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Original Article

Conjugated Linoleic Acid Stimulates Apoptosis in RH and Tehran Strains *of Toxoplasma gondii,* in Vitro

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Abstract

Background: The aim of this study was to evaluate the effects of conjugated linoleic acid (CLA) on apoptosis of tachyzoites of *T. gondii*, RH strain (type I) and the cyst-forming Tehran strain (type II) in vitro.

Methods: *Toxoplasma* strains were injected into the peritoneal cavity of BALB/c mice. The Tehran strain forms cysts in the brain of mice. Bradyzoites within the cysts are reactivated to proliferative tachyzoites, by dexamethasone. Tachyzoites were aspirated from the peritoneum of infected mice, and the percentage of viable parasites was estimated with trypan blue staining. Tachyzoites were inoculated into HeLa cells cultivated in DMEM medium. Different concentrations of CLA were evaluated on *T. gondii* in HeLa cells by the tetrazolium (MTT) colorimetric assay. Differentiation between apoptosis and cell death was determined by flow cytometry using Annexin V and propidium iodide (PI) double staining. The statistical analysis performed by GraphPad Prism version 6.00.

Results: CLA induces apoptosis in virulent (RH) and avirulent (Tehran) strains of *T. gondii*. The results of MTT indicated that CLA could decrease the proliferation of tachyzoites of both strains in HeLa cells.

Conclusion: Conjugated linoleic acid has anti-toxoplasmacidal activity on tachyzoites of *T. gondii*. Therefore, we recommended further studies on this component in order to achieve a new drug against the parasite.

Introduction

Toxoplasmosis is a serious global zoonotic disease caused by *Toxoplasma gondii*, an obligate intracellular protozoan parasite. Humans are often infected by eating raw or undercooked meat containing tissue cysts. Toxoplasmosis may be the most frequent food-related disease in some areas (1). *Toxoplasma gondii* infection can be critical in pregnant women, immunocompromised patients, those receiving high-dose immunosuppressive therapy, and solid organ transplant recipients (1, 2).

Available medications for prevention and treatment of toxoplasmosis have shown limited efficacy or substantial side effects (3). Although toxoplasmosis is one of the most prevalent parasitic diseases, affecting close to one billion people worldwide, its current chemotherapy is deficient and is only effective in the acute phase of the disease (1).

Conjugated linoleic acid (CLA) is a member of the octadecadienoic fatty acid family that has received attention in recent decades for its health benefits. Conjugated linoleic acid is a natural component of milk and dairy products. It possesses two conjugated double bonds, mainly localized on C-7 to C-14 in either a cis or trans configuration. This product was originally identified as an anti-cancer component in ground beef extract. There are various isomers of CLA, with the cis-9 and trans-11 isomers being the primary natural isomers in food (4, 5). CLA can induce apoptosis in tumor cells and in microorganisms such as yeasts and parasites (6-9), but to our knowledge there is no report of its effects on *T. gondii.*

This study was performed to evaluate the in vitro effects of CLA on apoptosis of *T. gondii* tachyzoites, RH and Tehran strains.

Materials and Methods

Preparation of T. gondii tachyzoites

We used RH strain tachyzoites (a virulent strain of T. gondii) and Tehran strain tachyzoites (an avirulent strain of *T. gondii*) propagated in BALB/c mice. The Tehran strain (type II) is a cyst-forming strain that was previously isolated from human lymphadenitis (10), and for the current study was passaged in mice. Dexamethasone injected intradermally 50 mg/kg, 3 times a week for 4 weeks for reactivation of bradyzoites inside brain cysts to proliferative tachyzoites by suppressing the mouse immune system, and tachyzoites were aspirated from the peritoneum of mice (11). The viability of tachyzoites of *T. gondii,* RH and Tehran strains, assessed with trypan blue staining was greater than 98%.

Preparation of conjugated linoleic acid (CLA)

Conjugated linoleic acid (Sigma-Aldrich, USA, Cat No. O5507) was dissolved in DMSO and prepared concentrations of 0, 10, 20, 30, 50, 100, 150, 200 and 250 μM. The amount of DMSO after adding to wells was lower than 1%.

In vitro cultivation of T. gondii *strains*

Toxoplasma gondii strains were harvested from peritoneal fluid of mice inoculated into a 1:1 tachyzoites: HeLa cell monolayer in flasks (12). Infection rates showed that tachyzoites of RH and Tehran strains infected about 80% of the host cells. The cells containing tachyzoites were transferred to 96-well plates (Sigma-Aldrich, USA) for evaluation of sensitivity to CLA by MTT viability assay.

MTT assay

The diphenyl tetrazolium bromide (MTT) assay is based on the conversion of MTT to formazan crystals by living cells, which determines mitochondrial activity. Since, for most cell populations, the total mitochondrial activity is related to the number of viable cells, this assay is used to measure the in vitro cytotoxic effects of drugs on cell lines or primary patient cells (13).

Tachyzoite infected HeLa cells (5×10^5 per well) were transferred to 96-well plates and allowed to attach to the well surface. Plates incubated in 37°C, 5-8% CO2 and 95% humidity. After 24 h, the supernatant was discarded and replaced with fresh medium and 2% fetal bovine serum (FBS) (Bovogen, Australia). Conjugated linoleic acid was dissolved in non-toxic concentration (<1%) of dimethyl sulfoxide (DMSO) (Merck, Germany) and added to wells at concentrations of 0, 10, 20, 30, 50, 100, 150, 200, and 250 µM. After 24, 48, and 72 h, MTT assays were performed according to Sigma in vitro Toxicology Assay Kit (USA, Cat No.5655). Plates were rotated for 10min and recorded with a 570nm filter by ELISA reader.

Statistical analysis

Analysis of data performed by GraphPad Prism version 6.00 software to predict viability of tachyzoites in MTT test and drawing graphs.

Flow cytometry

Apoptosis in *T. gondii* tachyzoites was evaluated using the BioVisioAnnexin V-FITC Apoptosis Detection Kit (BioVision, USA). This kit is based on the observation that, soon after initiation of apoptosis, cells translocate membrane phosphatidyleserine (PS) from the inner face of the plasma membrane to the cell surface. On the cell surface, PS can be detected by staining with a fluorescent conjugate of Annexin V, a protein with high affinity to PS (14). Annexin V is a calcium-dependent, phospholipid-binding protein that preferentially binds to PS that is normally confined to the inner (cytoplasmic) face of cells, but translocates to the cell surface in apoptotic cells. Therefore, PS exposure as detected by Annexin V-FITC binding appears to be an early event in apoptosis (14).

Annexin V-FITC Assay Protocol

One hundred thousand tachyzoites per well were suspended in DMEM medium with 5% FBS and poured into 96-well plates. Concentrations of 0, 10, 20, 30, 50, 100, 150, 200, and 250 µM of CLA were added to wells. Plates incubated in 37°C, 5-8% CO2 and 95% humidity. After 24, 48, and 72 h, cells were collected by centrifugation and re-suspended in 500 µL of 1x of binding buffer. Five µL of Annexin V-FITC and 5 µL of propidium iodide (PI) were added to each well. Doxorubicin, an anti-neoplastic drug, induces apoptosis in the human lymphoblastoid leukemia (REH) cell line; these apoptotic cells were used as positive control for the PI and Annexin dyes (15). Plates were incubated at 25 °C for 5min in dark. After incubation, cells were washed to remove unbound PI and Annexin, and tachyzoites were analyzed by flow cytometry (Partec, PAS, Germany).

Annexin V-FITC was analyzed by flow cytometry (Ex=488 nm; Em= 530 nm) using FITC signal detector (FL1) and PI staining by phycoerythrin emission signal detector (FL2) (14).

Results

MTT assay

Different concentrations of CLA were not affected on non-infected HeLa cells. The effects of CLA on cell viability of RH and Tehran strains of *T. gondii*, assessed by MTT are shown in Fig.1. Cell proliferation decreased 48 and 72 h post-treatment in RH and Tehran strains of *T. gondii* (Fig. 1), except in the case of 200 µM CLA at 24 and 48 h in the Tehran strain (Fig. 1A). Cell populations gradually declined in RH compared to the Tehran strain (Fig. 1). The IC_{50} value, the concentration at which parasite growth was inhibited by 50%, in the Tehran strain 24, 48, and 72 h post-treatment with CLA was 20, 30, and 100 µM, respectively. IC_{50} in the RH strain after 24 and 48 h was 100 µM and 150 µM after 72 h.

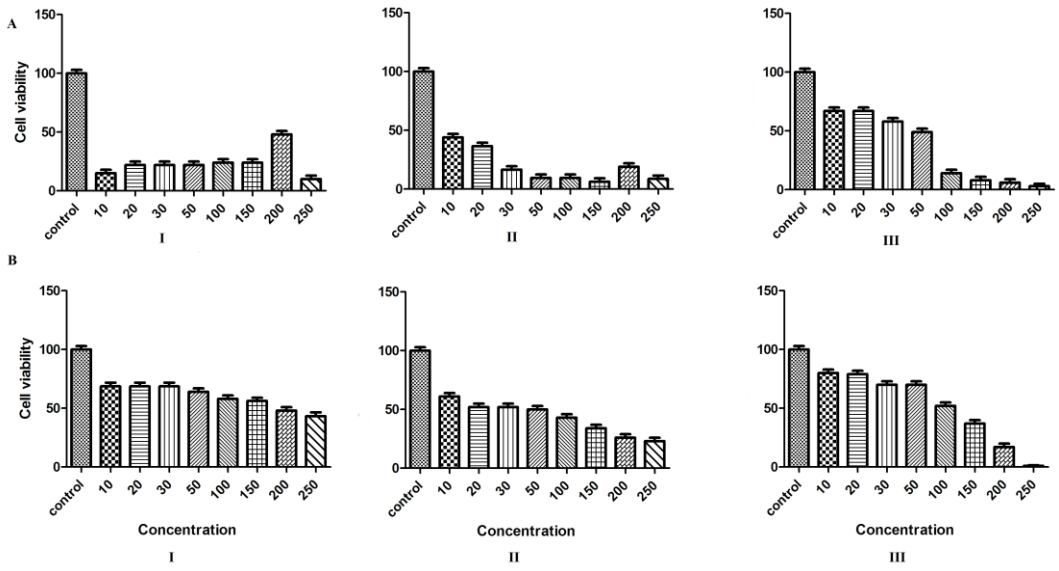

Fig. 1: Effects of various concentrations of conjugated linoleic acid on cell viability of *Toxoplasma* Tehran (A) and RH (B) strains after 24 (I), 48 (II), and 72 h (III). Data are the mean ± SE from at least three separate experiments ($P<0.0001$)

Flow cytometry assay

Flow cytometry assay showed that less than 5 % of tachyzoites dead in the control wells (0 µM CLA). After 24 h treatment of the RH strain tachyzoites, maximum early apoptosis (PS exposure detected by Annex V staining) (39.3%) was observed at 100 µM CLA. At higher concentrations, apoptosis decreased (approximately 20% at 150 to 250 µM). Late apoptosis (disintegration of other organelles of parasites detected by PI staining) was highest with the 50 µM treatment (36.1%) and was not considerable at other exposure times and concentrations in the RH strain (Table 1). After forty-eight hours exposure to CLA at 200 µM, apoptosis increased. The apoptosis rate was 9.8% at 30 µM CLA and increased to a maximum cell death of 54.0% at the concentration of 200 µM. After 72 h, the maximum rate of apoptosis (51.4%) was seen at 250 µM CLA (Fig. 2A).

In the Tehran *T. gondii* strain, early apoptosis was not pronounced (Fig. 2B). Twenty-four hours after treatment with CLA, the maximum rate of apoptosis occurred at 200 µM (34.3%). After 48 h, early apoptosis was the highest (31.4% at 10 µM) in lower concentrations, but with increasing levels of CLA, late apoptosis increased (30.6% at 250 µM). The pattern of apoptosis changed from early apoptosis to late apoptosis after 72 h of treatment (Table 1). Late apoptosis at 150, 200, and 250 µM was 18%, 29%, and 50%, respectively (Fig. 2B).

Table1: Maximum apoptosis rates after treatment of *Toxoplasma* strains with CLA

T. gondii **strain**	**Post-treatment time (h)**					
	24		**48**		**72**	
	[a]Annexin V	**[b]PI/Annexin**	**Annexin V**	**PI/Annexin**	**Annexin V**	**PI/Annexin**
RH	[c] 100	50	200	100	250	20
	[d] (39.3)	(36.1)	(54.0)	(7.5)	(51.4)	(9.0)
Tehran	[c] 200	10	10	250	150	250
	[d] (34.3)	(29.2)	(31.4)	(30.6)	(34.7)	(50.0)

[a]Annexin V: indicates early apoptosis with PS exposure to outer side of cells, stained by Annexin V/[b]PI/Annexin V: indicates late apoptosis that cells are double positive for both dyes/[c] Concentration of CLA (µM)/ [d] Apoptosi s percent

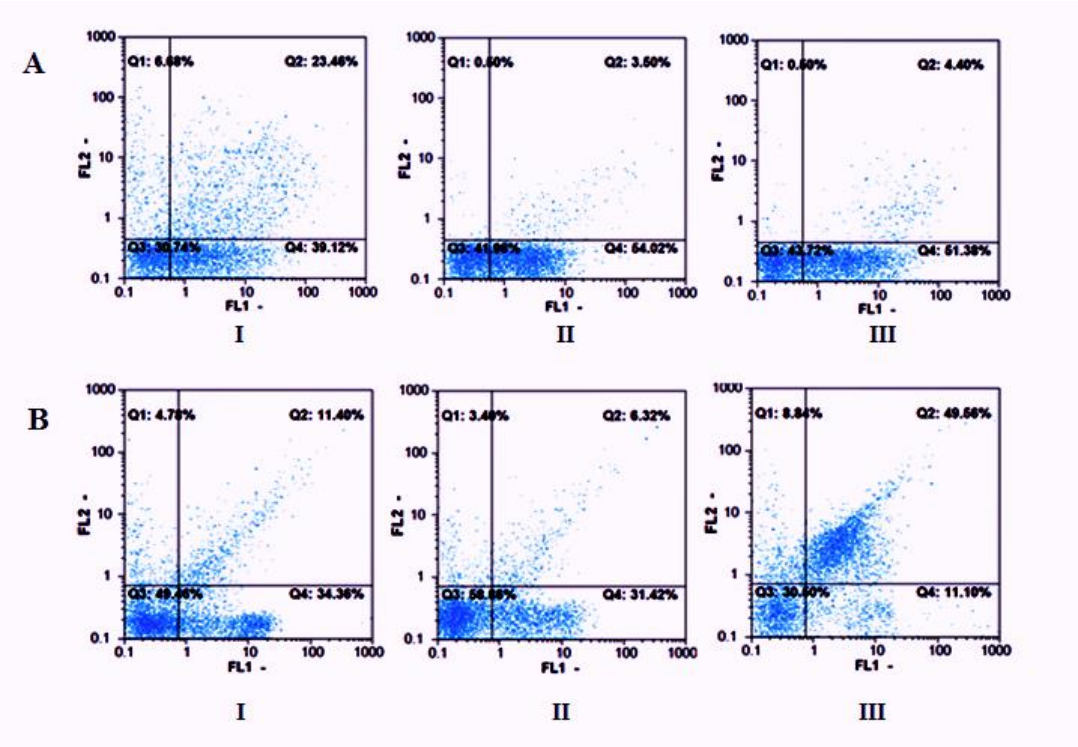

Fig. 2: Flow cytometry results. **(A)** Maximum apoptosis rates at 24 (I), 48 (II), and 72 (III) h post-treatment of RH strain *Toxoplasma* tachyzoites with CLA. **(B)** The highest level of apoptosis was with 200 µM CLA after 24 h post-treatment of Tehran strain (I). The highest apoptosis at 10 µM CLA was after 48 h (II). Late apoptosis at 250 µM CLA after 72 h is shown in Q2 of graph (III). The right lower quadrant of each chart shows cells stained with Annexin V

Discussion

Beneficial effects attributed to polyunsaturated fatty acids (PUFA) include improving heart disease related outcomes, decreasing tumor cell proliferation and metastasis, and enhancing of insulin sensitivity in some patients (5, 9). Biosynthesis of some essential fatty acids could be inhibited in tested parasites and microorganisms by CLA (6-8). Fatty acids have important roles in immune system function. Some are precursors of prostaglandins, leukotrienes, and compounds that play critical roles in inflammation and immunity (16). Human platelets have a cytotoxic effect on *T. gondii*, with thromboxane playing a key role in this process. Other lipid mediators released from platelets after incubation with *Toxoplasma* decrease parasite growth and reproduction (17). Fatty acids are important components of the human immune system, and therapeutic use lacks documented side effects.

Henderson and Chi (1992) studied toxoplasmacidal characteristics of linoleic acid, 13-hydroxyoctadecadienoic acid (13-HODE), the metabolite of linoleic acid and 12-hydroxyeicosatetraenoic acid (12-HETE) from arachidonic acid. They found that, concentrations of 1 µM or less (10^{-6}-10^{-10}M) of linoleic acid and 12-HETE, did not showed cytotoxic activity but 13-HODE at concentrations of 0.01 μM (10^{-8} M) rapidly induced cytotoxic changes in *T. gondii (18)*.

Kumaratilake et al. (6) showed that oxidized forms of fatty acids have higher activity against *Plasmodium falciparum* compared to unoxidized forms. Fatty acids vary in ability to inhibit parasite growth, depending in part on saturation. Fatty acids with two or more double bonds, such as CLA, show high anti-para-

sitic activity in culture (6).The decreased cell viability and induction of apoptosis observed in our study may be the result of CLA conversion to active agents inside the cells with ability to integrate into the cell membrane, causing cell permeability and death. The yeast-to-hypha transition in the opportunistic fungus *Candida albicans* that is essential for its pathogenicity is inhibited by CLA (8).Conjugated linoleic acid down regulates genes involved in signal transduction, including the GTPase gene *RAS1*, the adenylate cyclase gene *CYR1*, and mucin-like signaling protein gene *MSB2* in C. *albicans* (8). This may also be the source of inhibition of growth of *T. gondii* tachyzoites in culture.

The IC_{50} levels of the Tehran strain at 24, 48, and 72 h post-treatment with CLA are consistent with studies of methyl esters of linoleic acid on *P. falciparum* (7). Tachyzoites of the RH strain results showed low sensitivity to CLA, and flow cytometry and MTT showed the Tehran strain to be more susceptible to CLA than was the RH strain. Resistance to CLA can be attributed to differing expression of proteins involved in drug uptake and metabolism, as demonstrated by Doliwa et al. (19). The Tehran strain showed early apoptosis at low concentrations of CLA, as indicated by translocation of phosphatidyle serine (PS) from the inner surface of the cell membrane to the outer surface, as detected by Annexin V staining (20). Apoptosis is an active energy-dependent process that generally occurs without inflammation or injury to surrounding tissue (21).

Conclusion

CLA may be a candidate for treatment of toxoplasmosis. Further studies to investigate other characteristics of CLA are recommended.

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