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Original Article

In Vitro Effects of Ivermectin and Sulphadiazine on *Toxoplasma gondii*

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ABSTRACT

Objective: Ivermectin and sulphadiazine were tested individually to determine their *in vitro* effects on *Toxoplasma gondii* grown in human epidermoid larynx carcinoma (Hep-2) cell culture.

Study Design: *In-vitro* study.

Material and Methods: *Toxoplasma* growth was quantities by an enzyme immunoassay performed directly on the fixed cultures, using a rabbit anti-*T. gondii* immunoglobulin G as the first antibody and a phosphatase-labeled anti-rabbit immunoglobulin G as the second antibody. For each drug, regression models were used to quantify the relationship between optical density values and antimicrobial agent concentrations in the cultures.

Results: The 50% inhibitory concentrations (IC50) of ivermectin and sulphadiazine were found to be 0.2 µg/mL and 7.3 µg/mL after 48 h of exposure, respectively. None of the concentrations tested for each drugs demonstrated toxicity to Hep-2 cells after 72 h of incubation.

Conclusion: These results indicate that ivermectin significantly inhibited replication of the tachyzoites of *T. gondii* RH strain.

Key Words: *Toxoplasma gondii*, ivermectin, sulphadiazine, *in vitro*, Hep-2

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Introduction

Toxoplasma gondii is a protozoan parasite that infects up to a third of the world's population. In most individuals, acute infection with *T. gondii* is asymptomatic or causes mild symptoms. However, toxoplasmosis can cause severe diseases in fetuses if a seronegative pregnant woman is infected. Toxoplasmosis can also be fatal in immunodeficiency or immunocompromised individuals (1, 2).

The combination of sulphadiazine (SDZ) and pyrimethamine is frequently used for the treatment of *T. gondii* infections. These drugs have a remarkable synergistic activity against the replicating form of *T. gondii* through the sequential inhibition of parasite dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) (3). However, treatment fails to eliminate the encysted form of the parasite. Furthermore, treatment with these agents is associated with frequent and severe adverse reactions, especially in patients with acquired immunodeficiency syndrome (4). The development of new alternatives to sulphadiazine and pyrimethamine therapy requires better knowledge of the effects of antimicrobial agents on *T. gondii.*

Recently, Merli et al. (5) developed an enzyme immunoassay for evaluation of *Toxoplasma* growth in tissue culture and suggested its application to study the *in vitro* activity of anti-*T. gondii* compounds. With this method, the relationship between the inhibitory effect of a drug and its concentration in the culture medium can be determined by regression analysis (6).

The aim of this study was to investigate the *in vitro* anti-*Toxoplasma* activities of antiparasitic ivermectin and sulphadiazine, currently one of the most effective available therapeutic agents, using tachyzoit of *T. gondii* RH strain infected Hep-2 epithelial cells. For this purpose, Hep-2 cells in tissue culture plates were infected with tachyzoites isolated from peritoneal fluid of mice infected with *T. gondii.* Subsequently, ivermectin or sulphadiazine was added. Inhibitory activity was assessed after 24 h, 48 h and 72 h incubations using invert microscopy and at 48 h by enzyme-linked immunosorbent assay (ELISA). The toxicities of ivermectin, sulfadiazine and tachyzoit to Hep-2 cells were determined with Neutral Red Uptake (NRU) assay.

Material and Methods

Cultures of *T. gondii*

(i) Cells

Hep-2 (An1-92041501; Institute of Foot and Mounts Diseases, Virology Laboratory, Ankara, Turkey) were maintained in Minimum Essential Medium Eagle (MEM) (Sigma, Steinheim, Germany) containing penicillin (100 IU/mL), streptomycin (0.1 mg/mL) (Biochrom, Leonorenstr, Berlin), and 5% heat-inactivated fetal bovine serum (FBS) (Biochrom, Leonorenstr, Berlin). For the assays, Hep-2 cultures were prepared in 96-well cell culture plates (TPP, Switzerland). The cells were seeded onto each well ($2.0x10^4$ cells per well) and grown to confluence at 37°C in a moist 5% CO_2 -95% air atmosphere.

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(ii) Parasites

Tachyzoites of the virulent TR-RH strain maintained through serial intraperitoneal of mice passages were used. For experimental infections, tachyzoites were harvested from the peritoneal fluid of mice infected intraperitoneally 2-4 days earlier and purified by centrifugation. The parasites were washed with MEM. They were counted and adjusted to a concentration of $5.0x10^4$ parasites per mL.

(iii) Challenging the cultures

For the assays with antimicrobial agents, confluent monolayers were inoculated with 100 µL of the parasite suspension, i.e., 5000 trophozoites. This inoculum dose was determined in a preliminary experiment in which various parasite inputs concentrations ($2.0x10^5$, $1.0x10^5$, $5.0x10^4$, $2.0x10^4$, $1.0x10^4$, $5.0x10^3$, $2.0x10^3$ and $1.0x10^3$ parasites per well) were compared. The inoculums of $5.0x10^3$ parasites were found to be optimum for long incubation times. The cultures could be maintained up to 72 h after challenge, and infected monolayers were fully preserved for ELISA.

Three controls were included in each culture plate: (i) uninfected monolayers as a negative control, (ii) infected monolayers in which the replication of *T. gondii* was inhibited by the addition of 50 µL of 0.25% sodium azide (Serva, Heidelberg Carl-Benz-Str) per well at 4 h after challenge (inoculum's controls), and (iii) infected monolayers with culture medium alone as a positive control. After development of the coloration, spectrophotometric readings were performed at a λ of 450 nm using blanks (negative control) in addition to all two controls. The results were expressed as optical density values. Experiments were repeated two times and were duplicated for each drug dose.

Quantitation of *T. gondii* growth in cultures

For quantitation of *T. gondii* growth in the culture, the supernatant was removed by aspiration. The monolayers were fixed with cold methanol for 15 min and air dried.

Antimicrobial agents

Sulphadiazine (Sigma, Steinheim, Germany) was initially dissolved in 50% methanol-50% acetone (Riedel-de Haën, Germany) at a concentration of 2000 µg/mL. Ivermectin (Sigma, Steinheim, Germany) was dissolved in dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany) at a concentration of 1600 µg/mL.

Antimicrobial agents were added to the culture 4 h after challenge with *T. gondii*. For each antimicrobial agent, serial dilutions were prepared in MEM-FBS so that the addition of a 100 µL amount to the culture yielded the following final concentrations: SDZ, 0.7, 0.15, 0.3, 0.6, 12.5, 25, 50, and 100 µg/mL; ivermectin, 0.035, 0.07, 0.15, 0.3, 0.6, 1.25, 2.5 and 5 µg/mL. Each concentration was tested in eight replicate wells. Preliminary studies indicated that final concentrations of methanol and acetone in dilution of SDZ did not inhibit the growth of *T. gondii*. Cultures were incubated with antimicrobial agents up to 72 h after challenge and cultures were examined microscopically, and then fixed with methanol, air dried, and stored at +4°C, and ELISA was performed as described.

ELISA

An ELISA test was performed directly on the tissue culture test plates (TPP, Switzerland) by using (i) anti-*T. gondii* antibodies (immunoglobulin G fraction) (Abcam Inc, Kendall Square Stc, Cambridge/USA) as the first antibody and (ii) anti-rabbit immunoglobulin G (whole molecule) alkaline phosphatase antibody produced in goat (Sigma A3687, Steinheim, Germany) as the second antibody. Wells were postcoated with 200 µL of phosphate-buffered saline (PBS)-0.5% Tween 20-1% bovine serum albumin (Sigma, Steinheim, Germany) (PBS-T-BSA) for 1 h at 37°C to block nonspecific adsorption sites. The plates were washed five times with PBS containing 0.5% Tween 20 and then 100 µL of anti-*Toxoplasma* antibodies diluted 1/100 in PBS-T-BSA was added to the wells. Plates were incubated for 120 min at 37°C and then washed as described above. A 100 µL sample of the conjugate diluted 1/1000 in PBS-T-BSA was added, and the plates were incubated for 60 min at 37°C. After the plates were washed five times, 100 µL of paranitrophenyl phosphate (Sigma, Steinheim, Germany) 1 mg/mL in substrate solution (0.1 M Glisin, 5 M KOH, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH:10.4) (Sigma, Steinheim, Germany) was added, and the plates were incubated for 30 min at room temperature. For reading with a Microplate Reader (Rayto, RT-2100c) was used at 450 nm. The negative control wells were used as the blanks. The results were expressed as optical density (OD) values.

To assess the cytopathic effects and the reproduction of intracellular *T. gondii* tachyzoites and control cells in 24 h, 48 h and 72 h; the coverslips on 24 well tissue culture plates were stained with Giemsa and examined microscopically (Figure 1).

The plates prepared with 8 different concentrations of sulphadiazine and ivermectin were examined microscopically after 24 h, 48 h and 72 h.

Statistical analysis

A correlation coefficient was used to quantify the relationship between *T. gondii* counts and ELISA results. The effect of antimicrobial agents at various concentrations was described by data plotting. Optical density was plotted as a function of the logarithm of the concentration, which suggested models summarizing the concentration effect. Regression analysis was used to quantify the relationship between optical density values and antimicrobial agent concentrations in the cultures (6).

Results

The present study, after the microscopic examination of the plates it was determined that there were no inhibitory effects at all concentrations for ivermectin in 24 h. At 48 h the number of tachyzoites decreased relative to control wells at 0.31, 0.62 and 1.25 µg/mL. An important inhibitory effect was observed at 2.5 and 5 µg/mL. This inhibition of growth was associated with a reduction of the number of parasitized cells and intracellular parasites that were morphologically normal. In 72 h, because of the number of tachyzoites elevated in all wells, the inhibitory effect of ivermectin was not assessed except at 2.5 and 5 µg/mL concentrations.

For sulphadiazine, as for ivermectin, at 24 h there were no inhibitory effects at all concentrations tested. At 48 h, the

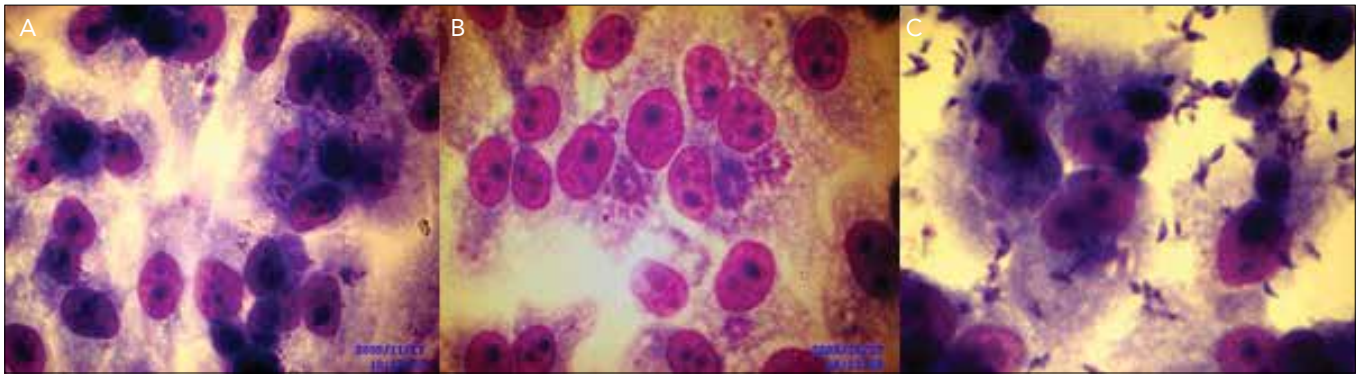

Figure 1. Reproduction of intracellular *T. gondii* tachyzoites A. one- two tachyzoites in 24 h, B. parasites formed rosettes or large pseudocysts in 48 h, C. free tachyzoites in extracellular fluid in 72 h

number of tachyzoites had decreased relative to control wells at 3.12, 6.25, 12.5 and 25 µg/mL. An important inhibitory effect was observed at 50 and 100 µg/mL. In 72 h, because of the number of tachyzoites elevated in all wells, the inhibitory effect of sulphadiazine was not assessed. Therefore, ELISA was performed directly on the culture plates at 48 h. The ELISA did not alter the structure of fibroblasts.

Effect of antimicrobial agents

For each antimicrobial agent, experimental OD values were plotted versus concentrations. Regression lines results are shown in Figure 2.

Sulphadiazine

Sulphadiazine in all concentration, except at a concentration of 0.75 and 1.5 µg/mL ($p>0.05$) showed significantly higher inhibitory activity against tachyzoites after 48 h incubations. The 50% inhibitory concentrations (IC_{50}) of sulphadiazine was estimated to be 7.3 µg/mL [OD=451.75-54.29 in concentration (c)] after 48 h of exposure. None of the concentrations tested for each drug demonstrated toxicity to Hep-2 cells after 72 h of incubation.

Ivermectin

Ivermectin was found to have significant inhibitory activity against tachyzoites after 48 h of incubation at a concentration of 2.5 and 5 µg/mL ($p<0.01$) and above dose. The 50% IC_{50} of ivermectin was estimated to be 0.27 µg/mL [OD=358.38-23.35 ln(c)] after 48 h of exposure. None of the concentrations tested for each drugs demonstrated toxicity to Hep-2 cells after 72 h of incubation.

Discussion

An ELISA performed directly on tissue culture was used to quantify the growth of *T. gondii* in Hep-2 fibroblasts. This assay was performed as described by Merli et al. (5) who initially showed that an ELISA performed on infected cells was a reliable method for studying *T. gondii* growth kinetics in tissue culture. Therefore, this method was used to evaluate the inhibitory effect of antimicrobial agents. The assessment was based on the relationship between OD values and log concentrations of antimicrobial agents in the cultures as shown by regression analysis in Figure 2.

Diab and El-Bahy (7) determined that the Hep-2 cell line appeared to be the most appropriate cell line for in vitro multiplication of *T. gondii* (RH strain) in maintenance media with or without fetal calf sera at different temperatures. Therefore, Hep-2 cell line was used in this assay.

With SDZ, we found a significant inhibitory effect for concentrations of 25, 50 and 100 µg/mL ($p<0.001$); a slight inhibitory effect for 3.12, 6.25, 12.5 µg/mL ($p<0.01$) and no inhibitory effect for 0.75 and 5 µg/mL ($p>0.05$) at 48 h incubation. The 50% IC_{50} of sulphadiazine was estimated to be 7.3 µg/mL [OD=451.75-54.29 ln(c)] after 48 h of exposure.

Similarly, Derouin and Chastang (6) investigated the *in vitro* inhibitory effect of antimicrobial agents on *T. gondii* grown on MRC5 fibroblasts. In their study, sulfadoxine was not found inhibitory at concentrations from 2 to 20 µg/mL. An inhibitory effect was observed at 30 µg/mL ($p<0.001$). Within the interval between 30 and 75 µg/mL, the inhibitory effect could be summarized by a linear function of the concentration [OD=2.35-0.5 ln(c)]. For higher concentrations, the inhibitory effect did not significantly increase.

In a study Meneceuri et al. (8) investigated the *in vitro* susceptibility of various genotypic strains of *T. gondii* to pyrimethamine, sulphadiazine and atovaquone. In their study they examined SDZ at 10 concentrations ranging between 0.0005 and 100 mg/liter. SDZ was inhibitory for 13 strains, with IC_{50} ranging between 3 and 18.9 mg/L.

Derouin and Chastang (9) investigated the *in vitro* effects of folate inhibitors on *T. gondii* grown in MRC5 fibroblast tissue culture found that three sulfonamides and four dihydrofolate reductase inhibitors have important inhibitory effects on *T. gondii*. They studied sulphadiazine at 0.001, 0.01, 0.05, 0.1, 0.2, 0.5, 2, 10, and 20 µg/mL concentrations, the %50 inhibitory concentration estimated from the regression model was 2.5 µg/mL for sulphadiazine.

Ivermectin is a derivative of the avermectins, a family of macrocyclic lactones produced by the filamentous bacterium *Streptomyces avermitilis*. With ivermectin, we found a significant inhibitory effect for concentrations 2.5 and 5 µg/mL ($p<0.001$), and a slight inhibitory effect for 0.15, 0.3, 0.6 and 1.25 µg/mL ($p<0.01$) and no inhibitory effect for 0.035 and 0.07 µg/mL ($p>0.3$) at 48 h incubation. The 50% IC_{50} of ivermectin was estimated to be 0.27 µg/mL [OD=358.38-23.35 ln(c)] after 48 h of exposure. Our *in vitro* results obtained with

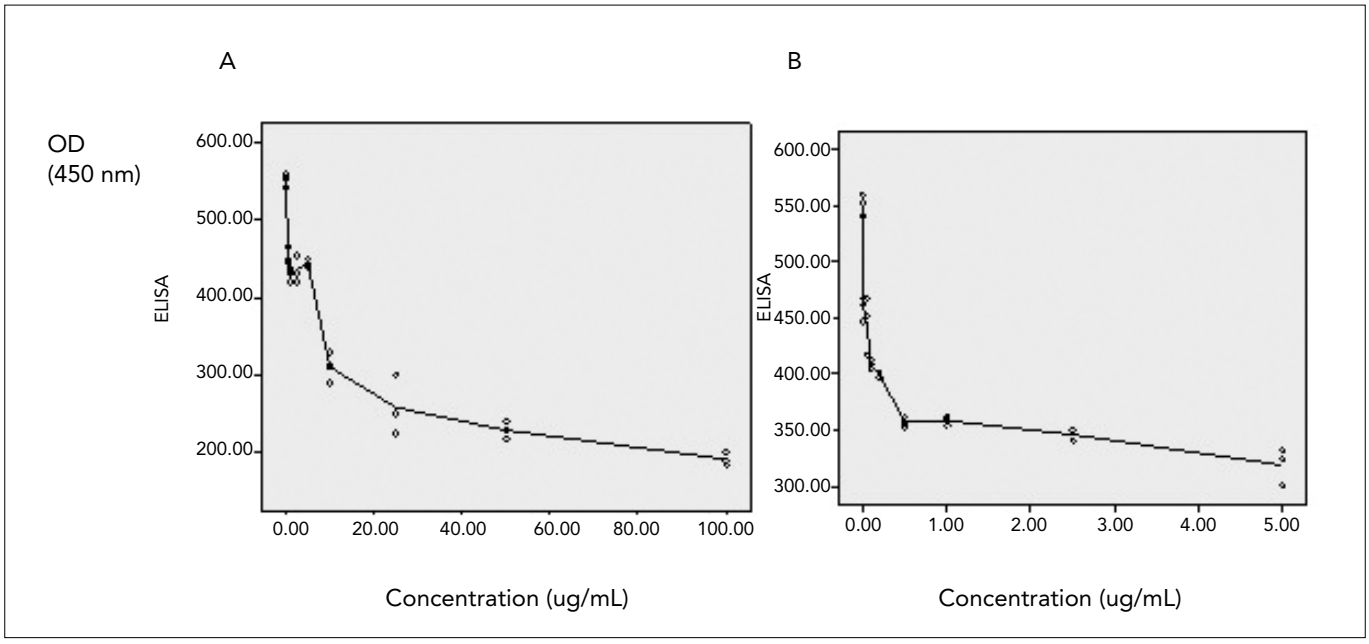

Figure 2. OD_{450} values for the ELISA with infected monolayers versus concentrations of antimicrobial agents. y axis, OD_{450} values; x axis, concentration of antimicrobial agent. A. SDZ, B. Ivermectin

ivermectin are in agreement with the studies performed by Guzzo et al. (10).

Our results indicated that ivermectin significantly inhibited replication of the tachyzoites of *T. gondii* RH strain. Therefore, the present study results may be useful for further studies in combination with other drugs and animal models to develop a better treatment model for toxoplasmosis in humans.

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