The cytopathic Effects of *Trichomonas vaginalis* on Fibroblast Cell Culture Alone and with *C. albicans* and *E. coli*

Klinik Örneklerden İzole Edilen *Trichomonas vaginalis*'in Tek veya *Candida* ve *E. coli* ile Birlikte L929 Fare Fibroblast Hücre Kültür Serileri Üzerine Sitopatik Etkisi

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ABSTRACT

Objective: In this study, the cytopathic effects of *Trichomonas vaginalis* were investigated in L929 mouse fibroblast cell cultures (FCC) under different conditions: only parasite, or coexistence with *Candida albicans* and *Escherichia coli*.
Methods: The parasite was isolated from a symptomatic patient and cultured in Cysteine-Peptone-Liver infusion Maltose medium (CPLM). *C. albicans* strain 10235 and *E. coli* strain 25922 were used in the experiments. Five groups were created and inoculated on FCC. The groups were as follows; only *T. vaginalis, C. albicans, E. coli, T. vaginalis+C. albicans* and *T. vaginalis+E. coli*. The plates were incubated for 24 hours and cell viability was examined under an inverted microscope. Each experiment was repeated 11 times.
Results: The fibroblast death rate was 19.1%, 21%, 40.9%, 96.5% and 89.6% in the five groups, respectively.
Conclusion: All fibroblasts were alive in the control group. *T. vaginalis* showed almost 100% cytopathic effects on FCC with *C. albicans* and parasites were very motile in this coexistence. *(Turkiye Parazitol Derg 2012; 36: 193-7)*

Key Words: *Trichomonas vaginalis*, fibroblast cell culture, *Candida albicans*, *Escherichia coli*, cytopathic effect

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ÖZET

Amaç: Çalışmada, *T. vaginalis*'in fibroblast hücre kültürlerinde hem tek başına hem de *E. coli* ve *Candida* ile oluşturabilecekleri sitopatik etkinin araştırılması amaçlanmıştır.
Yöntemler: Bu amaçla, L929 fare fibroblast hücre serisi ve *T. vaginalis*'in kültüründe ise CPLM besiyeri kullanılmıştır. Deneylerde semptomatik klinik örneklerden izole edilen yerel bir *T. vaginalis* suşu, *Candida* 10235 suşu ve *E. coli* 25922 suşu ile çalışılmıştır. Deneysel çalışmada; altı grup oluşturulmuş ve fibroblast kültürü üzerine inoküle edilmişlerdir. Bu gruplar; 1. *T. vaginalis*, 2. *Candida*, 3. *E.coli*, 4. *T. vaginalis+Candida*, 5. *T. vaginalis+E. coli* ve 6. Kontrol (fibroblast kültürü)'dür. Plaklar %5 CO_2'li etüvde 37°C'de 24 saat inkübe edildikten sonra inverted mikroskop altında gözlenerek canlılık sayımları yapılmıştır. Çalışmada grupların herbiri 11 kez çalışılmıştır. Kruskall-Wallis, Mann-Whitney U testi kullanılarak sonuçlar değerlendirilmiştir.
Bulgular: Kontrol grubundaki L929 fare fibroblast hücrelerinin tamamı canlı iken, 1. grupta bulunan yalnız *T. vaginalis*'in inoküle edildiği hücre kültürlerinde fibroblastların %19.09'unun, 2. grupta bulunan yalnız *Candida*'nın %21.00'inin, 3. grupta *E. coli*'nin tek başına olduğu kültürlerde %40.91'inin, 4. grupta *T. vaginalis* ve *Candida*'nın birlikte ekildiği hücre kültüründe %96.55'inin, 5. grupta *T. vaginalis* ve *E. coli*'nin birlikte inoküle edildiği godelerde ise hücrelerin %89.64'ünün öldüğü saptanmıştır.
Sonuç: *T. vaginalis*'in fibroblast hücre kültüründe patojen etkisi özellikle *Candida* varlığında çok daha belirgin olmaktadır.
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Anahtar Sözcükler: *Trichomonas vaginalis*, fibroblast cell culture, *Candida albicans*, *Escherichia coli*, sitopatik etki

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INTRODUCTION

Trichomoniosis is the most common non-viral sexually-transmitted disease around the world. Each year, approximately 170 million people are infected with the parasite (1). The most prominent complaint in trichomoniosis is vaginal discharge. The patients mostly complain of burning and itching. Upon examination of vaginal mucosa with a speculum, common hyperaemic, bright red lesions can be observed. The most common urinary symptom is dysuria; cystitis can also be seen in very few cases (2). Cervical carcinoma has been reported to show a relationship with *T. vaginalis* (3, 4). The infection is usually asymptomatic in men. *T. vaginalis* may play an important role as a cofactor in the transmission of the HIV virus. In some studies in Africa, levels of HIV positivity in *T. vaginalis* positive individuals were found to be more than two-fold (5). To touch on *in vitro* studies, in cultured mammalian cells *T. vaginalis* showed cytopathic effects (6-8). Parasites kill the target cells with only direct contact (9). Four different trichomonas surface proteins were identified adhering to cells easily (10). Also, *T. vaginalis* cell separation factor (cell-detaching-factor, CDF), mammalian cell culture cells leads to leave. A correlation was found between the severity of the infection and CDF in the pathogenesis of *T. vaginalis* (7). For many years, *T. vaginalis* was accepted as an apathogenic microorganism; however, both *in vivo* and *in vitro* studies have revealed that it is actually pathogenic (10-17). *T. vaginalis* was first grown by cell culture in the 1940s; Houge investigated the effect of the parasite on fibroblast cells in 1943. In those studies, it was attempted to determine the parasite's effect on cells, and whether there are any mechanical effects, toxins or enzymatic reactions (16). Heath worked on the effect of pathogenesis of the parasite on a single layer of vaginal epithelial cells in 1981 and found that approximately 10% of cells died in cultures (15).

However, there has been no study regarding the damage caused by *T. vaginalis* on cell lines with *C. albicans*. Therefore, this study investigated whether *T. vaginalis*, both alone and with *E. coli* and *C. albicans,* can play a role in the cytopathic effects on FCC.

METHODS

Test Microorganisms

T. vaginalis was isolated from a female patient with clinical symptoms of urogenital disease and cultured in CPLM. *C. albicans* 10231 and *E. coli* 25922 strains were used in the assays.

Cultivation of *T. vaginalis*

The medium was renewed every three or four days to keep the parasites alive. Before inoculation, the medium was heated at 37°C for a few minutes and 1 mL of inoculum was transferred to fresh medium. 20% inactivated human serum (heat inactivated at 56°C for 30 min and cooled), Penicillin G, streptomycin and Triflucan was added to each tube.

Fibroblast Cell Culture

The L929 mouse fibroblast cell line was used in this study. FCC passages were continued in order to ensure its sustainability and viability. Cells in the flasks were washed with PBS, and then rinsed with a trypsin/EDTA solution (0.05% trypsin+0.02 % EDTA). Trypsin was aspirated from flasks. The flasks were incubated 37°C in an incubator for 5 min and then cells were separated from the surface of flasks. The cell suspension was created by adding DMEM. The prepared cell suspension was divided into two flasks and was passaged. Cell proliferation was viewed in flasks. This process was repeated and continuity of cell culture was achieved.

Experimental Design

Six groups were formed in the experiments and were inoculated on FCC. The groups created were as follows**:**

1. *T. vaginalis* ($1.2x10^6$ parasite/mL)
2. *C. albicans*
3. *E. coli*
4. *T. vaginalis+C. albicans*
5. *T. vaginalis+E. coli*
6. Control FCC (no microorganism inoculation)

In the study, the cells collected from the L929 fibroblast cell series were placed in 24-well cell culture plates. The monolayer of fibroblast cells in the wells occurred within 24 hours. Solutions were inoculated in the wells: the first group contained 100 µL of *T. vaginalis*, the second group had 100 µL of *C. albicans*, and the third group contained 100 µL of *E. coli*. In the fourth group, both *T. vaginalis* and *C. albicans* (100 µL each) were inoculated, and the fifth group included both *T. vaginalis* and *E. coli* (100 µL). Cells in the last group formed the control group. Plates were incubated for 24 hours at 5% CO_2 and 37°C. Then, cells were examined under an inverted microscope (Eclipse TS 100, Nikon, Tokyo, Japan) and viability counts were performed with 0.01% neutral red. Each group was studied 11 times.

Statistical Analysis

SPSS 15.0 for Windows was used to analyse the statistical parameters. Significance between two means in an independent group was assessed by using Mann-Whitney U and Kruskal Wallis tests. The data was presented as mean±standard deviations and the p value was set at 0.01.

RESULTS

After incubation for 24 h, all of the control cells were attached and formed a normal monolayer (Figure 1A). In the inoculated *E. coli* group, a level of 40.91±8.03% was obtained for cell death (Figure 1B). At that time, the effects of *T. vaginalis* in the groups were clearly visible in L929 fibroblast cell cultures. In this first group of inoculated *T. vaginalis,* cell death was determined to be 19.09±4.74% (Figure 1C). The inoculated parasites were seen to attach to the monolayer and divide, producing a visible focal lesion. While the lesion was gradually expanded, the monolayer was destroyed and parasites became free-swimming.

When inoculated with *C. albicans* and *T. vaginalis,* there was almost no monolayer of fibroblasts and all cells were round. A large amount of *T. vaginalis* was observed and 96.55±2.91% fibroblast cells were counted as dead (Figure 1D). When *T. vaginalis* and *E. coli* were inoculated together in fibroblast cells, the cells left the base and had a round appearance; 89.64±4.38% of fibroblasts were dead (Figure 1E). In the group containing only *C. albicans,* cell death was determined to be 21.00±4.72% (Figure 1F). The percentage values of dead cells are presented in Table 1.

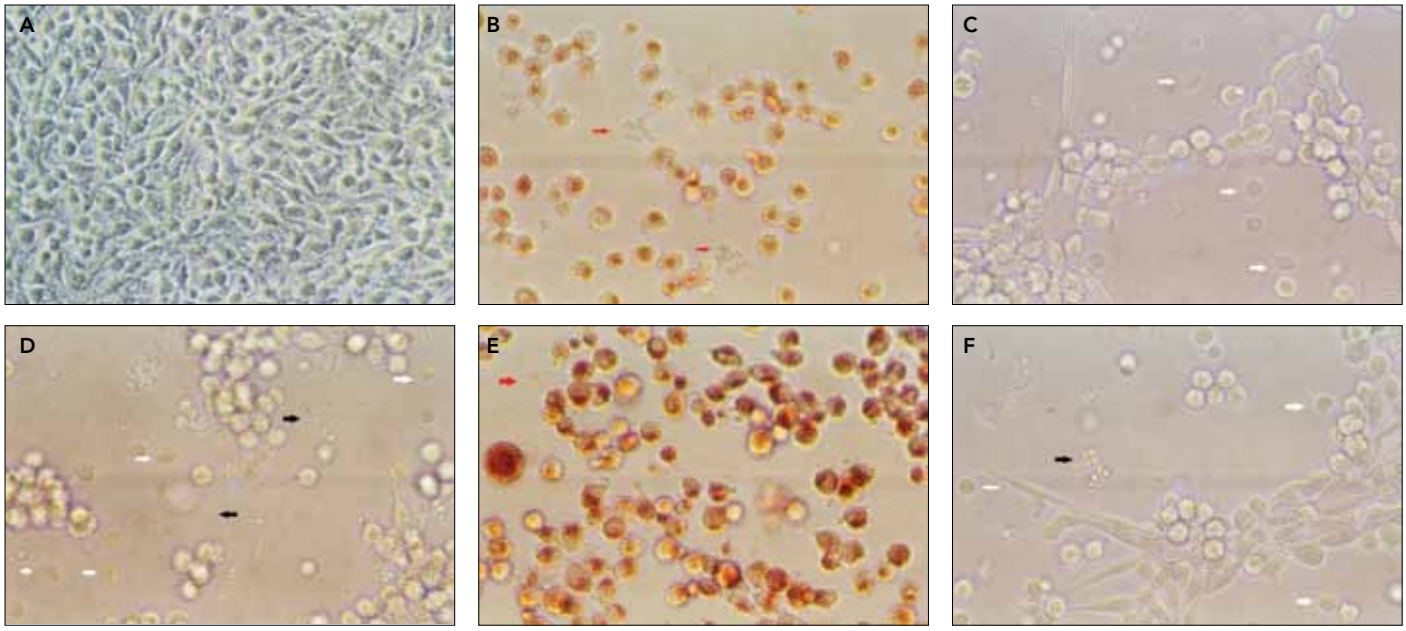

Figure 1. The changes in FCC under different conditions. A) Control-fibroblasts, B) *E. coli* and fibroblasts, C) *T. vaginalis*+fibroblasts, D) *T. vaginalis+C. albicans*+fibroblasts, E) *T. vaginalis+E. coli*+fibroblasts, F) *C. albicans*+fibroblasts (white arrows: *T. vaginalis*, black arrows: *C. albicans*, red arrows: *E. coli*)

Table 1. Cytopathic effects of infectious agents on fibroblast cells

Organisms	% Live cells (n=11)	% Dead cells (n=11)
T. vaginalis	79.09±5.15	19.09±4.74
C. albicans	78.91±4.78	21.00±4.73
E. coli	59.09±8.03	40.91±8.03
T. vaginalis+C. albicans	3.45±2.91	96.55±2.91
T. vaginalis+E. coli	9.45±3.61	89.64±4.38
Control	99.18±1.25	0.82±1.25

A significant difference between live and dead cell groups was determined by Kruskal-Wallis analysis of variance. The Mann-Whitney U test was used to determine which groups caused the difference. According to this test, while there was no significant difference between the first and second groups ($p>0.01$), significant differences were determined between all of the other groups ($p<0.01$). *T. vaginalis* and *C. albicans* showed a similar effect on the fibroblast cells; however, the two organisms together were found to make the cytopathic effect about 100%.

DISCUSSION

Trichomoniosis is one of the most important forms of protozoan parasitosis that is transmitted by sexual intercourse. Depending upon the social conditions, the methods used in diagnosis and the sexual habits of people, its prevalence varies from one country to another. Donne first described the parasite; the question of its pathogenicity has been subject to some degree of controversy. *T. vaginalis* causes vaginitis in women and urethritis and balanitis in men. During trichomoniosis, significant changes may be observed in the epithelial cell layers of the vagina (2). After Houge's observation that CPE was associated with *T. vaginalis* in embryonic human and chicken tissue explants, it was estimated that tissue cultures might become useful tools for the demonstration and identification of at least some aspects of the virulence of the organism (16).

To date, virulence factors such as adhesion molecules, proteolysis, haemolysis, cell separation factors and cytotoxicity factors were determined (6, 17-28). Adhesion of *T. vaginalis* to the vaginal epithelial cells was found to be effective. Parasite cell surface proteins and glycoproteins provide the host-cell adhesion. To date, four adhesion molecules have been identified (AP65, AP51, AP33 and AP23) (29). In the adhesion process of parasites, the target protein is laminin (28). Parasite surface carbohydrates, such as lectin-binding D-lactose and N-acetyl-D-glucosamine, also have important roles in virulence (30). Parasites are unable to synthesise lipids, so lipids are obtained from erythrocytes. Parasites can haemolyse erythrocytes via cysteine proteases; the effect of this activity in virulence has also been reported (21). Krieger et al. (31), who correlated beta-haemolytic activity by *T. vaginalis* with the symptoms of the patient and with the mouse assay, found that the pathogenic strains had higher haemolytic activity. The parasite has a maximum cysteine protease (23 pieces) (13, 32), which reduces immunoglobulin levels in the vagina (27). *In vitro* studies showed that the cell separation factor decreases in the presence of beta-oestradiol. The cell separation factor is capable of separation without killing the host epithelial cells. This effect was also observed in the present study. Cells separating from the monolayer fibroblast series took the vital dyes, but they became round and small. Other molecules that play a role in the pathogenesis of the parasite show a pore forming molecule perforin-like domain (9). In addition, some of *T. vaginalis* strains were a virus carried by double-stranded and that the virus was found in the majority of clinical isolates. This virus is thought to play a role in the pathogenesis of the parasite (33).

The cytopathic effects of *T. vaginalis,* both alone and with *C. albicans* and *E. coli,* on fibroblast cell culture series were stud-

ied, and the striking findings of this study were presented. In some earlier studies, the effect of *T. vaginalis* on the cell series was found to be 10% (15); in our study, this rate was higher (20%). This effect increased when *C. albicans* or *E. coli* was co-incubated with the parasite; in particular, *C. albicans* revealed more disruption of FCC, which was almost 100%. Additionally, the trophozoites were very active and motile in this situation. The findings showed that mixed vaginal infections due to *T. vaginalis* and *C. albicans* may result in more complicated clinical symptoms and require a different treatment. Candidiasis is reported as the most common cause of vaginitis in Europe and the second most common cause of vaginitis in the United States (34). According to the World Health Organization (WHO), the worldwide prevalence of trichomoniosis is 174 million and accounts for 10% to 25% of vaginal infections (35). The prevalence of trichomoniasis is reported to be approximately 5% in Turkey (36, 37). These two common infections are found together in many cases, which implies the importance of the present study. Alderete and Pearlman reported an extensive disruption in different monolayers (human urogenital and vagina, human epithelial, normal baboon testicular, and monkey kidney cells) with exposure to *T. vaginalis* (12). The explanation of the cellular disruption in cell monolayers is the key point for understanding the pathogenesis of the parasite. For instance, the presence of Zn^{2+} downregulates the transcriptional levels of a protein and has a negative effect on trichomonal cytotoxicity, while lipophosphoglycan mutants of *T. vaginalis* shows reduced adherence and cytotoxicity to human ectocervical cells (38, 39). Another factor that can affect the cytotoxicity is the source of the parasite; fresh isolates were more cytotoxic and can easily attach to cell layers than laboratory strains that have been cultivated for a long time in axenic cultures (40). In the study, the *T. vaginalis* strain was isolated from a symptomatic clinical case and freshly used, so the cytopathic effect was not decreased due to long-term cultivation.

CONCLUSION

The other microorganisms in the vagina may affect the cytotoxic potential of T. vaginalis; in particular, the presence of *C. albicans* may increase the disruption of epithelial cell layers.

Conflict of Interest

No conflict of interest was declared by the authors.

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