Original Article

The anti-proliferative and anti-invasive effects of the estrogen receptor modulator (Tamoxifen) and aromatase inhibitor (Letrozole) on *T. gondii* (RH strain) tachyzoites *in vitro*

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ABSTRACT

Background: Tamoxifen is the first line of treatment in hormone receptor-positive breast cancer. Letrozole is developed for postmenopausal patients and metastatic breast cancer. Patients with concomitant chronic toxoplasmosis are at risk of reactivation of tissue cysts. It is still unknown whether these drugs will cause protection against reactivated toxoplasmosis or exacerbated symptoms.
Objective: This study aimed to investigate the possible effect of Tamoxifen and Letrozole on *T. gondii* freed bradyzoites in case of rupture tissue cyst with their subsequent reactivation to tachyzoites.
Material and Methods: The study was conducted using *in vitro* cultured *T. gondii* tachyzoites in a cultured *Vero* cell line. Different concentrations of Tamoxifen and Letrozole were used (0.24-125 ug/ml). Anti-proliferative effect was determined by methyl thiazolyl tetrazolium (MTT) cytotoxicity assay estimated as cell line viability% reflecting the inhibition of tachyzoites proliferation after 24 and 48 h treatment. Anti-invasive effect was determined by counting Giemsa-stained intracellular tachyzoites inside infected *Vero* cells using light microscopy. Induced cytotoxic morphological changes in the tachyzoites was determined by transmission electron microscopy (TEM).
Results: By MTT, cytotoxicity of both drugs to the infected cultured *Vero* cells indirectly assayed the decreased tachyzoites proliferation compared to the control drug-free parasites (P<0.01). Letrozole proved to be more cytotoxic than Tamoxifen after 48 h treatment (P<0.05). Both drugs decreased the ability of tachyzoites' invasion compared to drug-free tachyzoites (P<0.05) with no statistical difference between the used drugs. Additionally, TEM demonstrated tachyzoites damage caused by both drugs.
Conclusion: Tamoxifen and Letrozole demonstrated anti-toxoplasmic activity, indicating that administering these drugs in cancer patients may also prevent the possibility of reactivated toxoplasmosis. *In vivo* studies are recommended to evaluate their efficacy.

Keywords: cancer breast; *in vitro;* letrozole; protective therapy; reactivated toxoplasmosis; tamoxifen; TEM.

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INTRODUCTION

Toxoplasmosis is a zoonotic infection caused by *T. gondii*, an obligate, intracellular, apicomplexan protozoan with worldwide distribution[1]. It can affect 30%–50% of the world's population[2]. The outcome of the infection depends on patients' immune status. The acute phase generally produces either asymptomatic or flu-like symptoms in immunocompetent adults[3]. In contrast, the latent stage of infection is characterized by the presence of bradyzoites within tissue cysts formed in the skeletal muscle and central nervous system[4]. Contini[5] advocated that immunosuppression markedly increased the risk of reactivated toxoplasmosis with cyst rupture, conversion of latent bradyzoites to rapidly dividing tachyzoites, and development of reactivated disease, resulting in a recrudescent acute infection and causing a potentially lethal toxoplasmic encephalitis. Individuals with compromised immune systems, like cancer patients receiving immunosuppressive drugs and HIV/AIDS patients, might experience neurologic, ocular, or systemic toxoplasmosis with widespread organ damage and a high mortality rate[6,7].

A combined pyrimethamine and sulfadiazine therapy, targeting folate metabolism, is commonly used in treatment for toxoplasmosis[8]. Other available lines of treatment include atovaquone and clindamycin, targeting mitochondrial enzymes and protein synthesis, respectively. Unfortunately, they are poorly tolerated and inefficient against bradyzoites. In addition, drug resistance was commonly developed. Bradyzoites exacerbations may occur in immunocompromised patients. This encouraged the investigation of the impact of drugs administered for control of the original malignant disease on the reactivation of bradyzoites[9-11].

In fact, the protocols for treatment in patients with malignancy is a challenge leading to immunosuppressive states that might exacerbate

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other latent diseases, e.g., toxoplasmosis[12,13]. Activation of latent toxoplasmosis was confirmed by reports after the use of anticancer drugs as chemotherapy in patients with malignancy[14,15]. However, hormonal therapy with Tamoxifen in toxoplasmosis was evaluated in experimental animals with no conclusive effect[16].

According to the national population-based cancer registry in Egypt, the most common cancer in females is breast cancer[17]. The hormone receptor-positive breast cancer is the most prominent type expressing estrogen or progesterone receptors[18]. Tamoxifen is commonly used to prevent and treat breast cancer as well as to decline morbidity risk. It is a selective estrogen receptor modulator used as an adjuvant treatment in breast cancer[19,20]. It has an antagonistic effect on α and β estrogen receptors[21] besides its immunomodulatory effect[22]. Due to its low cost and safety profile, its use is now generalized worldwide[23]. Furthermore, Tamoxifen was reported to exhibit antibacterial, antiviral, and even anti-parasitic effects[24]. It exhibited anti-protozoan activity *in vitro* against *T. cruzi*[25], and different *Leishmania* spp.[26]. It was used as topical therapy in a cutaneous leishmaniasis experimental model and efficiently decreased lesion size and parasite load[27]. Antimalarial activity was also observed *in vitro* against *P. falciparum*[28]. Scientists recorded considerable evidence that steroid hormones affect the course of toxoplasmosis in humans and mice. Accordingly, estrogen administration in mice was found to increase the disease severity[29]. Few studies conducted to study the effect of Tamoxifen on toxoplasmosis demonstrated its role in decreasing *T. gondii* load[30], while others incriminated its use because it caused disease progression[16,31].

Other lines of cancer breast treatment evolved mainly for postmenopausal women, included third-generation aromatase inhibitors like Letrozole and Anastrozole. Aromatase inhibitors are critical for preventing the progression of estrogen-related diseases such as breast cancer[32]. Letrozole acts by preventing the conversion of androgens to estrogens and decreasing estrogen levels in tissue and plasma without affecting estrogen levels in ovaries. It is the more effective treatment for metastatic breast cancer and in the neoadjuvant setting than Tamoxifen[33]. Fadrozole, another drug with the same mechanism of action, and investigated as anti-parasitic, was found to inhibit the parasite burden of *T. crassiceps* cysticercosis in mice[34].

Due to the worldwide increased incidence of breast cancer and *T. gondii* opportunistic nature, there is fear for the increasing risk of complication with toxoplasmosis in those patients[16]. Moreover, the controversial results of Tamoxifen raised concerns about its actual effect on reactivated toxoplasmosis[16,30]. Besides, metastatic patients who are at a greater risk due to their increased immunocompromised state are shifting their line of therapy to another drug, Letrozole[33]. To our knowledge, no data is available about the possible effects of Letrozole in chronic toxoplasmosis and its impact on reactivated toxoplasmosis. Accordingly, our study aimed to investigate the possible effect of Tamoxifen and Letrozole on *T. gondii* tachyzoites simulating freed bradyzoites in case of rupture of tissue cyst and their subsequent reactivation as tachyzoites.

MATERIAL AND METHODS

This descriptive analytical study was conducted in Diagnostic and Research Laboratories, Parasitology Department, Faculty of Medicine, Ain-Shams University during the period from August 2021 to February 2022.

Study design: Virulent *T. gondii* (RH strain) was used to evaluate the anti-proliferative and anti-invasive effects of Tamoxifen and Letrozole. The anti-proliferative effect of different drug concentrations was evaluated in infected *Vero* cell line cells using MTT assay. The anti-invasive effect was evaluated by counting number of intracellular tachyzoites in Giemsa stained smears using light microscopy. Induced morphological changes of tachyzoites was studied using TEM.

Parasites: Virulent *T. gondii* (RH strain) was kindly obtained from Medical Parasitology Department, Faculty of Medicine, Ain Shams University. The strain was maintained by serial intraperitoneal injection of Swiss albino mice every 3-4 days. An inoculum of approximately $2x10^6$ tachyzoites was adjusted in 0.2 ml sterile saline using a hemocytometer counted under a light microscope. The tachyzoites were harvested under sterile conditions, and the peritoneal aspirate was washed three times with RPMI 1640 media (Sigma-Aldrich, USA) containing 1% penicillin and streptomycin (Sigma-Aldrich, USA)[35].

Cell line preparation[11]: The *Vero* cells were provided from VACSERA company, Egypt, and maintained in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml Penicillin, and 100 µg/ml Streptomycin (Sigma-Aldrich, USA) in 75 cm^2 flasks, and incubated in 5% CO_2 at 37°C[10]. The *in vitro* study was performed at the International Center for Training and Advanced Research (ICTAR, Egypt), Al-Azhar University, Cairo, Egypt.

Pilot cytotoxicity assay: Cytotoxicity of both drugs to *Vero* cells was evaluated by MTT assay[36]. *Vero* cells (2×10^5/ml, 100 ul/well) were seeded in 96-well plates (Nunc; Fisher Scientific, Pittsburgh, USA) and cultured in 10% FBS-RPMI for 24 h to obtain a monolayer. *Vero* cell monolayers were washed and directly subjected to different concentrations of both drugs. The concentrations were started from a very high dilution to obtain a wide range and then further diluted from

1000 to 0.24 ug/ml to determine the concentration that will be used for the following experiments. Drug-free control wells were enrolled in the study as negative control. The MTT assay is based on the conversion of MTT to formazan crystals in living cells which determines mitochondrial enzyme activity. The assay was performed according to instructions of the *in vitro* Toxicology Assay Kit (Sigma-Aldrich, USA). An ELISA reader recorded the optical density (OD) at a 570 nm filter (ELX-800 Biotek, USA)[36]. The MTT assay revealed that different concentrations of Tamoxifen had no cytotoxicity on *Vero* cells compared with the negative control. In contrast, Letrozole showed the beginning of cell death at 250 ug/ml. Accordingly, a concentration starting from 125 ug/ml with different dilutions was chosen for evaluating both drugs.

Drugs preparation: Tablets of Tamoxifen (Nolvadex™, 10 mg), and Letrozole (Femara™, 2.5 mg) were obtained commercially. One tablet of each drug was crushed, and dissolved in one ml 99% dimethyl sulfoxide (DMSO) obtained from Sigma-Aldrich, USA. An equal amount of medium was added to 0.2 ml dissolved drug followed by serial two fold dilutions until reaching the desired concentration (125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.9, 0.95, 0.47, and 0.24 mg/ml) in cell culture experiments[37].

Proliferation assay: Cultured host cell viability was assessed by MTT to indirectly reflect the drug's inhibitory effect on the parasites, whose proliferation caused damage to the cultured cells. *Vero* cells were transferred to 96-well tissue culture plates and grown to confluence in a humidified incubator at 37°C with 5% CO_2. Infection with *T. gondii* RH strain tachyzoites was performed at a concentration of 5 tachyzoites for each *Vero* cell[11]. After three hours of infection, cells were washed to remove nonadherent parasites and then treated with serial dilutions of the drugs (125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.9, 0.95, 0.47 and 0.24 ug/ml) for 24 h and 48 h. Drug-free *T. gondii*-infected *Vero* cells were used as a positive control, while *Vero* cells without tachyzoites were used as a negative control. The *Vero* cell viability% was calculated as: [percentage of the mean OD of treated wells/mean OD of negative control]. Additionally, inhibition rate (%) of *T. gondii* proliferation was calculated for each concentration by the following equation: Inhibition rate (%) = [(mean OD of treated wells – mean OD of positive control)/ mean OD of negative control)] x 100[38]. The experiment including positive and negative controls was performed in triplicate.

Invasion assay: Evaluation of invasion was conducted using three concentrations, 0.24, 3.9, and 125.0 ug/ml of both drugs to represent low, intermediate, and high concentrations, respectively. They were randomly chosen as every 5th sample concentration. Tachyzoites were incubated with these concentrations for two hours and were allowed to infect the *Vero* cell culture monolayer on coverslips placed inside 6 well culture plate ($2x10^4$ cells/well) using the previously mentioned ratio (5 tachyzoite/each cell)[39]. After two hours of incubation, the cells were washed twice with PBS to remove the extracellular parasites. Coverslips were transferred to small petri dishes for fixation with methanol for 5 min and staining with Giemsa for 15 min. The invasion rate was estimated by counting tachyzoites invading 200 *Vero* host cells, depicted by their nuclei, under light microscopy. The total number of tachyzoites in 200 cells divided by 200 was calculated[39]. Counts were done in triplicate to obtain mean and SD for each concentration.

Induced cytotoxic morphological changes determined by TEM[39]: To evaluate the effect of both drugs on the morphology of *T. gondii* tachyzoites, the lowest concentration of both drugs (0.24 ug/ml) that gave a significant difference with drug-free (control tachyzoites) was used. The effect of both drugs on tachyzoites morphology was evaluated by allowing *Toxoplasma* tachyzoites to infect *Vero* cells for three hours, followed by incubation with each drug in 75 cm2 flask for 24 and 48 h. TEM image was also taken after allowing pretreated tachyzoites for two hours, to infect *Vero* cells in 75 cm^2 flasks for 60 min. The cells in all experiments were fixed and examined using TEM at the Regional Center for Mycology and Biotechnology, El Azhar University, Cairo, Egypt.

Statistical analysis: Data were analyzed using IBM SPSS statistics (ver 18.0) and Rstudio program (ver 1.4.1106). All values were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA, post hoc Tukey's tests, and *t*-test. IC50 was calculated using online LC 50 calculator (AAT Bioquest). Statistical significance was considered when *P* value ≤0.05.

Ethical consideration: The study was conducted following the ethical guidelines of animal experiments, and after the Ethical Committee approval of the Medical Research Institute, Ain Shams University.

RESULTS

Anti-proliferative activity: Tamoxifen and Letrozole ability to inhibit intracellular tachyzoite proliferation within *Vero* cells was evaluated using the MTT assay at 24 and 48 h post-treatment. Cell viability can indirectly reflect the drug's inhibition effect against parasites, as *T. gondii* tachyzoites cause damage to *Vero* cells after proliferation. Tamoxifen's IC50 (50% anti-parasitic concentration) was 2.04 ug/ml at 24 h and 0.119 ug/ml at 48 h. While IC_{50} of Letrozole was 2.82 ug/ml at 24 h and 0.52 ug/ml at 48 hr. There was a significant statistical difference between treated tachyzoites with different concentrations of Tamoxifen and Letrozole in comparison to drug-free (positive control) (P<0.01) (Figs. 1 and 2).

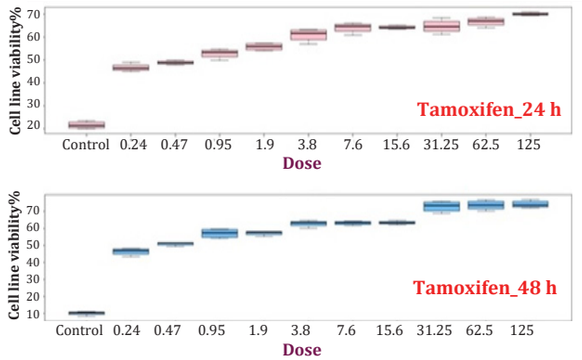

Fig. 1. The effect of different concentrations (0.24-125.0 ug/ml) of Tamoxifen on *T. gondii*-infected *Vero* cells after 24 and 48 h treatment using MTT assay. *Vero* cells infected with drug-free tachyzoites were used as a positive control. Cell line viability% reflects the inhibition of parasite proliferation. Data represent mean±SD. There is a significant difference between all concentrations and the control parasite (P<0.01). Data were analyzed using one-way ANOVA and post hoc Tukey's tests for comparison between groups.

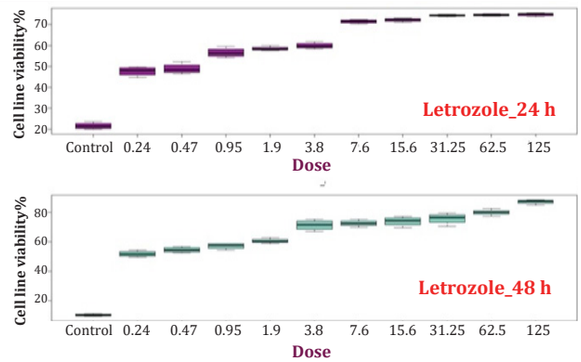

Fig. 2. The effect of different concentrations (0.24 ug/ml-125ug/ml) of Letrozole on *T. gondii*-infected *Vero* cells after 24 and 48 h treatment using MTT assay. *Vero* cells infected with drug-free tachyzoites were used as a positive control. Cell line viability% reflects the inhibition of parasite proliferation. Data represents mean±SD. There is a significant difference between all concentrations and the control parasite (P<0.01). Data were analyzed using one-way ANOVA and post hoc Tukey's tests for comparison between groups.

Three doses of each drug were compared and found to have a statistically significant difference between low, median, and high concentrations (Fig. 3). The percentage of tachyzoites inhibition was calculated in comparison to the positive non treated control group (Fig. 4). Tamoxifen inhibited *T. gondii* proliferation in a dose-dependent matter, starting from 25.08% inhibition using 0.24 ug/ml and reaching 48.50% inhibition using 125 ug/ml at 24 h. Similar results were obtained using Letrozole, with a percentage

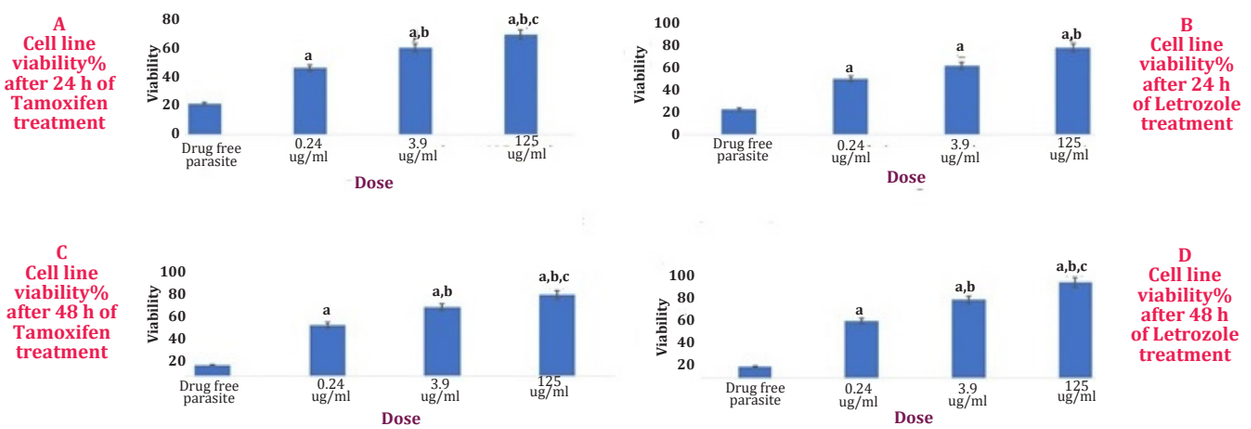

Fig. 3. Comparative effect of different concentrations (low, intermediate, high) of Tamoxifen and Letrozole on *T. gondii*-infected *Vero* cells compared to the control drug-free parasites using MTT assay. **(A)** Effect of Tamoxifen on *T. gondii* infected *Vero* cells after 24 h treatment. **(B)** Effect of Letrozole on *T. gondii* infected-*Vero* cells after 24 h treatment. **(C)** Effect of Tamoxifen on *T. gondii* infected-*Vero* cells after 48 h treatment. **(D)** Effect of Letrozole on *T. gondii* infected-*Vero* cells after 48 h treatment. Data represents mean±SD: **"a"** means statistically significant difference with respect to the drug-free parasite group's value (P≤0.05), **"b"** represents statistical significance compared to the 0.24 µg dose group (P≤0.05), and **"c"** means statistical significance compared to the 3.9 µg dose group (P≤ 0.05). Data were analyzed using one-way ANOVA and post hoc Tukey's tests for comparison between groups.

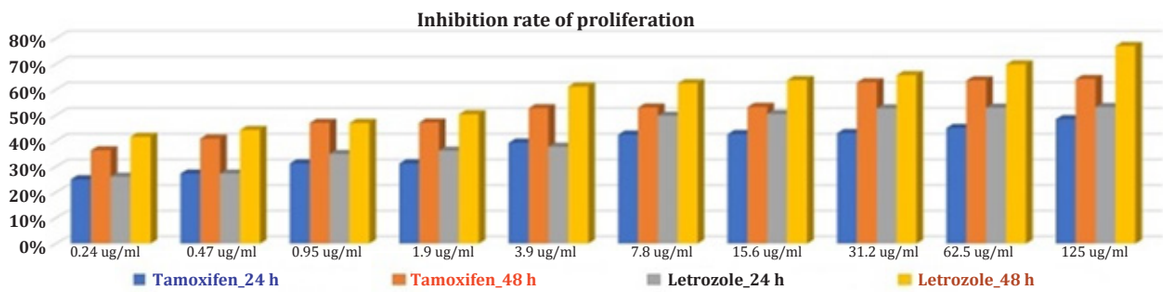

Fig. 4. Effect of Tamoxifen and Letrozole on *T. gondii* proliferation relative to cultured host cell viability after treatment with different concentrations of both drugs for 24 and 48 h using MTT assay.

of inhibition starting from 26.06% using 0.24 ug/ml and reaching 53.16% at 24 h. At the same time, the percentage of inhibition ranged from (36.35%-64.07%) and (41.63%-76.98%) using Tamoxifen and Letrozole for 48 h, respectively (Fig. 4). Letrozole was found to have an inhibitory effect better than Tamoxifen in concentrations >7.8 ug/ml after 24 h treatment. It had a better inhibitory effect in almost all concentrations at 48 h treatment (Tables 1 and 2).

Table 1. Comparison between the inhibition of tachyzoites proliferation, indirectly assessed by cultured host cell viability, after 24 h treatment with different concentrations of Tamoxifen and Letrozole assayed by MTT.

Dose µg/ml	Inhibition of tachyzoites proliferation after 24 h		Statistical analysis
	Tamoxifen	Letrozole	*P* value
0.24	25.08 ± 0.58	26.06 ± 0.43	0.501
0.47	27.26 ± 0.83	27.27 ± 1.01	0.997
0.95	31.24 ± 0.45	34.93 ± 1.25	0.052
1.90	34.23 ± 1.05	36.22 ± 1.11	0.784
3.9	39.34 ± 0.81	37.75 ± 6.62	0.715
7.8	42.5 ± 4.42	49.8 ± 3.8	**0.004***
15.6	42.66 ± 1.13	50.5 ± 0.5	**0.00***
31.25	43.07 ± 0.36	52.67 ± 0.43	**0.006***
62.5	45.09 ± 1.5	52.91 ± 0.3	**0.002***
125	48.5 ± 0.96	53.16 ± 0.3	**0.00***

Data are represented as mean±SD and analyzed using *t*-test.
***:** Significant (*P*≤ 0.05).

Table 2. Comparison between the inhibition of tachyzoites proliferation indirectly assessed by cultured host cell viability after 48 h treatment with different concentrations of Tamoxifen and Letrozole assayed by MTT.

Dose µg/ml	Inhibition of tachyzoites proliferation after 48 h		Statistical analysis
	Tamoxifen	Letrozole	*P* value
0.24	36.35 ± 1.05	41.63 ± 1.02	**0.011***
0.47	40.91 ± 0.56	44.3 ± 0.98	**0.032***
0.95	47.02 ± 1.35	46.99 ± 1.06	0.987
1.90	47.16 ±0.7	50.38 ± 0.83	**0.026***
3.9	52.75 ± 0.93	61.18 ± 1.77	**0.01***
7.8	53.04 ± 2.09	62.43 ± 1.1	**0.013***
15.6	53.26 ± 0.54	63.68 ± 1.6	**0.005***
31.25	62.82 ± 1.65	65.64 ± 1.68	**0.303**
62.5	63.55 ± 1.4	69.84 ± 1.02	**0.013***
125	64.07 ± 1.1	76.98 ± 0.77	**0.00***

Data are represented as mean±SD and analyzed using *t*-test.
***:** Significant (*P*≤ 0.05).

Invasion assay: The control non treated parasites culture comprised the highest number of intracellular parasites (40.6%±1.24), detected by light microscopy (Fig. 5). In contrast pretreated tachyzoites with different Tamoxifen and Letrozole concentrations (0.24, 3.9 and 125 ug/ml, respectively) showed statistically significant decrease in invasion (*P*<0.05) (Fig. 5). There was no significant difference between the two drugs. Tamoxifen mean invasion assays were 25.0±0.14, 22.0±0.81, and 18.6±0.94, while Letrozole mean invasion assays were 23±1.63, 19±0.81, and 16.6±0.47.

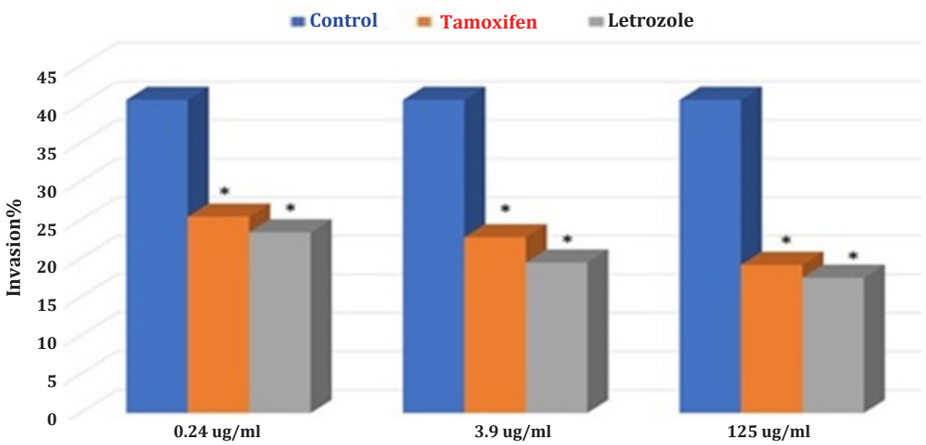

Fig. 5. Invasion% of *T. gondii* tachyzoites treated with different concentrations of Tamoxifen and Letrozole compared to drug-free parasites. Evaluation was by counting tachyzoites in Giemsa-stained preparation.
***:** Significant (*P*≤0.05) compared to control non treated parasites.

Evaluation by TEM

Evaluation of the effect of both drugs after incubation for 24 h and 48 h: Cells infected with tachyzoites treated with the lowest concentration (0.24 ug/ml) of both drugs for 24 h and 48 h were compared to cells infected with control drug-free tachyzoites. Control parasites demonstrated the normal morphology of tachyzoites (Fig. 6a) and also showed more than one intracellular parasite within parasitophorous vacuoles (PVs) (Figs. 6 b-d). The number of tachyzoites present in PVs was lower in almost all pictures taken by TEM in treated samples (Figs. 7 b-f). Moreover, treated parasites with Tamoxifen (Fig. 7a) and Letrozole (Fig. 7b) for 24 h showed swelling of the tachyzoite with accumulation of intra-parasitic vacuoles and alteration in cell membrane, which increased after 48 h incubation with Tamoxifen (Figs. 7 c-d) and Letrozole (Figs. 7 e-f). These findings confirmed the lethal effect of both drugs on tachyzoites affecting their replication. TEM taken after one-hour incubation with *Vero* cells

demonstrated more of the intracellular drug-free tachyzoites (control) present inside parasitophorous vacuoles (Fig. 8a), than in the 0.24 ug/ml Tamoxifen and Letrozole treated cultures. Tachyzoites were seen only outside or attached to the cell membrane (Figs. 8 b, c).

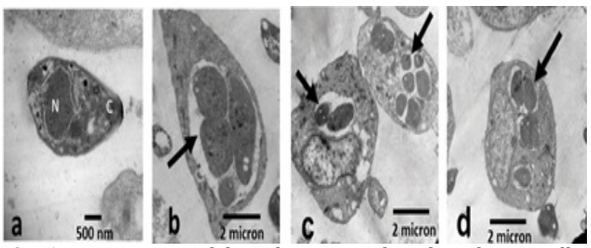

Fig. 6. TEM images of drug-free *T. gondii*-infected *Vero* cells. **(a):** A tachyzoite showing normal morphology (x10300); **(b-d):** presence of multiple tachyzoites inside PVs (arrow) (x3480). **N:** Nucleus, **C:** Conoid.

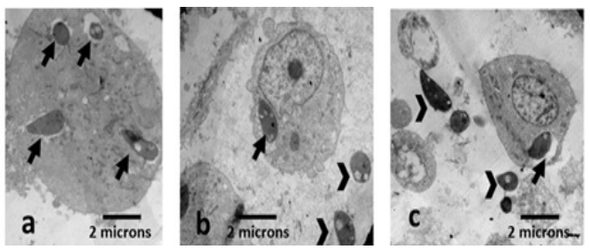

Fig. 8. TEM images of *T. gondii* tachyzoites after one hour incubation with *Vero* cells. **(a):** Drug-free tachyzoites (control) showing more than one parasite visible in PVs (arrow) (x2610). **(b):** Treated tachyzoites with Tamoxifen 0.24 ug/ml. **(c):** Treated tachyzoites with Letrozole 0.24 ug/ml. Both **(b)** and **(c)** showed few intracellular tachyzoites (arrow), while most were only outside or attached to the cell membrane (arrowhead) (x2610).

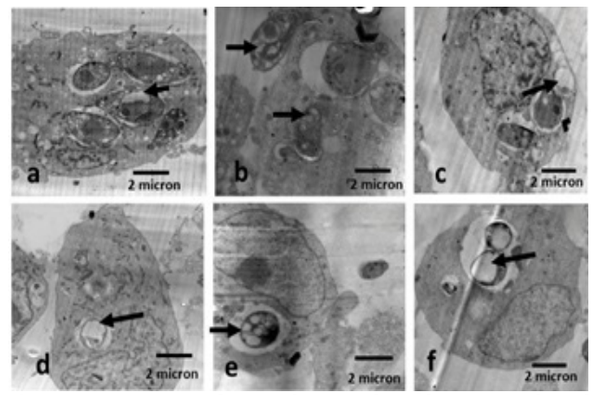

Fig. 7. TEM images of treated *T. gondii* tachyzoites-infected Vero cells. Treated tachyzoites with Tamoxifen **(a)** and Letrozole **(b)** after 24 h showing swelling of the tachyzoite with vacuoles starting to accumulate inside the tachyzoites (arrows) and alteration in the cell membrane (head of arrow) **(b)**. There is extensive damage with replacement of normal morphology of tachyzoites by vacuoles after 48 h treatment by both Tamoxifen **(c-d)** and Letrozole **(e-f)** (arrow) with alteration of the membrane (arrowhead) **(c)** (x3480).

DISCUSSION

Since *T. gondii* is an opportunistic parasite, it represents a significant cause of morbidity in cancer patients. Most cancer patients are in a state of impaired immune response either from the cancer itself, or the anticancer treatment that usually includes chemotherapy and radiotherapy administration. All of which may lead to progression and exacerbation of toxoplasmosis[13]. It was suggested that some hormones as progesterone, and estradiol, induce *Toxoplasma* pathogenicity, which explains the increased prevalence among women. The presence of an estradiol regulatory factor-hydroxysteroid dehydrogenase (HSD) gene, in *T. gondii* was implicated[40]. Our study investigated the possible effect of Tamoxifen and Letrozole on *T. gondii* trophozoites *in vitro*, simulating the risk of increase or decrease of disease progression in cancer patients with exacerbated toxoplasmosis.

Both drugs showed anti-proliferative and anti-invasive effect on treated *T. gondii* in comparison to drug-free (control) parasite. *In vitro* studies done on *T. gondii* demonstrated the same results. Zhang *et al*.[40] stated that Tamoxifen decreased the number of tachyzoites by approximately 50% in host cells. Galván Ramírez *et al*.[30] noted a decrease in *Toxoplasma* tachyzoites inside neurons. Halonen *et al*.[41] observed a similar effect in astrocytes infected by *T. gondii*.

On the other hand, *in vivo* studies showed controversial results. Barakat *et al*.[16] found that Tamoxifen increased the parasite burden in animals treated with the drug compared to the control group. It induced a series of histopathological and immunohistochemical changes in many organs. Also, Cervantes-Candelas *et al*.[42] stated that Tamoxifen could exacerbate symptoms in animals infected with *P. berghei*. This controversy in results between *in vitro* and *in vivo* studies could be attributed to the immunomodulatory role of Tamoxifen in experimental animals[16,42].

Tamoxifen's role as an anti-microbial, and anti-parasitic drug is due to boosting innate immunity. It activates macrophages through estrogen receptor-independent targets of Tamoxifen which potentiate an inflammatory response that could lead to tissue damage[43]. Tamoxifen can induce a shift from cellular (Th1) to humoral (Th2) immunity. It decreases interferon gamma (IFNγ) production (a Th1 cytokine) and generates an antibody class switch indicative of a Th2 response. Also, Cervantes-Candelas *et al*.[42] stated that Tamoxifen has a potent immunomodulator

effect in mice infected with malaria and recommended attention when administering it to malaria-infected women with breast cancer.

In contrast, aromatase inhibitors such as Letrozole and Anastrozole have different mechanisms of action. They can increase Th1 response by increasing the levels of the proinflammatory cytokines IFNγ and interleukin (IL)-12. At the same time, Th2 is suppressed with decreased IL-4 and IL-10 cytokines levels. They also suppress the differentiation of *naïve* T cells into T-regulatory cells and reduce the production of immunosuppressive cytokines[44]. Morales-Montor *et al*.[34] also stated that drugs acting by inhibiting aromatase enzyme were found to have a protective effect from cysticercosis through the recovery of the specific cellular immune response and the increased production of IL-6. This difference may boost the possibility of a better effect of Letrozole on *Toxoplasma*-infected animals.

In our study, both drugs inhibited the invasion of *T. gondii* significantly as compared to control parasites ($P<0.05$). In contrast, there was no statistical difference between the effect of Tamoxifen and Letrozole on invasion. Numerous drug-free parasites were found inside PVs compared to treated parasites with Tamoxifen and Letrozole. TEM analysis showed that Tamoxifen and Letrozole significantly destroyed the tachyzoite structure, resulting in alteration of the cell membrane and vacuolization of the cytoplasm after 24 and 48 h treatment in comparison to the control parasite. Zhang *et al*.[45] reported similar results using Licarin-B in their experiment. Their results were attributed to a process called autophagy, in which there is damage of mitochondria and cell membrane. Dittmar *et al*.[10] also explained that Tamoxifen could reduce the parasite burden through the same process.

In conclusion, Tamoxifen and Letrozole used in the treatment of patients with breast cancer, demonstrated anti-proliferative and anti-invasive effects on *T. gondii* tachyzoites. Letrozole showed a better outcome than Tamoxifen, especially after 48 h treatment, which should be further evaluated using *in vivo* studies. Administration of these drugs in cancer patients may decrease the possibility of disease reactivation.

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Author contribution: Zahran F proposed the study conception and design, analyzed and interpreted the results. Meselhey RA collected the data, and performed the experiments. Both authors wrote the manuscript, approved the final version, and confirmed the order of authorship.

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