JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Toxoplasma gondii destroys Her2/Neu-expressing mammary cancer cells *in vitro* using a continuous feed medium approach

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Abstract

Introduction: *Toxoplasma gondii* is an opportunistic protozoan and can be grown using several human cell lines. Breast cancer is the second leading cause of cancer death in women. Her2/Neu-expressing mammary cancer cell lines called TUBO can be grown *in vitro*. In recent years, protozoan parasites have become popular means of use in cancer therapy research. In this study, we analyzed whether *T. gondii* tachyzoites can destroy TUBO cells using a novel continuous feed medium approach.

Methodology: Two sets of flasks (each containing four groups) containing TUBO cells were inoculated with *T. gondii* Ankara strain tachyzoites. First set containing 5×10^6 TUBO cells were inoculated with TUBO-tachyzoite ratios of 1:2, 1:1, 2:1, and 4:1 and second set containing 1×10^6 TUBO cells were inoculated with TUBO-tachyzoite ratios of 10:1, 100:1, 1000:1, and 2000:1. Thereafter, culture supernatants were harvested at various days until TUBO cells were destroyed and tachyzoites were counted.

Results: In the first and second sets of flasks, TUBO cells were destroyed between days 8 to 12 and 12 to 25, respectively. In addition, the amount of tachyzoites increased 7- 43 and 595 to 112500 times in the first and second set of flasks, respectively.

Conclusions: These results show that *T. gondii* tachyzoites successfully destroy Her2/Neu-expressing mammary cancer cells using a continuous feed medium approach. Although this idea may be too premature for the moment, the approach defined herein may support future researchers investigating the relationship between cancer and parasites which can make important progress toward saving cancer patient lives.

Key words: *Toxoplasma gondii*; cancer; breast cancer; *in vitro*; Her2/Neu; TUBO.

J Infect Dev Ctries 2020; 14(10):1204-1209. doi:10.3855/jidc.12820

(Received 15 April 2020 – Accepted 07 October 2020)

Introduction

Toxoplasma gondii is an opportunistic protozoan parasite that can infect all warm-blooded animals, birds, and humans [1-3]. *T. gondii* tachyzoites can be continuously grown *in vitro* using HFF (Human foreskin fibroblasts) cells for biological and structural studies [4].

In recent years, *T. gondii* has been analyzed for its anticancer properties [5-9]. *T. gondii* has shown to increase survival against lung ovarian cancer, melanoma, and pancreatic cancers in murine models [5, 6, 9]. These studies show the importance of *T. gondii* as a possible bioagent to fight against cancer. According to Zloza, 2018; researchers have begun to realize the limitlessness of the undiscovered area covering the interactions between pathogens and cancer [10]. Moreover, there are new knowledge and novel tools to uncover these interactions which can make important progress toward saving cancer patient lives [10].

T. gondii tachyzoites can invade and destroy various cell human cancer cell lines including HeLa (cervical cancer), Her-2 (human larynx carcinoma cells), and A549 (human lung carcinoma) [11-13]. *T. gondii* vaccine containing tachyzoites was found to effectively stimulate potent immunity, increasing survival, and preventing the recurrence of cancer types (6, 7, 9). Based on these data, we aimed to investigate whether *T. gondii* tachyzoites can invade and destroy

breast cancer cells which has not been tested yet in *in vitro* or *in vivo* models.

The reason why we choose breast cancer cell line is that breast cancer is the most frequent diagnosed cancer in women worldwide [14]. Cancer occurs when the host immune system is not able to destroy or eliminate the abnormally dividing cells and fails to enclose the tumor [15]. HER2 (human epidermal growth factor receptor 2) or HER2/Neu is an oncogene that can be overexpressed in breast cancer and associated with recurrence and death [16]. The breast cancer cell line that we will use in this study is the Her2/Neu-expressing mammary cancer cell lines called TUBO [17]. Overall, we aimed to analyze whether *T. gondii* tachyzoites can invade and destroy Her2/Neu-expressing mammary cancer cells and determine the date TUBO cells were destroyed using a novel continuous feed medium approach.

Methodology

T. gondii Ankara strain tachyzoites and TUBO cell culture

T. gondii Ankara strain tachyzoites used in the present study belongs to Africa 1 genotype [18]. The *T. gondii* Ankara strain tachyzoites were cryopreserved and thawed to be used for this study with minor modifications as previously described [12,19]. Briefly, *T. gondii* Ankara strain tachyzoites continuously grown in HeLa cell line [12] were aspirated from the cell culture supernatant and centrifuged at 1500 rpm for 10 min. Then, the supernatant is discarded and the pellet is resuspended in 500 µL RPMI. The amount of *T. gondii* tachyzoites was counted with a haemocytometer and viability was determined using 0.4% trypan blue dye (Applichem, Darmstadt, Germany). Thereafter, at least 10^8 tachyzoites in 0.5mL RPMI medium were added to 1 mL cryopreservation solution [15% (v/v) DMSO, 15% (v/v) fetal bovine serum (FBS) containing RPMI medium] and transferred to -80°C freezer. To thaw the cryopreserved tachyzoites, the tube containing cryopreserved material was immersed in to 37°C water bath until the solution dissolved. Next, the solution was immediately added to 5 mL of DMEM and centrifuged at 1500 rpm for 10 min. Thereafter, the supernatant was discarded and the pellet was resuspended in 500 µL DMEM. After thawing, the amount of tachyzoites was counted with a haemocytometer and viability was determined using 0.4% trypan blue dye (Applichem, Darmstadt, Germany).

TUBO (a cell line cloned from a lobular carcinoma in BALB-neuT mice) cells kindly obtained from Prof. Federica Cavallo (University of Torino, Torino, Italy) were cultured in DMEM containing 20% fetal bovine serum (FBS) and penicillin (10U/mL) and streptomycin (10µg/mL) at 37°C in a 5% CO_2 atmosphere as previously described with some modifications [20]. As the monolayer formed in growth medium containing 20% FBS, 25-cm^2 flasks were washed once with 1×PBS, incubated with 0.25% trypsin for 5 min at 37°C and tapped sharply to release the cells from the bottom of the flasks (Greiner, Frickenhausen, Germany). Subsequently 5 mL growth medium containing 20% FBS was added to the trypsinized cells and homogenized until a homogeneous cells suspension formed using a sterile Pasteur pipette. The viability of TUBO cells were counted with a haemocytometer and viability was determined with 0.4% trypan blue dye (Applichem, Frickenhausen, Germany). Based on the amount of viable cells, an appropriate amount of TUBO cell was subcultured every 3-4 days into new 25-cm^2 flasks with growth medium containing 20%FBS and incubated at 37°C in a 5% CO_2 atmosphere to form the confluent monolayer. Unless noted otherwise, the entire cell culture media were obtained from Cegrogen, Germany (Stadtallendorf).

Inoculation of TUBO cells with T. gondii Ankara strain tachyzoites

As the monolayer formed and TUBO cells were released with trypsin and viability was determined with 0.4% trypan blue dye. Then, TUBO cells in 5 mL maintenance medium containing 2% FBS were added to 25-cm^2 flasks and incubated for an additional 16 hours. Next, two sets of 25-cm^2 flasks (each set contains four groups and each group contains three flasks) containing TUBO cells in maintenance medium were inoculated with *T. gondii* Ankara strain tachyzoites. The first set of four flasks containing 5×10^6 TUBO cells were inoculated with TUBO-tachyzoite ratios of 2:1, 1:1, 1:2, and 1:4 at 37ºC in a 5% CO_2 atmosphere. In the second set of four flasks, 1×10^6 TUBO cells were inoculated with TUBO-tachyzoite ratios of 10:1, 100:1, 1000:1, and 2000:1 at 37ºC in a 5 % CO_2 atmosphere.

In order to assess whether *T. gondii* Ankara strain tachyzoites can destroy TUBO cells, a feed medium approach was used. For this purpose, the cell supernatants were collected at days 3, 5, 7, 8, 10, 11, and 12 for the first set of flasks and at days 3, 5, 7, 11, 12, 13, 15, 18, 22, and 25 for the second set of flasks. The supernatant containing *T. gondii* Ankara strain tachyzoites were initially centrifuged at 250 rpm for 10 minutes and the pellet was discarded to remove host cell debris. The remaining supernatant containing *T. gondii* Ankara strain tachyzoites was centrifuged at 3000 rpm

for 10 minutes. After centrifugation, the supernatant was discarded until 1 mL maintenance medium was left and then homogenized. Thereafter, the amount of TUBO cells and viable *T. gondii* Ankara strain tachyzoites were determined with a haemocytometer using 0.4 % trypan blue dye under phase-contrast inverted microscope (Nikon, Melville, USA). After counting, the homogenate was fed up to 5 mL with new maintenance medium.

For the flasks that the supernatant was aspirated, the remaining TUBO cells attached to each flask were washed with 1×PBS and then *T. gondii* Ankara strain tachyzoites in 5 mL feed maintenance medium were added to each corresponding flask. This approach was applied as we observed TUBO cells in each flask by microscopy after washing with 1×PBS. Negative control flaks that were not inoculated with *T. gondii* tachyzoites were also include to the study to assess the efficiency of feed maintenance medium approach in terms of TUBO cells attachment to the flasks. Experiments were performed in triplicate to obtain a mean ± SD for each set of experiments.

Statistical analysis

Data obtained during the study were processed using Microsoft Excel programme.The amount of tachyzoites obtained from three individual trials were calculated by microscopy and compared based on the average tachyzoite amount with standard deviation results.

Results

According to the results, we showed that *T. gondii* Ankara strain tachyzoites successfully invade TUBO cells *in vitro* as shown in Figure 1. In the first set of flasks, 5×10^6 TUBO cells were inoculated with *T. gondii* tachyzoites at ratios of 1:2, 1:1, 2:1, and 4:1 using a continuous incubation with feed medium approach.

In the flasks that have TUBO-tachyzoite ratio of 1:2, the supernatant was aspirated and feeding of TUBO cells with new medium was performed at days 3 (Figure 2A), 5, and 7. TUBO cell culture was finalized at day 8 due to the absence of attached TUBO cells. The total amount of *T. gondii* tachyzoites was 69.5×10^6 which was almost 7 times higher than the initial concentration (10×10^6).

In the flasks that have TUBO-tachyzoite ratio of 1:1, TUBO cell culture was finalized at day 10 due to the absence of attached TUBO cells and the total amount of *T. gondii* tachyzoites were 66.25×10^6 which was almost 11 times higher than the initial concentration (5×10^6) (Figure 2A).

In the flasks that have TUBO-tachyzoite ratio of 2:1, TUBO cell culture was finalized at day 11 due to the absence of attached TUBO cells and the total amount of *T. gondii* tachyzoites were 55×10^6 which was almost 22 times higher than the initial concentration (2.5×10^6) (Figure 2A).

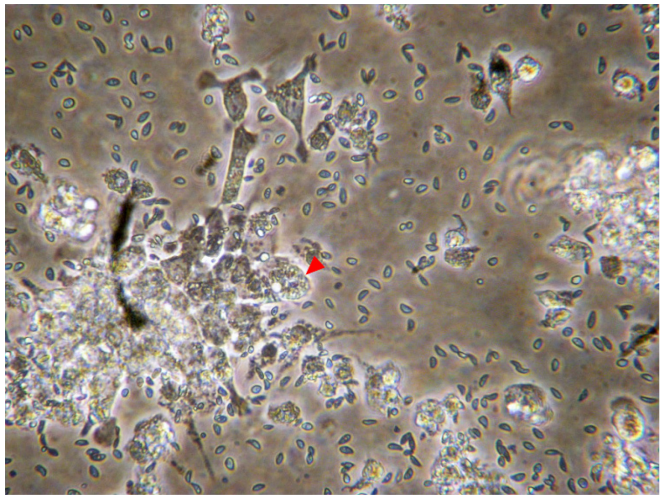
In the flasks that have TUBO-tachyzoite ratio of 4:1, TUBO cell culture was finalized at day 12 due to the absence of attached TUBO cells and the total amount of *T. gondii* tachyzoites were 54.25×10^6 which was almost 43 times higher than the initial concentration (2.5×10^6) (Figure 2A).

Accordingly, in the first set of flaks, as the amount of *T. gondii* Ankara strain tachyzoites that were inoculated to each flask decreased, the time that the TUBO cells were attached to each flask increased but at the same time the amount of viable tachyzoites increased compared to initial inoculate amount (Figure 2A and 2B).

In order to determine the survival time of TUBO cells using lower amount of *T. gondii* Ankara strain tachyzoites, a new set of flaks containing 1×10^6 TUBO cells were inoculated with *T. gondii* Ankara strain tachyzoites at ratios of 10:1, 100:1, 1000:1, and 2000:1.

During continuous incubation with feed medium approach, in the flasks that have TUBO-tachyzoite ratio of 10:1, the supernatant was aspirated and feeding of TUBO cells with new medium was performed at days 3, 5, 7, and 11. TUBO cell culture was finalized at day 12 due to the absence of attached TUBO cells (Figure 2C). The total amount of *T. gondii* tachyzoites was

Figure 1. Flask image of TUBO cells inoculated with *T. gondii* Ankara strain tachyzoites at a ratio of 1:1 at the third day of culture. Arrowhead showing a rosetta formation in TUBO cells typical for *T. gondii* invasion.



59.5×10^6 which was almost 595 times higher than the initial concentration (1×10^5).

In the flasks that have TUBO-tachyzoite ratio of 100:1, TUBO cell culture was finalized at day 13 due to the absence of attached TUBO cells and the total amount of *T. gondii* tachyzoites were 72.25×10^6 which was almost 7250 times higher than the initial concentration (1×10^4) (Figure 2C).

In the flasks that have TUBO-tachyzoite ratio of 1000:1, TUBO cell culture was finalized at day 22 due to the absence of attached TUBO cells and the total amount of *T. gondii* tachyzoites were 55×10^6 which was almost 55000 times higher than the initial concentration (1×10^3) (Figure 2C).

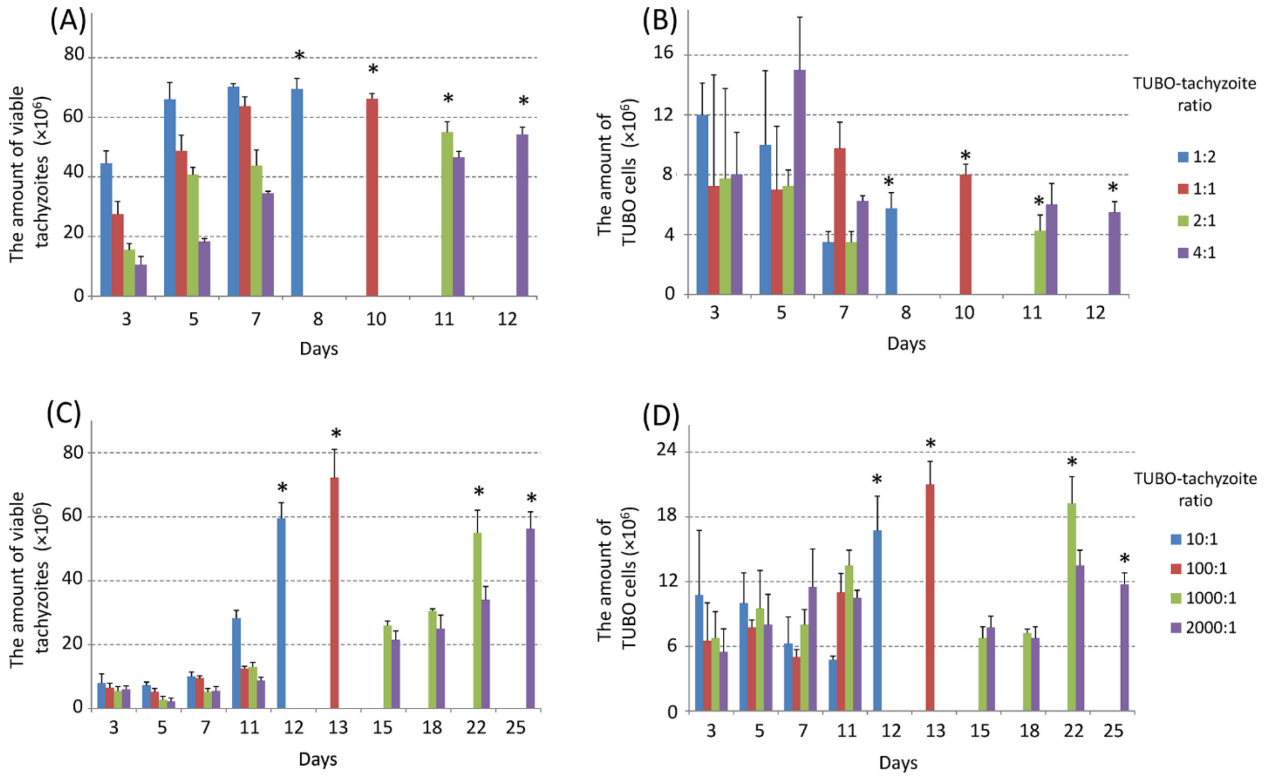
In the flasks that have TUBO-tachyzoite ratio of 2000:1, TUBO cell culture was finalized at day 25 due to the absence of attached TUBO cells and the total amount of *T. gondii* tachyzoites were 56.25×10^6 which was almost 112500 times higher than the initial concentration (5×10^2) (Figure 2C).

Accordingly, in the second set of flaks, as the amount of *T. gondii* Ankara strain tachyzoites that were initially inoculated to each flask decreased, the time that the TUBO cells were attached to each flask increased but at the same time the amount of viable tachyzoites increased compared to initial inoculate amount (Figure 2C and 2D). Although maintenance medium containing 2% FBS was used feed medium, TUBO cells also grew at a slower rate as shown in Figure 2 B and D. The amount of TUBO cells in cell culture supernatant were higher in second set of flasks possibly due to lower amount of *T. gondii* Ankara strain tachyzoite inoculation (Figure 2D). In the negative control flasks, TUBO cells were visualized in flasks forming a confluent layer up to 25 days using feed medium approach.

Discussion

T. gondii has been used in several studies to treat *in vivo* cancer models [5-9]. For example, a mutant strain called "Nonreplicating *Toxoplasma* uracil auxotrophs" has been found to effectively stimulate potent immunity and increased survival against ovarian cancer, melanoma, and pancreatic cancer bearing mice models [6,9]. In another study, *T. gondii* ME49 strain infection in mouse model inhibits tumor growth of Lewis lung carcinoma through the induction of Th1 immune responses and antiangiogenic activity [5]. *T. gondii*

Figure 2. The amount of viable *T. gondii* Ankara strain tachyzoites and TUBO cells obtained from cell culture supernatants collected at various days. The amount of (A) viable *T. gondii* Ankara strain tachyzoites and (B) TUBO cells counted in the first set of flasks using TUBO-tachyzoites ratios of 2:1, 1:1, 1:2, and 1:4. The amount of (C) viable *T. gondii* Ankara strain tachyzoites and (D) TUBO cells counted in the second set of flasks using TUBO-tachyzoites ratios of 10:1, 100:1, 1000:1, and 2000:1. (*) sign represents the days that the cell culture was finalized due to absence of TUBO cells in the flasks as observed by microscopy.



lysate antigen has also been used against tumor models in mice and showed suppression of angiogenesis as well as induction of IL-12 [21]. In addition to these in vivo studies, various *in vitro* studies showed that *T. gondii* tachyzoites can invade and destroy cervical cancer, larynx carcinoma and lung cancer cells [11-13].

In this study, we aimed to analyze whether *T. gondii* tachyzoites can destroy Her2/Neu-expressing mammary cancer cells *in vitro* using a novel continuous feed medium approach. During the continuous cell culture, the condition of TUBO cells was continuously observed and cell culture supernatants were collected at various days. *T. gondii* Ankara strain tachyzoites were collected from the supernatant and inoculated again in to corresponding flaks using fresh feed maintenance medium approach. Using this approach, we were able to extend culture of TUBO cells inoculated with *T. gondii* tachyzoites up to 25 days in the flasks in which TUBO-tachyzoite ratio was 2000:1.

According to the results, in the first and second sets of flasks, TUBO cells were finalized between days 8 to 12 and 12 to 25, respectively as detected by microscopy. In addition, the amount of *T. gondii* Ankara strain tachyzoites increased 7 to 43 times in the first set of flasks and 595 to 112500 times in the second set of flasks. The most important result of this study was, as low as 500 tachyzoites were able to destroy 1×10^6 TUBO cells in 25 days. Altogether these results show that *T. gondii* Ankara strain tachyzoites successfully destroy Her2/Neu-expressing mammary cancer cells. Moreover, the continuous medium feed approach described in this study has not been tested before in any *in vitro* studies conducted by *T. gondii* tachyzoites.

In addition, our results as well as findings obtained from previous studies indicate that *T. gondii* tachyzoites have an affinity for cancerous cells. This relation between T. gondii and cancer cells have been showed by glycosylation studies. For example, cancer cells have excessive sialylation [22] and during penetration of *T. gondii* to these cancer cells, microneme protein 1 (TgMIC1) forms a complex with TgMIC4 and TgMIC6 and binds to sialylated glycolconjugates on cancer cell surface. It has also been reported that invasion efficiency of *T. gondii* tachyzoites reduces 90% when the host cells were treated by neuraminidase, an enzyme that cuts sialic acids present on cell surface [23].

Conclusion

The present study first time showed that *T. gondii* Ankara strain tachyzoites can successfully invade and destroy Her2/Neu-expressing mammary cancer cells *in vitro* using a continuous feed medium approach. It has also been showed that *T. gondii* tachyzoites have an affinity for cancerous cells, stimulate potent immunity and as a result increased survival against cancer in animal models. Although this idea may be too premature, future analyses investigating the relationship between *T. gondii* and cancer cells in terms of invasion and termination of cancer cells may shed important therapeutics tools in the future to save cancer patient lives. Overall, future studies aiming to use the termination power of *T. gondii* tachyzoites as showed in this study as well as immunostimulant efficacy of the parasite may have additive efficacy against cancer types.

Acknowledgements

The authors would like to acknowledge Prof. Federica Cavallo and Irene Fiore Merighi (University of Torino, Torino, Italy) for providing TUBO cells and technical assistance for the maintenance of TUBO cells in our lab. This study was partly supported by the grant given by the Scientific Research Projects Branch Directorate of Ege University, Turkey (Grant No: 2016TIP082) to L.Y.

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Conflict of interests: No conflict of interests is declared.