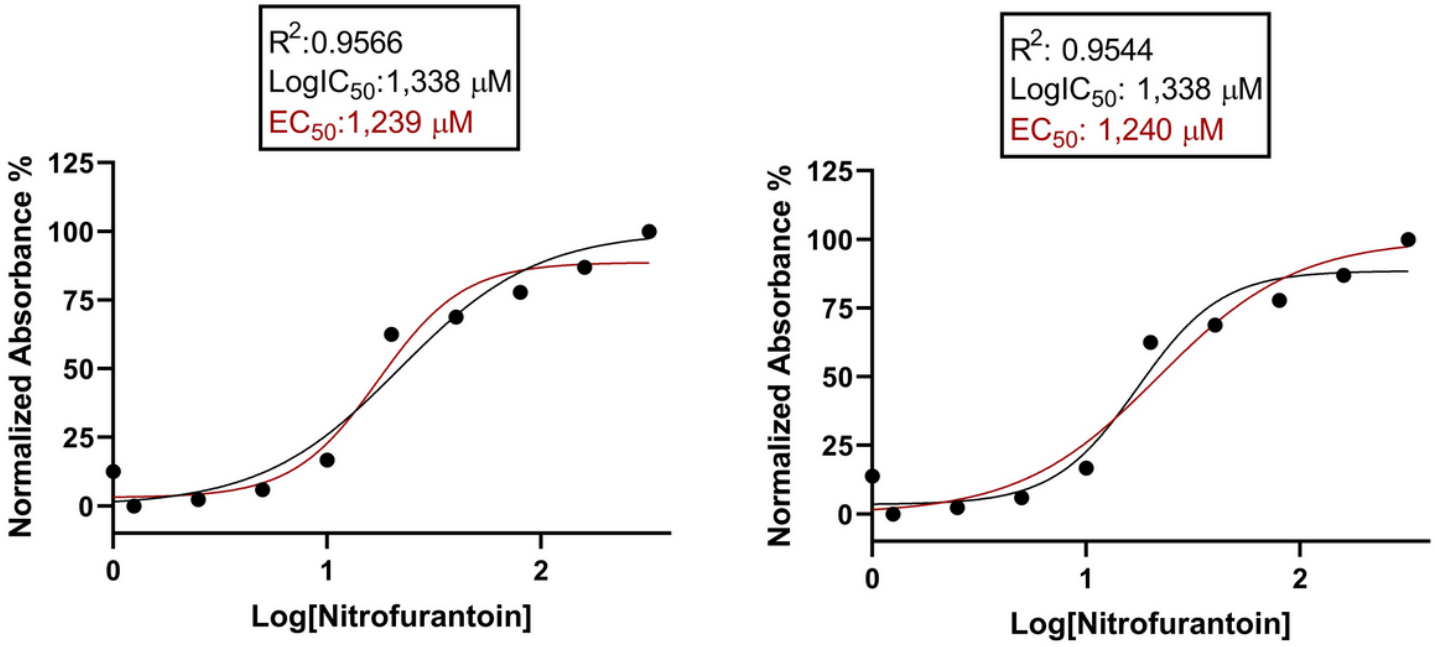

Figure 3

a) IC_{50}, EC_{50} and R^2 value accprding to negative control group **b)** IC_{50}, EC_{50} and R^2 value accprding to positive control group.

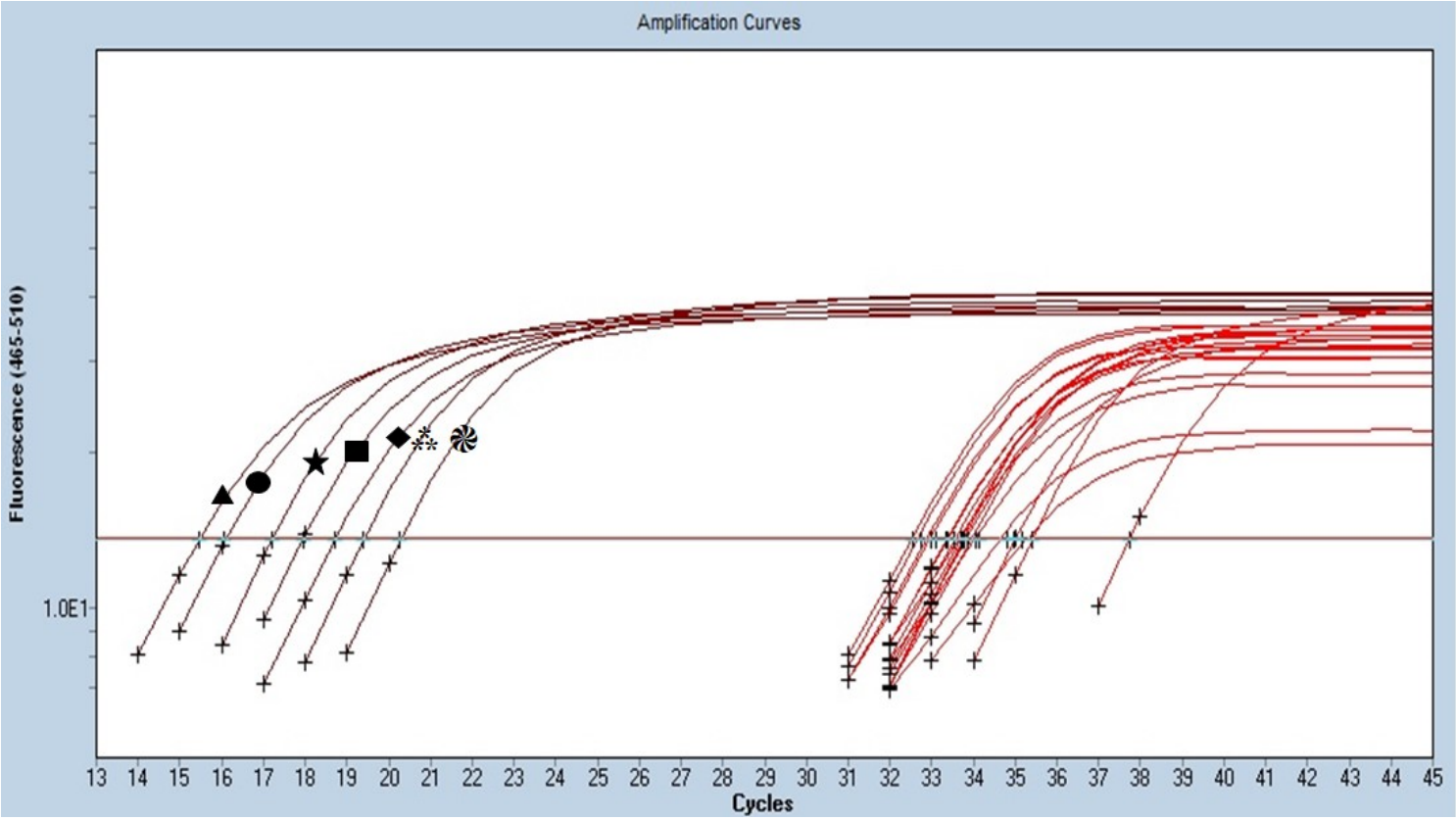

Figure 4

▲: dilution in a 1:1 ratio, ●: dilution in a 1:10 ratio, *: dilution in a 1:100 ratio, ■: dilution in a 1:1000 ratio, ♦: dilution in a 1:10000 ratio, : dilution in a 1:100000 ratio, : dilution in a 1:1000000 ratio.

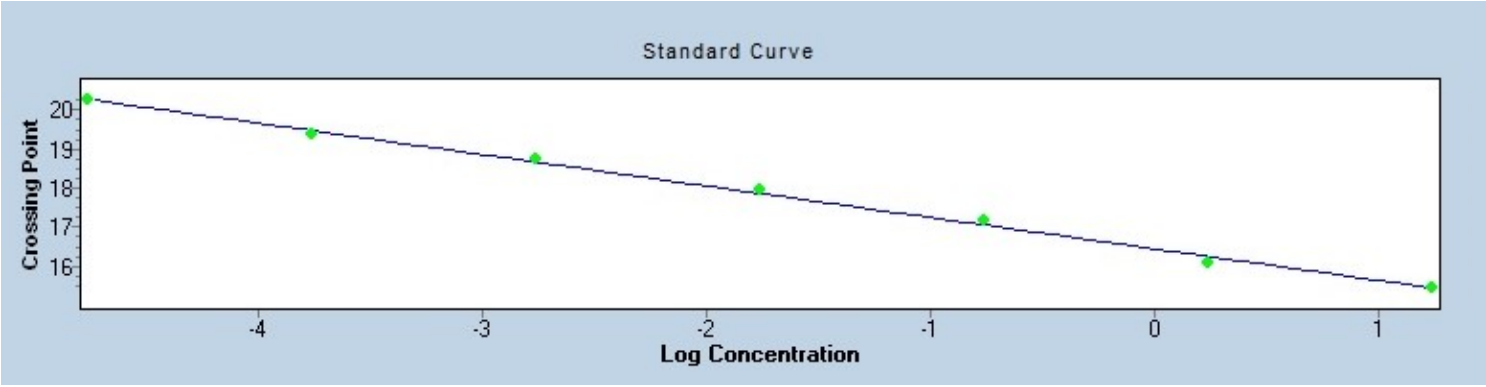

Figure 5

Standart curve of samples diluted according to B1 primers.

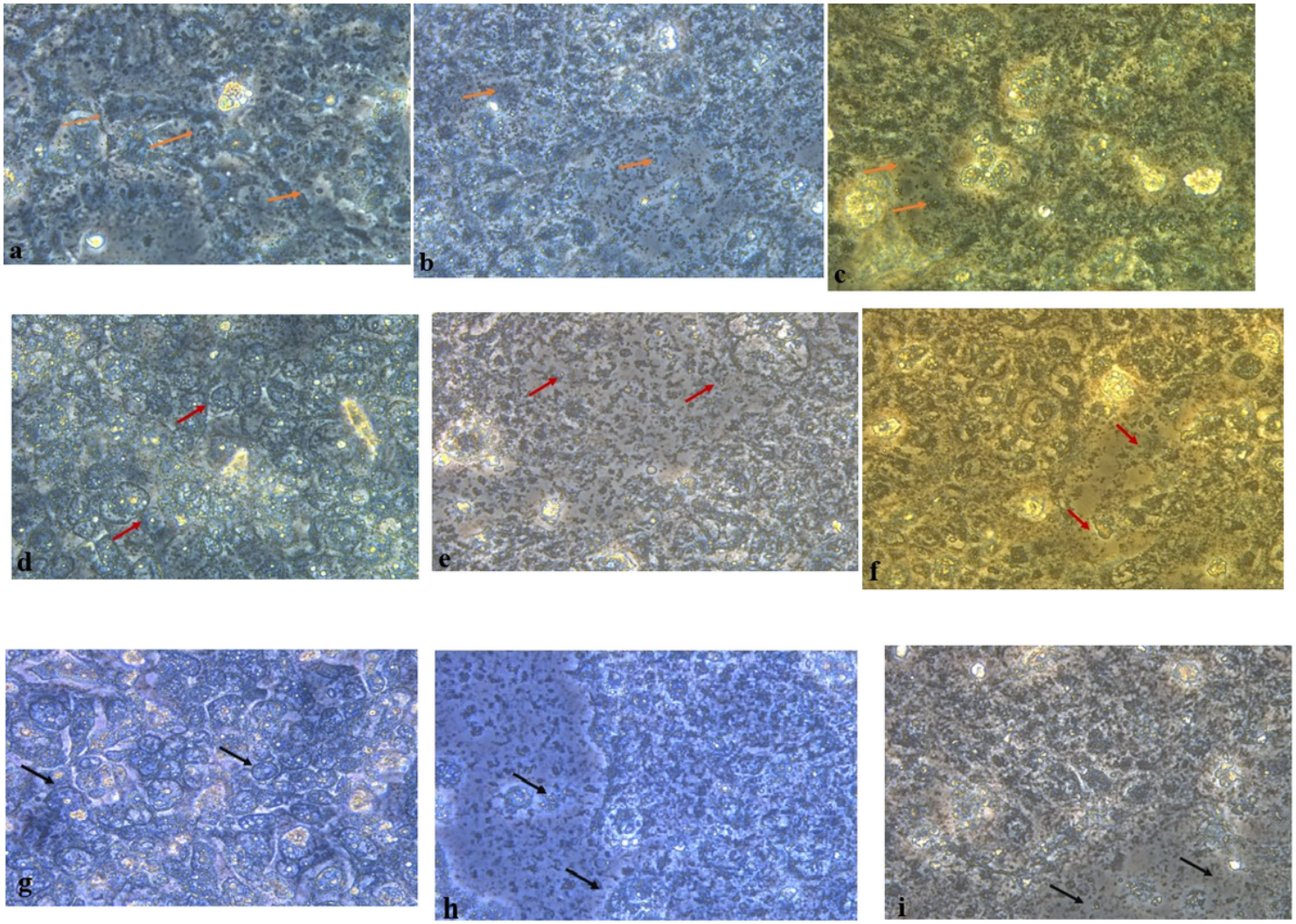

Figure 6

Image of cells with 1.25 µM NF added at 24 hour in inverted microscope **a)** Negative control group **b)** Positive control group **c)**1,25 µM NF.Image of cells with 1.25 µM NF added at 48 hour in inverted microscope **d)** Negative control group **e)** Positive control group **f)**1,25 µM NF. Image of cells with 1.25 µM NF added at 72 hour in inverted microscope **g)** Negative control group **h)** Positive control group **i)**1,25 µM NF.

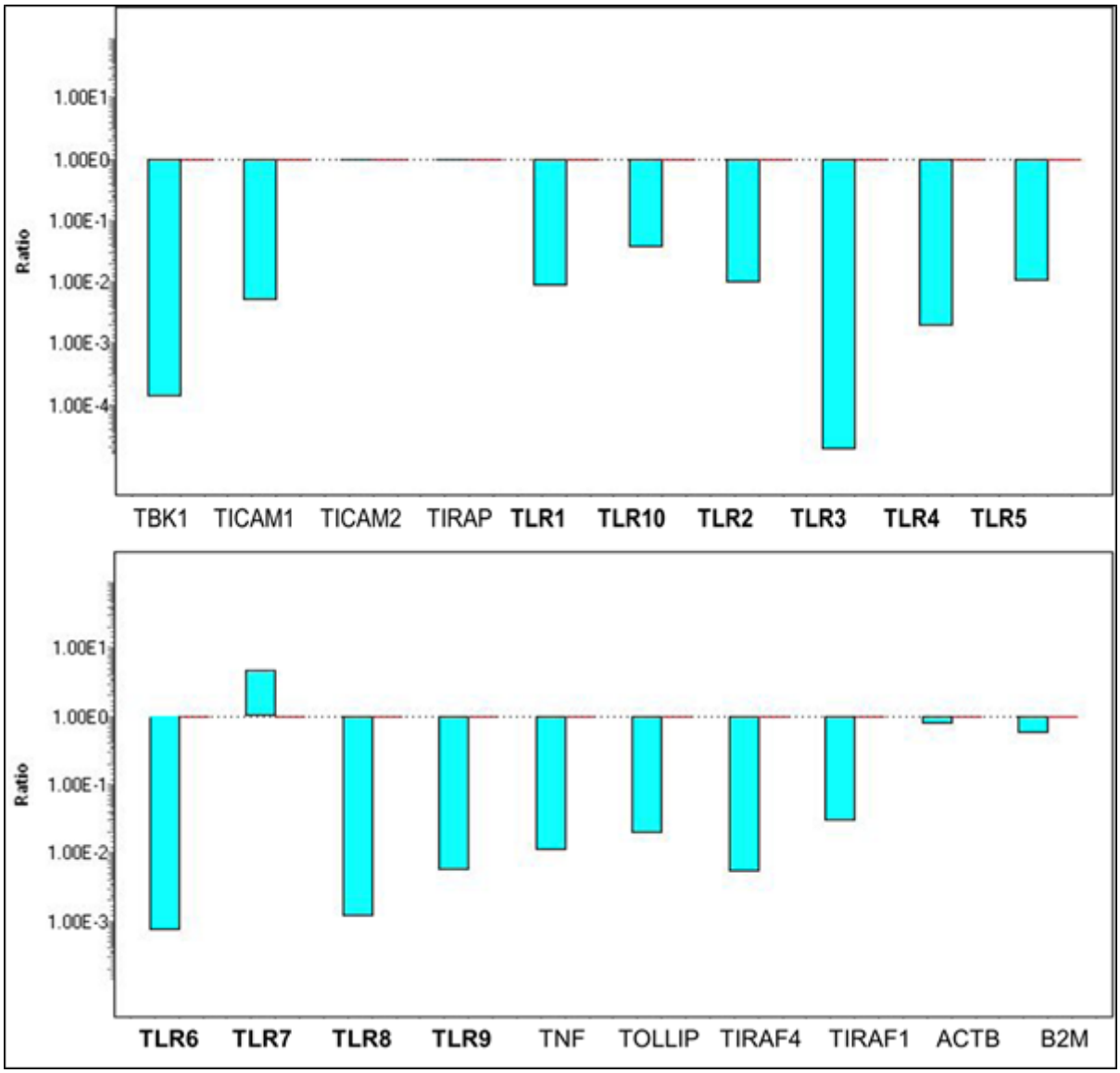

Figure 7

Relative quantification of TLR related genes of cells with 1.25 μM NF added at 24 hour.

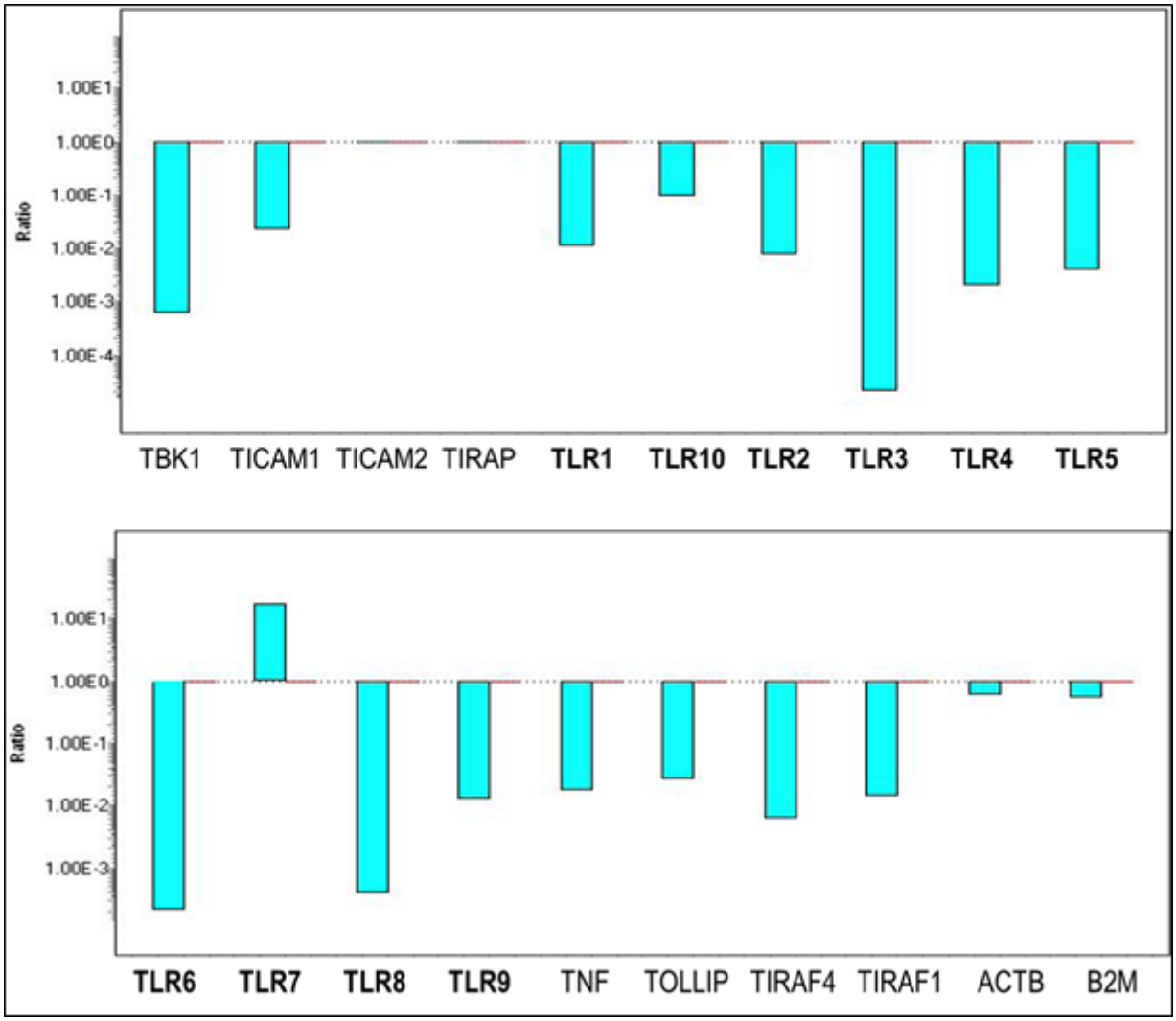

Figure 8

Relative quantification of TLR related genes of cells with 1.25 μM NF added at 48 hour.

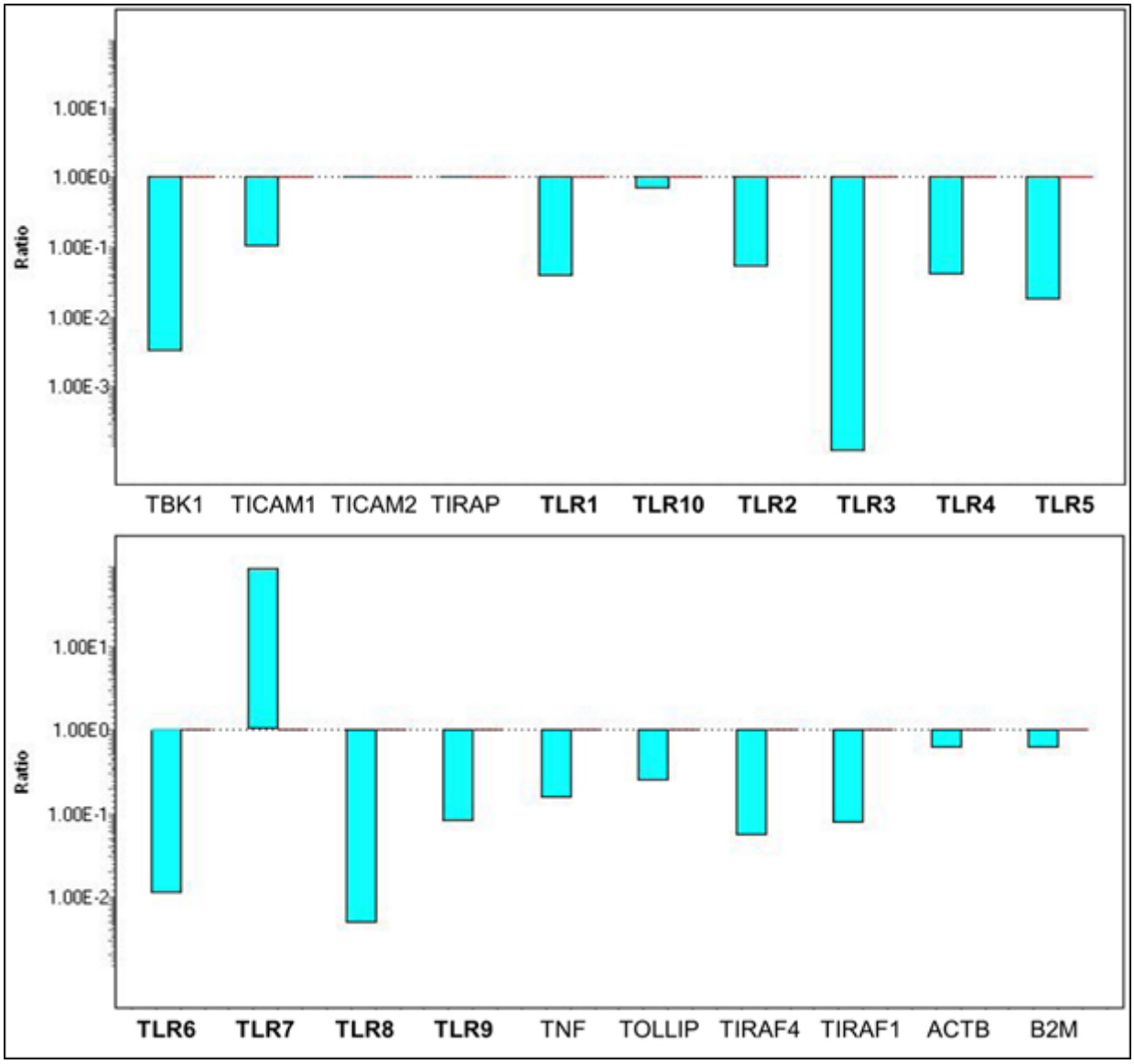

Figure 9

Relative quantification of TLR related genes of cells with 1.25 μM NF added at 72 hour.