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Research Article

THE EFFECT OF *ENCEPHALITOZOON INTESTINALIS* ON OXIDATIVE STRESS AND CYTOKINE LEVELS IN U937 CELLS: AN *IN VITRO* STUDY

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ABSTRACT. This *in vitro* study was performed to investigate the changes in nitric oxide (NO), malondialdehyde (MDA), total antioxidant capacity (TAC), tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) levels in human monocytic (U937) cells infected with *Encephalitozoon intestinalis*. *E. intestinalis* was first cultured in the African green monkey renal epithelial (Vero) cells to obtain sufficient amount of parasite for the study. U937 human macrophage cell line and *E. intestinalis* 50506 (ATCC) strain were used in the study. U937 macrophages were infected with $5x10^6$ spores after stimulation with Phorbol 12-Myristate 13-Acetate (PMA) and uninfected U937 cells were used as control. Culture media were collected at the 6th, 12th, and 24th hour after infection to determine NO, MDA, TAC, TNF-α and IL-10 levels. NO levels significantly increased at the 6th, 12th and 24th hour. There was no significant difference in MDA and TAC levels at the 6th and 12th hour, but a significant increase in MDA, and a significant decrease in TAC was observed at the 24th hour. TNF-α levels did not differ in all sampling times, and IL-10 level decreased only at the 6th hour. In conclusion, *E. intestinalis* caused oxidative stress by increasing the levels of oxidants (NO and MDA) and by suppressing TAC level without no significant changes in cytokine levels in U937 cell line.

Keywords: *Antioxidant, cytokine, Encephalitozoon intestinalis, oxidative stress, U937.*

INTRODUCTION

Microsporidian parasites are obligate intracellular parasites that infect many vertebrates and invertebrates. There are one hundred forty genera and more than one thousand two hundred species of the parasite described to date. Of these, only fourteen species in seven genera cause infection in humans. One of these species, *Encephalitozoon intestinalis*, which is the most common species in Turkey, causes many different clinical symptoms in addition to life-threatening diarrhea in immunocompromised patients [1, 2, 3, 4]. When the parasite infects the cells, it develops in a vacuole formed by the host cells and the infected cells reach 2-3 folds bigger in size than the healthy cells. In this process, which lasts approximately 10 days, the parasite prevents apoptosis, cell division and multiplication [5, 6].

Reactive oxygen species are fundamental molecules for macrophages to eliminate invasive microorganisms [7] but when they are produced excessively, they begin to be harmful rather than beneficial [8]. It has been reported that oxidative stress, which is known to cause changes in many structures and functions in the organism [9], may occur as a result of suppression of the antioxidant system due to increased free radicals or decreased antioxidant defence capacity [7, 10]. It has been claimed that free radicals can cause metabolic disorders and cell damage in many ways [9]. It is well known that malondialdehyde (MDA), a lipid peroxidation product, increases under oxidative stress conditions [11, 12]. It has been demonstrated in different studies that antioxidants reduce lipid peroxidation [13, 14, 15]. Nitric oxide (NO), a reactive nitrogen species, has also a dual effect. It has very important physiological functions at low concentrations, but excessive and uncontrolled NO synthesis is harmful for cells [16].

Cytokines are the substances in protein structure that play important roles in intercellular communication, and in regulation of various biological functions particularly in immune response, and inflammation [17, 18, 19]. It is stated that TNF-α, one of these cytokines, is required for nitric oxide induction in macrophages, and plays an important role in defense against intracellular pathogens by promoting phagocytosis and intracellular death [17]. Interleukin-10 (IL-10), an important immune-regulatory cytokine, is produced by numerous cell populations. It was reported that IL-10 mediates immunostimulatory properties by helping the elimination of infectious and non-infectious agents with limited inflammation [20].

Although many previous studies in human and animals investigated the several aspects of microsporidiosis caused by *E. intestinalis,* to the authors' knowledge there have been very limited studies investigating the changes in biochemical parameters [21, 22, 23]. Since *E. intestinalis* affects the basic mechanisms so much, this study was performed to determine the effects of this parasite on biochemical parameters *in vitro* to find an answer to the question of how it acts in the host. For this purpose, in this study, the effects of *E. intestinalis* on NO, MDA, total antioxidant capacity (TAC), tumor necrosis factor-α (TNF-α) and IL-10 levels were investigated in human monocytic (U937) cells.

MATERIALS AND METHODS

Culture of Parasite

In this study, *E. intestinalis* (strain ATCC 50506) was used. The parasite was first cultured in the African green monkey renal epithelial (Vero) cell line to obtain sufficient amount of parasite for the study. For parasite culture, Dulbecco's Modified Eagle Medium (DMEM, Biochrom, FG 0445) medium, which was enriched with 2mM L-glutamine (Sigma, 59202), 100 IU /ml penicillin + 100 µg/ml streptomycin (Life, 15140-122) containing 10% inactivated fetal bovine serum (FBS) (Thermo, 10270-106) was used. Vero cells were seeded in a 75 cm^2 cell culture flask and incubated overnight at 37 °C under 5% CO_2. After incubation, the non-adhered cells were removed together with the medium. Then the cells were infected with the parasite when they covered approximately 1/3 of the cell culture flask. The medium on the cells was replaced with 7 ml of 10^7/ml spore-containing medium and incubated overnight at 37 °C under 5% CO_2 for the infection. After incubation, non-adherent spores were removed from the environment together with the medium. The medium was changed twice a week after infection. The

medium was filtered through a 5 μm mesh filter and collected by centrifugation at 4000 rpm for 10 minutes. Spores were stored at +4 ºC until use.

Preparation and Infection of U937 Cells with Parasite and Study Groups

U937 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 growth medium enriched with 1% L-glutamine, 10% inactivated fetal bovine serum, 1% penicillin/streptomycin and 1% 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES). When the cell concentration reached a sufficient number, they were stimulated with pPhorbol12-myristate 13-acetate (PMA) (10 ng/ml) in 6-well cell culture plates for 120 hours. After stimulation, the cells were infected with E. *intestinalis* spores. For infection, 2 ml of medium containing 5x106 spores was added to the cells before leaving them to incubate overnight. The next day, non-adherent parasites were removed along with the medium and then the fresh medium was added to the cells. The medium contents used after infection was different in that it contained only 2% FBS. Cell media were collected at the 6^{th}, 12^{th}, and 24^{th} hour after infection. The U937 cells that were not infected with parasites were used as control in parallel with the parasite-infected group.

Biochemical Parameters

The NO (Sunred Bio, 201-12-1511, China), MDA (Cayman, 10009055, USA) TAC (Rell assay, 0017, Türkiye), TNF-α (Sunred Bio, 201-12-0083, China), and IL-10 (Sunred Bio, 201-12-0103, China) levels in cell culture media were determined using commercial kits in a Bio-Tech Brand (Quant, USA) ELISA reader.

Statistical Analysis

Statistical analyses of the data were performed with the SPSS 20.0 package program (Microsoft, USA). The conformity of the data to the normal distribution was evaluated with the Shapiro Wilk test, histogram and Q-Q graph. Homogeneity of variances was evaluated with Levene's test. The significance control of the difference between groups was tested with the one-way analysis of variance (ANOVA), when the F value was found to be significant Duncan's new multiple range test was applied to determine which group caused that difference. Independent sample t-test was used to compare two independent groups. Values were given as the means and the standard error of the means (SEMs).

RESULTS AND DISCUSSION

In the study, the NO levels of the infected group were significantly higher at the 6^{th}, 12^{th} and 24^{th} hours ($p<0.005$, Table 1) than the control group. Compared to the control group, when the MDA levels of the infected group were considered, no statistically significant difference was observed at the 6^{th} and 12^{th} hours of the treatment ($p>0.005$, Table 1), but a significant increase ($p<0.005$, Table 1) was observed at the 24^{th} hour. While the TAC did not differ between the control group and the infected group at the 6^{th} and 12^{th} hour ($p>0.05$, Table 1), a significant decrease in the infected group ($p<0.05$, Table 1) was observed at the 24^{th} hour.

It was reported that many intracellular pathogens can inhibit macrophage activation by inhibiting signal transduction [24, 25]. Moreover, it has been demonstrated that Microsporidia can regulate cell cycle and apoptosis in human lung fibroblasts [5] and suppress apoptosis in human macrophages [26]. Nitric oxide, which is cytotoxic to some

intracellular bacteria, fungi and protozoan parasites [27], is produced from L-arginine, a semi-essential amino acid, by nitric oxide synthase (NOS) enzymes [28]. Nitric oxide synthase is a group of enzymes as structural NOS (cNOS) and inducible NOS (iNOS) [29, 30]. Inducible NOS was first identified in macrophages [31] and it can be expressed in response to cytokines and lipopolysaccharides [28]. It has been suggested that NO is produced by immune system cells as a result of immunological interference and mediates the non-selective cytotoxic effects of these cells [32, 33]. In a study conducted by Franzen et al. [22] to determine the cytokine and NO levels in human macrophages inoculated with three different *Encephalitozoon* spp. (*E. cuniculi*, *E. hellem* and *E. intestinalis*) and *Vittaforma corneae*, slight but not significant increase in nitrate production and decrease in nitrite production in infected macrophages compared to uninfected macrophages were reported. Concerning NO production, these authors found that microsporidian species had no effects on the amount of NO. However, in the present study, significant increases occurred in NO levels of the infected cells at 6^{th}, 12^{th} and 24^{th} hours of the treatment. The regulation of NO by intracellular microsporidia may contribute to the survival of microsporidia within the macrophage [21].

It was reported that the infection of the animal body with internal or external parasites is associated with the excessive release of free radicals, which may be due to the reduction of nutrients consumed by the body to synthesize antioxidants and the destruction of cells by the activity of parasites [34]. It is well known that oxidative stress and free radicals cause damage especially in lipids. Free radicals attack the lipid membrane of the cell and form lipid hydroperoxides, and their destruction causes the formation of bioactive aldehydes. The main aldehyde among the formed aldehydes was MDA [35, 36]. In this study, compare to the control group, no statistically significant differences were observed at the 6^{th} and 12^{th} hour, but a significant increase was observed at the 24^{th} hour in the MDA levels of the infected cells similar to the results of Chandramathi et al. [23] who reported elevations in the levels of oxidative stress markers such as hydrogen peroxide, free radicals and lipid peroxides in patients infected with microsporidia. A time dependent increase was found in the MDA level, which is one of the indicators of the oxidative stress due to the parasite infection, whereas a decrease was observed in the TAC of the cells in a similar pattern to the MDA level in the present study. These results suggest that *E. intestinalis* may either cause overproduction of free radicals or the increase in MDA level at the 24^{th} hour may be resulted from the decreased TAC of the cells at the 24^{th} hour.

In the study, there was no statistically significant difference concerning the TNF-α levels of the control and the infected groups in all sampling times ($p>0.05$, Table 1). When the IL-10 level of the infected group compared to the control group, it was determined that there was a significant decrease ($p<0.05$, Table 1) only at the 6^{th} hour. After the *E. intestinalis* infection, IL-10 production by macrophages showed a time dependent increase. However, these increases were not found to be significant at the 12^{th} and 24^{th} hour compared to the control groups (Table 1).

Cytokines are the molecules that play significant roles in signal exchange between cells. They are known as mediators produced by many types of cells involved in immune responses. It was reported that the presence of parasites is usually associated with significant cytokine production. The parasites can trigger cytokine secretion by cells of the innate immune system directly through parasitic products or indirectly through stimulation of T cells [37].

Franzen et al, [22] reported a significant increase in TNF-α level in the supernatants of infected macrophage cultures with the microsporidian species compared to uninfected

cultures. And also, these authors found significantly higher amounts of IL-10 with the most noticeable increase in cultures infected with *E. cuniculi* spores. In contrast, in this study, *E. intestinalis* had no effect on TNF-α levels at all sampling times.

In addition to the limited number of *in vitro* studies available on this subject, few *in vivo* studies have been encountered. In studies conducted in New Zealand rabbits, the brain and kidney NO levels of animals infected with *E. cuniculi* were statistically higher than healthy animals [38], but there was no difference in IL-10 levels [39] is indicated. In this study, as stated in the literature, it was determined that NO levels increased after infection, and IL-10 values increased significantly at the 6th hour, but this increase reached control values depending on time.

It was reported that macrophages require TNF-α for the full induction of NO, and TNF-α plays an important role in defense against intracellular pathogens, phagocytosis and intracellular death [22]. Similarly, in this study, significant increases in NO levels were observed after infection with *E. intestinalis*. In the present study, although the IL-10 level was lower after 6 hours in the infected cells compared to the control group a time-dependent, slight but not significant, increases occurred in IL-10 levels of the infected cells which suggests that this cytokine may cause a defensive response against infection as indicated by Franzen [21].

***Table 1**. The NO, MDA, TAC, TNF-α, and IL-10 levels in U937 cells infected with E. intestinalis and uninfected U937 cells.*

Parameters	**6th hour**	**12th hour**	**24th hour**	**P (One-Way ANOVA)**
NO (µmol/L)				
Control	144.60±18.72	162.69±10.74	160.93±19.56	-
E. intestinalis	171.78±24.11	183.81±21.73	184.56±16.26	-
P (Independent Sample t-Test)	**	*	*	
n=8				
MDA (µmol/L)				
Control	2.25±0.36	2.00±1.27	1.56±0.73	-
E. intestinalis	2.09±0.49	2.96±0.97	3.23±1.62	-
P (Independent Sample t-Test)	-	-	**	
n=8				
TAC (mmol/L)				
Control	1.13±0.08	1.13±0.04	1.16±0.07	-
E. intestinalis	1.16±0.04^{a}	1.13±0.05^{a}	1.06±0.09^{b}	*
P (Independent Sample t-Test)	-	-	**	
n=8				
TNF-α(ng/L)				
Control	207.03±11.33	218.76±37.38	227.55±24.33	-
E. intestinalis	217.74±11.97	217.14±14.74	233.61±20.36	-
P (Independent Sample t-Test)	-	-	-	
n=8				
IL-10 (pg/ml)				
Control	650.00±53.29	606.81±119.02	761.41±140.57	-
E. intestinalis	463.43±267.17^{b}	666.91±160.71^{a}	712.95±118.24^{a}	*
P (Independent Sample t-Test)	*	-	-	
n=8				

*^{a,b}: The difference between values with different superscripts in same row is significant. -: not significant; *: p>0.05; **: p<0.01*

CONCLUSION

The results of this study have shown that *E. intestinalis* increased the amounts of oxidants (NO and MDA) whereas decreased the antioxidant capacity (TAC) without no significant changes in cytokine (TNF-α and IL-10) levels in the U937 cells.

Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. Concept: M.Ş., Ü.Ç., M.E., Design: M.Ş., Ü.Ç., M.E., A.C., Data Collection or Processing: M.Ş., Ü.Ç., Analysis or Interpretation: M.Ş., Literature Search: M.Ş., Ü.Ç., Writing: M.Ş., Ü.Ç., M.E.

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