

Full Length Research Paper

Investigation of *in vitro* effects of estrogens and selective estrogen receptor modulators (serms) on *Trichomonas vaginalis*

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The aim of this study was to investigate if there is a relationship between the hormones and *Trichomonas vaginalis* growth. A strain of *T. vaginalis*, was incubated and cultivated to compare the *in vitro* effects of 17 β -Estradiol, Estriol, Raloxifene, Tamoxifen on *T. vaginalis* trophozoites in terms of different concentrations. High concentrations of 17 β -Estradiol and Estriol (20 ng/ml), and Tamoxifen and Raloxifene (100 ng/ml) promoted the reproduction of *T. vaginalis*, conversely, in low concentrations (0.5 and 10 ng/ml) respectively; they reduced the reproduction of *T. vaginalis*. This concentration related effect may not be explained purely by the effect on pH.

Key words: *Trichomonas vaginalis*, 17 β -Estradiol, Estriol, Raloxifene, Tamoxifen.

INTRODUCTION

Hormones regulate growth of organisms such as differentiation, cellular and physiological functions. Recently, research is being done with the effects of hormones on the host parasites (Escobedo et al., 2005). Trichomoniasis is the most common sexually transmitted disease, caused by a motile flagellate non-invasive parasitic protozoan, *Trichomonas vaginalis* (*T. vaginalis*) (WHO, 2007; Grodstein et al., 1993; Inceboz et al., 2004). Trichomonads also affect the bladder, urethra and paraurethral glands and cause urinary tract infection. Women with trichomoniasis may have several associated problems such as premature labour, low birth weight, greater risk of tubal infertility, atypical pelvic inflammatory disease, amplified HIV transmission/acquisition, and increased risk of cervical cancer (Sood and Kapil, 2008). Men are usually asymptomatic carriers, only occasionally

affected by self-limited urethritis and prostatitis. Thus, in the case of clinically indicated partner therapy, compliance of men is low so that reinfection of their partners can rapidly occur (Mitteregger et al., 2011).

Many sex hormones affect the host immune system and sensitivity to protozoa (Grossman, 1984; Olsen and Kovacs, 2005; Roberts et al., 2001; Petrin et al., 1998). In the different periods of the human body the increased or decreased level the amount of estrogen in the body affects *T. vaginalis* differently. Many studies showed that in cases with trichomoniasis during pregnancy the amount of high estrogen increased the infectivity and/or symptoms (Brown, 1972; Silva-Filho and Bonilha, 1992). It has been found that 17 β -Estradiol decreased virulence of *T. vaginalis*, whereas especially α -Estradiol and 17 β -Estradiol increased the adhesivity of parasites. Martinotti et al. reported that β -Estradiol increased the growth of *T. vaginalis* at 20 h while it decreased the growth at 28, 48 h (Martinotti et al., 1986). However, Sugarman and Mummaw showed that 17 β -Estradiol increased the growth of *T. vaginalis* (Sugarman and Mummaw, 1990). Selective estrogen receptor modulators (SERMs) are

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non-steroidal compounds and have estrogenic and antiestrogenic effects in different tissues. According to our literature search, we were unable to find any study on SERMs and *T. vaginalis*. Thus, this study is aimed to investigate the *in-vitro* effects of different forms of estrogens and SERMs on the growth and reproduction of *T. vaginalis* trophozoites, and pH of the culture media.

MATERIALS AND METHODS

Hormones and preparation of dilution of hormones

Hormones were prepared as stated below:

1. 17 β -Estradiol (17 β -Estradiol-16, 16, 17-d3) (Sigma, E8875 Lot No: 079K0131) (0.5, 1, 5, 1 and 20 ng/ml)
2. Estriol (Sigma E1253 lot 038 K1602) (0.5, 1, 5, 10 and 20 ng/ml)
3. Tamoxifen (Sigma T5648 lot 089K1381) (10l, 20, 50, 75 and 100 ng/ml)
4. Raloxifene hydrochloride (Sigma –Aldrich R1402 lot 019K1788) (10, 20, 50, 75 and 100 ng/ml)

Culture of *T. vaginalis* and preparation

A strain of *T. vaginalis*, isolated from a patient complaining vaginal discharge, was incubated in 10-ml glass tubes containing 5 ml of Trypticase-yeast extract-maltose (TYM) medium complemented with 10% heat-inactivated horse serum and a combination of 100 IU of streptomycin per ml and penicillin at 37°C as previously described (Diamond, 1957).

T. vaginalis strain was incubated in the TYM culture medium at 37°C in incubator and allowed to reproduce (Diamond, 1957; Singh et al., 1999). Concentration of environmental bacteria (*Candida spp*, *Enterobacteriaceae spp*, *Staphylococcus aureus*, Group B *Streptococcus*, *Escherichia coli*) was minimized by recurrent passages as previously described (Oh et al., 2003). The samples from the flask were evaluated under the microscope and moving trophozoites were counted by using Thoma counting chamber. The study was completed in two periods due to prepared medium: In the first period, different concentrations of 17 β -Estradiol and Raloxifen were added in the prepared medium that has *T. vaginalis* trophozoites at a count of 7.0×10^4 ml. In the second period, different concentrations of Estriol and Tamoxifen were added in the prepared medium that has *T. vaginalis* trophozoites at a count of 9.0×10^4 ml.

Hormones

Series of Raloxifene, Tamoxifen, Estriol and 17 β -Estradiol (Sigma) were dissolved initially in absolute ethanol, then diluted in phosphate-buffered saline (PBS; 0.15 M sodium chloride, 0.15 M sodium phosphate; pH 7.3) to provide stock solutions at concentrations 100 times the test concentrations. Then these solutions were prepared at concentrations specified in this study by TYM culture medium containing 10% Foetal Calf Serum (FCS) (Biochrom Ag) (Sugarman and Mummaw, 1990).

In this study, one plate (Garnier 24 well-plate) was used for each hormone (estradiol, raloxifen, estriol, tamoxifene), and on each plate 3 wells were used for controls as TYM culture medium and PBS and ethanol. 5 different concentrations were used for each hormone as shown in Figures 1 to 4. Total of 8 wells (5 different concentrations of hormones plus 3 controls) were evaluated at 4 different times (at the beginning 1, 24, 48th h). First, 250 μ L of TYM

culture medium containing 10% FCS were put in each well. Then 250 μ L of 17 β -Estradiol, Raloxifene, Tamoxifen, and Estriol at different concentrations, were added into each well. For each aforementioned concentrations of substances and plus for control, TYM culture medium, PBS and ethanol were distributed to 250 ml of three different wells. Finally, 250 μ L of *T. vaginalis* solution were added into each prepared well, and then were incubated at 37° C in the 5% CO₂ incubators. At 1, 24 and 48 h of incubation, by using Thoma counting chamber, the number of *T. vaginalis* was counted in the sample taken from the wells including controls, and the pH of each well was noted simultaneously.

RESULTS

Examinations at first, 24, 48 h revealed that 0.5 ng/ml concentration of 17 β -Estradiol provided the best reproductivity but at the 20 ng/ml concentration, 17 β -Estradiol inhibited the reproduction (Figure 1). 0.5 ng/ml concentration of 17 β -Estradiol yielded a suitable environment for the growth of *T. vaginalis* on the pH scale. When we examined the effects of Estriol on the growth of *T. vaginalis*, at the 20 ng/ml concentration, Estriol provided the best reproductivity but 0.5 ng/ml concentration of Estriol inhibited the reproduction (Figure 2). It was indicated that all of the concentrations of Estriol has not significant change on the pH values. When we examined the effects of Tamoxifen on the growth of *T. vaginalis*, at the 100 ng/ml concentration, Tamoxifen provided the best reproductivity whereas at 10 ng/ml concentrations, Tamoxifen inhibited the reproduction (Figure 3). It was shown that the different concentrations of Tamoxifen generally increased the pH values. When we examined the effects of Raloxifene on the growth of *T. vaginalis*, at the 100 ng/ml concentration, Raloxifene provided the best reproductivity; however, at 10 ng/ml concentrations Raloxifene inhibited the reproduction (Figure 4). It was revealed that the 100 ng/ml concentration of Raloxifene caused a decrease in pH value.

Effects of tested substances with different concentrations on the count of *T. vaginalis* trophozoites were shown in Table 1. As seen, the growth and reproduction were highest at 1 and 24 h for the concentrations of 20 ng / ml for 17 β -Estradiol and Estriol, 100 ng/ml for Tamoxifen and Raloxifene 100 ng/ml. The statistical analysis was performed by using SPSS 15.0 for Windows. P value of less than 0.05 was considered significance. The count of *T. vaginalis* trophozoites in different hormone concentrations and control groups at different times were compared by analysis using General linear model with repeated measures covariates. To compare the drug groups, one way ANOVA was used with Bonferoni correction. Comparisons of drug concentrations revealed the statistically significant difference between the Estriol concentrations of 0.5 and 20 ng / ml. Regardless of the drug type, the difference of *Trichomonas* counts at first and 24 h was statistically

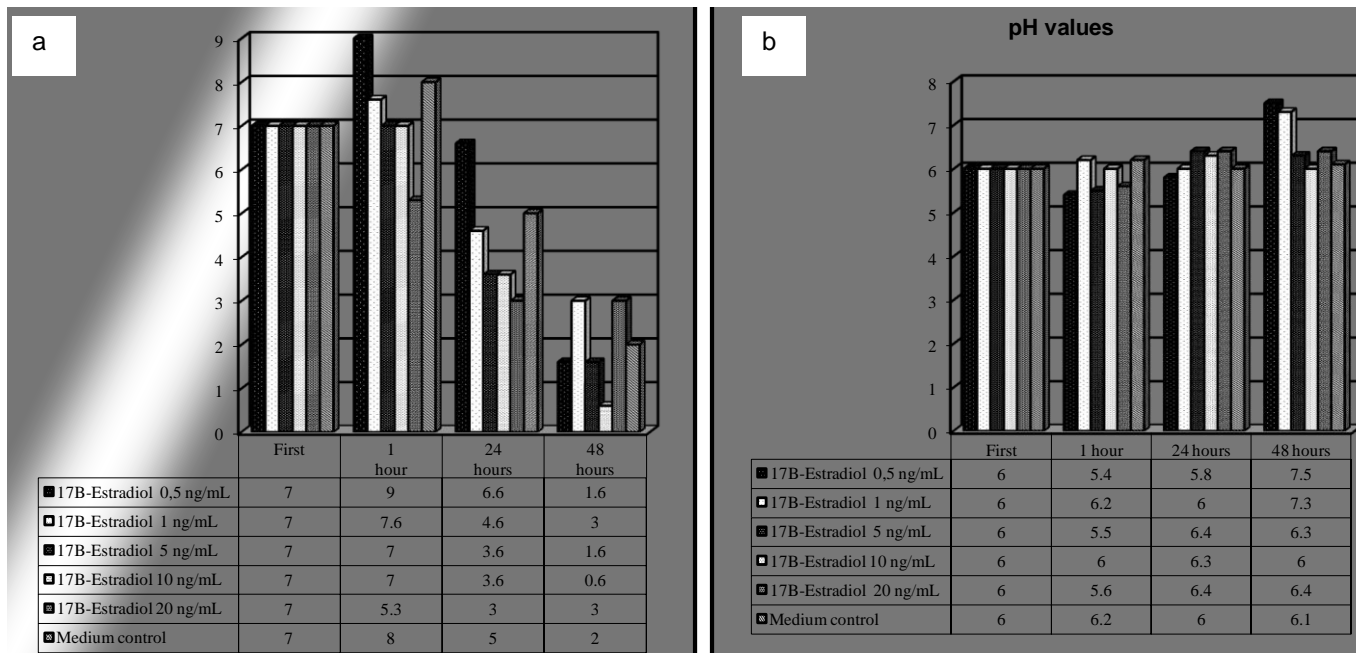


Figure 1. The effects of 17β-Estradiol in different concentrations at different times (at the beginning and at 1, 24, 48 h) on a) the count of *T. vaginalis* trophozoites (7.0×10^4 /ml) and b) pH values.

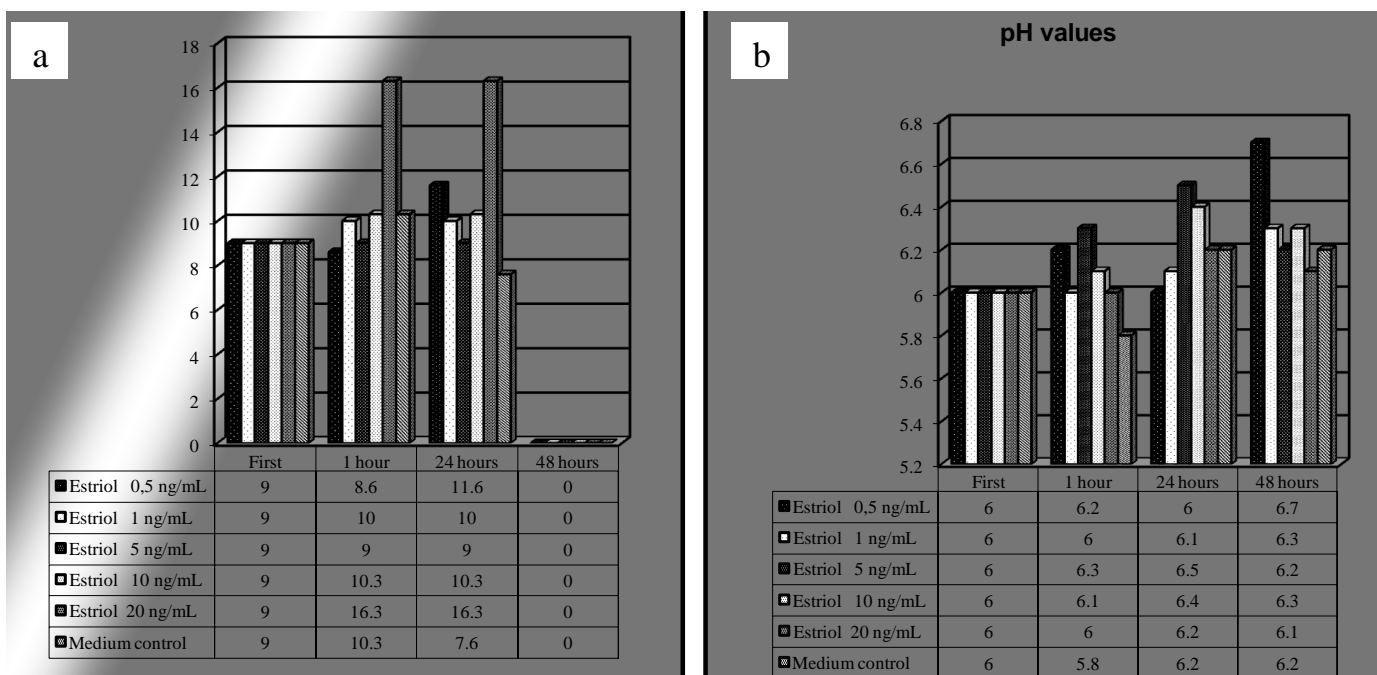


Figure 2. The effects of Estriol in different concentrations at different times (at the beginning and at 1, 24, 48 h) on a) the count of *T. vaginalis* trophozoites (9.0×10^4 /ml) and b) pH values.

different ($p=0.035$). We did not find any statistical difference between the drug groups in terms of time (0.197).

DISCUSSION

Due the variety of the social values, the sexually

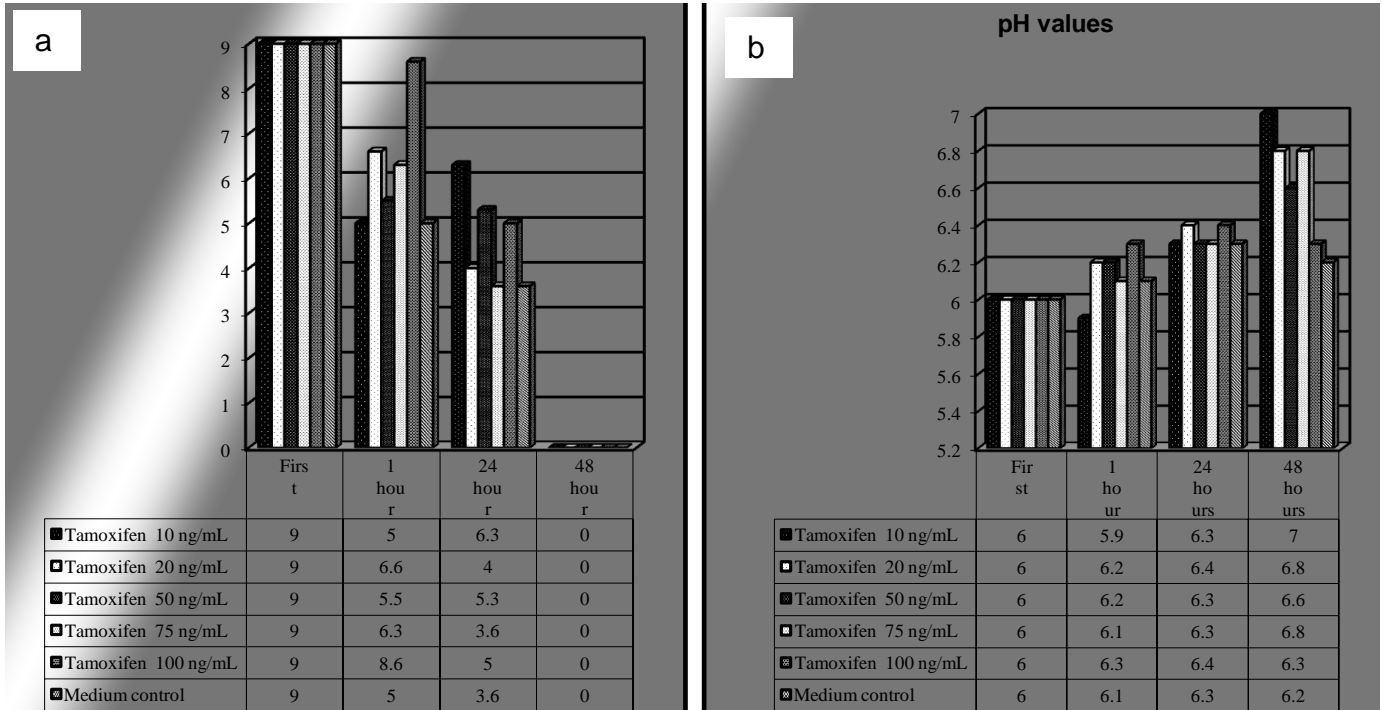


Figure 3. The effects of Tamoxifen in different concentrations at different times (at the beginning and at 1, 24, 48 h) on **a)** the count of *T. vaginalis* trophozoites (9.0×10^4 /ml) and **b)** pH values.

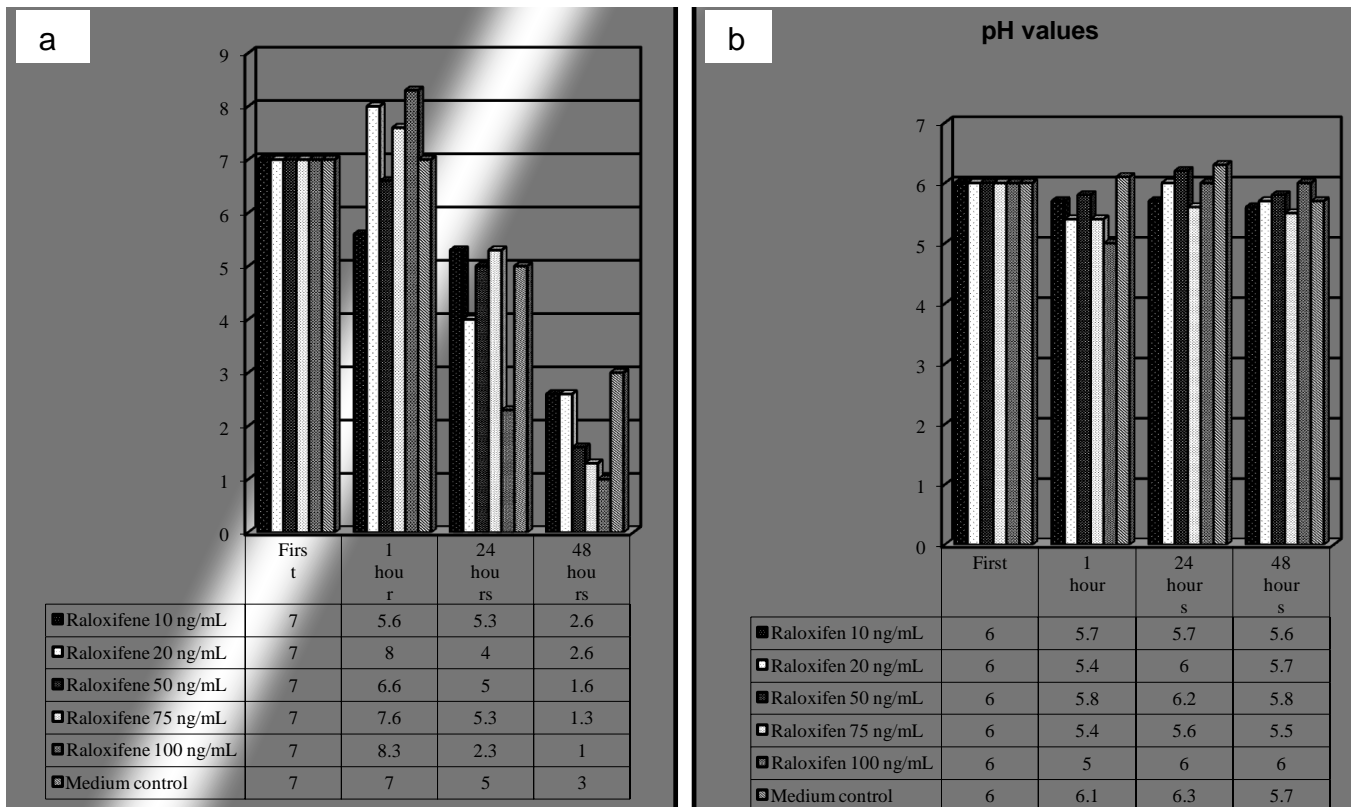


Figure 4. The effects of Raloxifene in different concentrations at different times (at the beginning and at 1, 24, 48 h) on **a)** the count of *T. vaginalis* trophozoites (7.0×10^4 /ml) and **b)** pH values.

Table 1. Effects of tested substances with different concentrations on the concentrations of *T. vaginalis* trophozoites.

Hormone	Concentrations (ng/ml)	Effects
17 β -Estradiol Estriol	20	Growth \uparrow , reproduction \uparrow
	0.5	Growth \downarrow reproduction \downarrow
Raloxifene	100	Growth \uparrow , reproduction \uparrow
	10	Growth \downarrow reproduction \downarrow

transmitted diseases have increased in recent years. Unfortunately, the possibility of this increase seems somewhat unavoidable (Sugarman and Mummaw, 1990). In this study we have investigated the effects of selected hormones, namely 17 β -Estradiol, Estriol and also two major SERMs (Tamoxifen and Raloxifene), on the *in vitro* reproduction of *T. vaginalis*. As far as we know this is the first study that evaluated the effects of SERMs on this issue. For different indications, many of hormonal medications are given to patients by clinicians or at least some hormonal variations occur physiologically. Since hormones may change the vaginal pH, it may also affect the reproduction of some infection agents. There have been some *in vitro* researches on this topic. When the *in vitro* effect of Estradiol on *Plasmodium falciparum* was investigated, it has been shown that it has had both reproductive and growth effects (Lingnau et al., 1993). Escobedo and study workers have shown that Estradiol increased the growth, viability, infectivity and reproductivity of *Taenia crassiceps* that are multi cellular parasites (Escobedo et al., 2005). The effects of anti-estrogenic and Estradiol on the *S. haematobium* infection were researched and were found that it could be alternative treatment (Botelho et al., 2009).

In our study, we have investigated both 17 β -Estradiol and Estriol. We have showed that both types of estrogens have affected the growth of *T. vaginalis* in *in vitro*, especially at first hour and 24 h. Both hormones positively affected the reproductivity of *T. vaginalis* at high concentrations whereas both have negative effect on *T. vaginalis* at low doses. This situation can be explained by pH changes for 17 β -Estradiol, however, not for Estriol. Since the effect on the growth of the parasite may not solely be explained by the change of pH, we believe that direct effect via the specific hormone receptors on the surface of the parasite may play a role on the growth as previously suggested (Ford et al., 1987).

Prototype of trifeniletalen is Tamoxifen which is the first discovered compound of SERMs (Jordan, 2006). At first, Tamoxifen was developed for "contraception", but especially in women with subfertility, it increased to fertility by inducing the ovulation. Tamoxifen was also established for cancer treatment of estrogen receptor (ER) positive breast cancer due to its anti-estrogenic effect in the breast tissue. Tamoxifen is known to be as

effective as clomiphene- citrate for the induction of ovulation (Steiner et al., 2005). Prototype of benzothiophenes is Raloxifene (original name is keoxifene). It is effective as "antiresorptive" on the bone. Raloxifene hydrochloride was also shown to have protective effect for breast cancer and its effect is as comparable as Tamoxifen. New researches with SERMs are directed to different application areas. For example, an interesting study by Miguel et al showed that Tamoxifen was effective against *Leishmania* in *in vitro* conditions (Miguel et al., 2007). In that study, it was indicated that there was a therapeutic effect of Tamoxifen on *L. amazonensis*, *L. braziliensis* and *L. chagasi* in *in vivo*. In our study, we have examined if there was any effect of Tamoxifen or Raloxifene on *T. vaginalis* *in vitro*. We have demonstrated converse concentration-related effect of Tamoxifen and Raloxifene on *T. vaginalis*' reproduction in *in vitro* conditions. Our study showed that the most effective hormone that promoted *T. vaginalis* growth was Estriol 0.5 and 20 ng/ml at first hour and 24 h. This study also investigated the effect of Raloxifene on *T. vaginalis* for the first time; Raloxifene at 100 ng/ml promoted the reproduction of *T. vaginalis*.

In summary, we found that high concentrations of 17 β -Estradiol and Estriol hormones promoted the reproduction of *T. vaginalis*, conversely, in low concentrations; they reduced the reproduction of *T. vaginalis*. The concentration-dependence was also similar for Tamoxifen and Raloxifene. There may be some other factors for promoting *T. vaginalis* reproduction with high concentrations these substances. Future studies on this issue will shed light on this interesting interaction.

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