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The Effects Parabens on the Estrogenic Receptors Behavior in Human Breast Adenocarcinoma MCF-7 Cell Line.

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ABSTRACT

Parabens are a group of preservatives that have vast indications in pharmaceuticals, cosmetics and food industries. Recently, there have been reports about the estrogenic effects of these compounds which raised concerns about the possibility of involvement of parabens in estrogen-responsive cancers. In the current study, the effect of methylparaben (MP) and propylparaben (PP) was assessed on the growth pattern of human breast adenocarcinoma cell line, MCF-7. 17-ß-estradiol (EST) and tamoxifen (TAM) were used as estrogenic and anti-estrogenic compounds. EC_{50} of EST and IC_{50} of TAM were calculated to be 1.206 and 7.017 µg/ml, respectively, based on trypan blue dye exclusion assay in a 5-day exposure. Proliferation kinetics of MCF-7 cells in a 10 day exposure to these compounds showed that EST (1.206 µg/ml) could induce cell proliferation, while exposure of MP or PP (at the same dose) did not change the growth curve of MCF-7. TAM, on the other hand could decrease the proliferation rate of MCF-7. This inhibition was more exacerbated when MP and PP were added to the culture media, suggesting a competitive binding of these compounds to receptors. In conclusion, the data propose the probable estrogenic effect of MP and PP with less potency compared to EST and less competitive binding to ERs compared to TAM.

Keywords: MFC-7, Methylparaben, Propylparaben, 17-ß-estradiol, Tamoxifen

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INTRODUCTION

Parabens are a group compounds with the general structure of p-hydroxybenzoic acid ester (figure 1) that have wide applications in the field of cosmetics, pharmaceuticals and food industries [1]. The antimicrobial properties of these compounds had made them a favorable preservative agent to be used in the above mentioned fields. Based on the structure of side chain in parabens (depicted as R in figure 1 different physicochemical properties are expected. Lipid solubility of these compounds, for example, increases as the length of the side chain increases [2] and therefore, higher penetration through the epidermis is expected [3]. Higher penetration can also be correlated with higher effects of these compounds.

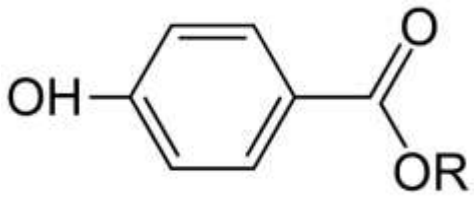

Figure 1: General structure of parabens. Side chains with different lengths can be placed at R position.

Estrogens and xenoestrogens exert their effects through estrogen receptors (ER), which are located and active inside certain cells. The receptors are widely distributed throughout the body including central nervous system, cardiovascular system, liver, gastrointestinal tract, urinary tract, bones and the breasts [4]. The receptors also are believed to play roles in certain cancers such as breast cancer. In fact wide experimental and epidemiological data suggest the correlation between such cancers and estrogens [5]. Studies show that estrogens can increase breast cell proliferation and affect breast cancer etiology [6].

Parabens have also been classified as a group of xenoestogens and therefore wide range of effects, including interference with male reproductive system or increase in the incidence of breast cancer are associated with these compounds [7]. As the length of the side chain of parabens paly role in their effects, and since breast cancer is still one of the growing concerns of the health system, the aim of the current study was to investigate the effect of two commonly used parabens with different side chain lengths on the growth pattern of human breast adenocarcinoma cell line.

MATERIALS AND METHOD

Materials

Human breast adenocarcinoma (MCF-7) cell line was obtained from National Cell Bank of Iran (NCBI C135). Methyl paraben and propylparaben were gift obtained from Darou Pakhsh®

Pharmaceutical Company (Iran). Tamoxifen and 17-ß-estradiol were prepared from Osveh® Pharmaceutical Company (Iran). Phenol red free RPMI-1640 was obtained from Sigma® and other used chemicals and reagents were prepared from Gibco BRL®.

METHODS

Cell Culture

Following thawing MCF-7 cells, they underwent 3 consecutive passages in 5% CO_2 incubator at 37°C. At the start of the experiment, the cells were cultured in 12-well plates (Orange®) at the density of 1.5×10^4 and 2.5×10^4 cells per well for stimulatory and inhibitory effects of compounds, respectively.

Preparation of Reagents

The reagents were dissolved in a 50% (v/v) ethanol-distilled water solution to prepare the stock solution. The stock solution was then filtered in aseptic conditions using 0.2 µM filters and serial dilutions were made using serum-free media to obtain the final and desired concentrations of compounds. Final concentration of ethanol exposed to cells was less than 1%.

Calculating the Effective Concentrations of Chemicals

In order to calculate the half maximal effective concentration (EC_{50}) of EST and half maximal inhibitory concentration (IC_{50}) of TAM, the cultured cells were exposed to 0-100 µg/ml of TAM and EST for 5 days. After the exposure period, stimulatory and inhibitory effects of EST and TAM were measured as described below.

Cell Viability Measurement

At defined times, the supernatant media on the cells was discarded and the cells were washed with normal saline twice. The cells were then trypsinized and suspended in media. Cell viability was measured using trypan blue dye exclusion assay. Briefly, the cells were trypsinized and detached from the culture surface. Further cell suspension was obtained with adding complete media to the trypsinized cells. Then 0.1 ml of trypan blue was added to 0.1 ml of cell suspension, a hemocytometer was loaded and immediately examined under an inverted microscope. The test was performed in triplicate and the number of viable cells per ml of the suspension, showing a clear and not blue cytoplasm (8), was calculated based on the below formula:

$$\text{Viable cells/ml} = \text{Average counted viable cells} \times 2 \times 10^4$$

Growth Curve Construction

In order to study the effect of chemicals on this cell line, the cells were seeded at the density of 2.5×10^4 (for TAM exposure) and 1.5×10^4 (for EST, MP, and PP exposure) and incubated overnight. The measured IC_{50} amount of TAM and EC_{50} amount of EST were exposed to cells.

The cells were also exposed to MP and PP at the EC_{50} concentration of EST. Single or simultaneous effect of these compounds was studied in a 12- day period. At 24 hour intervals, the wells were trypsinized and the cell viability was measured based on trypan blue assay.

Statistical Analysis

Statistical analysis of IC_{50} and EC_{50} of TAM and EST for non-linear regression and the related graphs were measured and plotted using GraphPad PRISM®, version 5. Other graphs are plotted using Microsoft Excel®, version 2013. Two way analysis of variance, repeated measurement was performed using GraphPad PRISM®, version 5 and $p<0.05$ was considered as the limit for significant differences.

RESULTS AND DISCUSSION

Parabens, as preservatives, have had wide use in various industries for years. Finding remains of parabens in human breast tissues has raised concerns about the possibility of involvement of such compounds in the incidence of breast cancer [9-11]. Studies suggest that parabens have affinity for ERs, however the affinity seems to be low. Investigators believe that at high enough concentrations, parabens can cause breast cell proliferation [12], leading to cancer. On the other hand, there are controversies about the concentrations of parabens in human breast samples [9, 12]. These footprints leave few questions about the effects of parabens on human breast.

MP and PP are two of the paraben family members, with short and relatively long side chains, respectively. The differences in the lengths of the side chains are expected to cause different results in experiment, as the physicochemical properties of the compounds changes. In the current study, the effect of PM and PP is compared to EST to investigate the estrogenic effect of these compounds. In this regard, human breast adenocarcinoma cell line, MCF-7, was chosen. This cell lines in known to be an estrogen responsive cell line, and thus can provide a good model for this study. In order to prove the involvement of ERs in the observed effects of parabens, TAM was also applied as an antagonist. To have a good comparison between the chemicals, an appropriate concentration was required. EC_{50} and IC_{50} of EST and TAM, respectively, could provide such standard concentration for comparison. Based on the aim of the study, cell proliferation was the method of choice to calculate such concentrations. As shown in figure 2, IC_{50} of TAM was 7.017 µg/ml (95% confidence interval of 4.01 to 12.26) and EC_{50} of EST was 1.206 µg/ml (95% confidence interval of 0.37 to 3.86). In another study, Ashtarinez had *et al.* conducted a similar study on the ovarian cell line, C13 [13]. They found IC_{50} of TAM to be 3.125 µg/ml and EC_{50} of EST to be 12.5 µg/ml. This simple comparison can imply that the human breast cancer cell line is more

sensitive to the inhibitory effect of TAM and stimulatory effect of EST. This might be suggestive of higher concentration of ERs in breast tissue and their higher sensitivity of the cell line to the estrogenic/antiestrogenic effects.

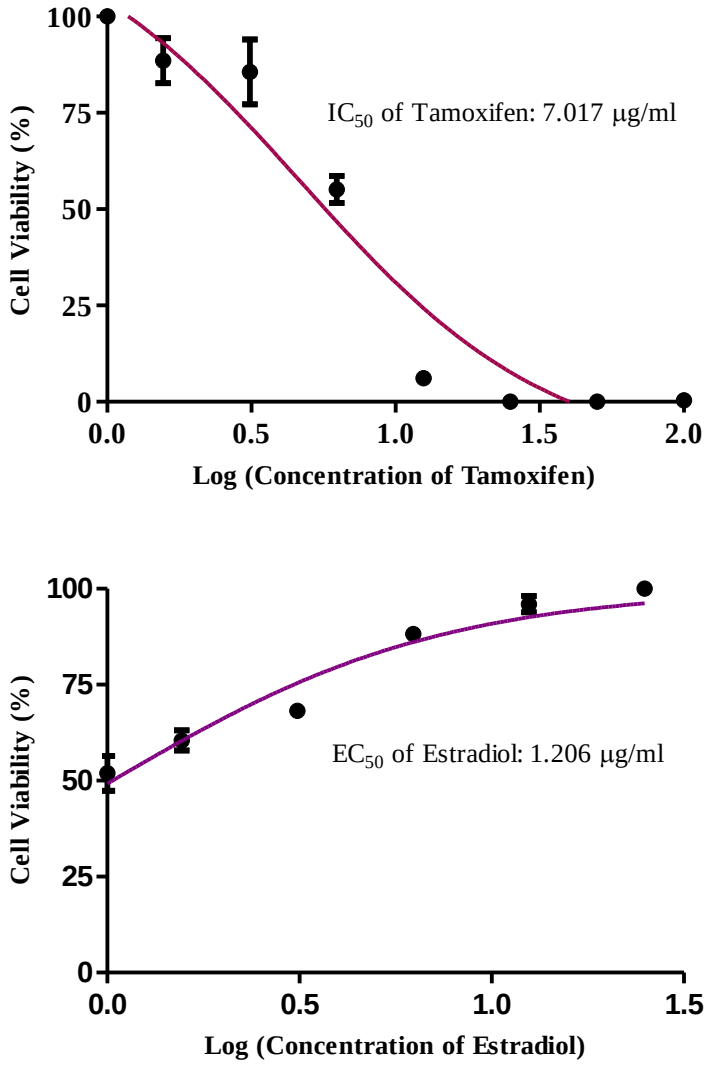

Figure 2: Half maximal inhibitory (IC_{50}) and effective (EC_{50}) concentrations of tamoxifen and 17-ß-estradiol, respectively. The cells were seeded in 12-well plates at 2.5×10^4 and 1.5×10^4 cells/well and exposed to 0-100 µg/ml of each compound for 5 days. Cell viability was measured based on trypan blue assay for three separate experiments.

Later, in order to study the effect of parabens on MCF-7 cell lines, EST, MP and PP were added to culture dishes at the concentration of 1.206 µg/ml (figure 3). The cells were exposed to these compounds for 10 days and each day, cell proliferation was assessed. As it is depicted in figure 3, at this concentration, EST seems to have the most proliferative effect on MCF-7 cells. The compounds caused a different growth pattern in C13 cell line [13]. The compounds caused a dramatic

increase in cell proliferation in the ovarian cell line while in breast cell line, the most significant increase in cell proliferation was due to EST (figure 4). Based on statistical analysis, control and treated cells, all showed a sudden increase in cell count on day 5 ($p<0.01$) and the increases went on to the peak of the curve on day 6 ($p<0.0001$). Then control cells kept a plateau state for two days, until the day 8. Cell treated with MP also kept the same pattern and a plateau state, while the cells treated with EST ($p<0.01$) and PP ($p<0.0001$) showed a decreased cell count on day 8. The entire cells, whether treated or control, go through the death phase of the growth curve on days 9 and 10 of the study ($p<0.0001$).

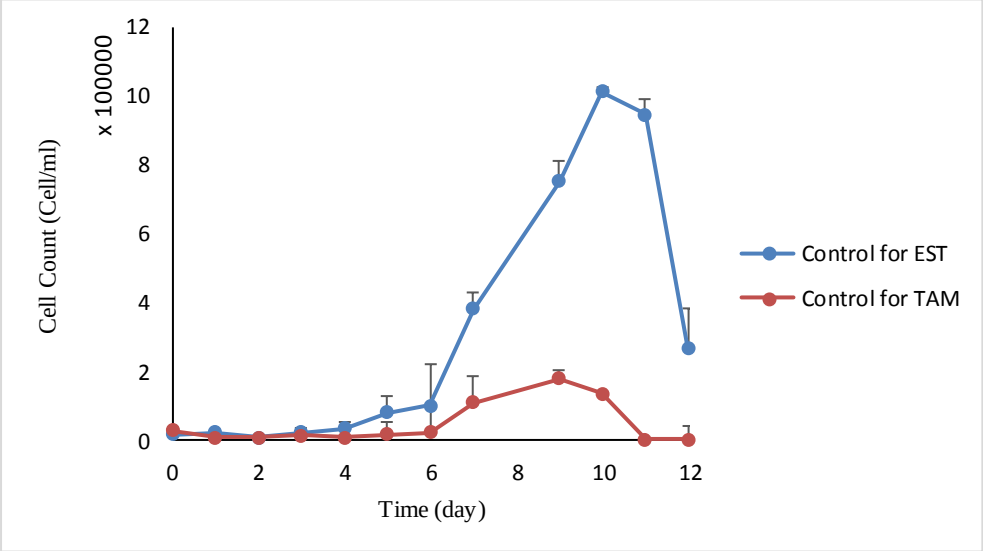

Figure 3: Growth curve comparison of MCF-7 at 2 different seeding densities. The cells were seeded at 1.5×10^4 and 2.5×10^4 cell/ml for testing the effects of 17-ß-estradiol (EST) and tamoxifen (TAM), respectively.

Statistical analysis also shows that there are significant differences between the curves of EST and control cells on days 7 and 8 ($p<0.0001$) of the study. Meanwhile, curve of the cells treated with MP and PP had no statistically significant difference with control cells. These data suggest no observable estrogenic effect for MP and PP on this cell line.

In order to prove the involvement of ERs in parabens effects on cell growth, TAM was used (figure 5). For the study of inhibitory effects cells were seeded at higher density of 2.5×10^4 cell/well, instead of 1.5×10^4 cell/well. It is noteworthy to indicate that statistical analysis showed that cell seeding density could cause significant differences on growth curve patterns only on days 7 ($p<0.05$) and 9 ($p<0.001$). In other words, higher cell seeding density caused a higher peak of cell density on day 7, which later resulted in faster cell death, as observed in day 9 of the study (figure 5). Otherwise, two curves are statistically identical.

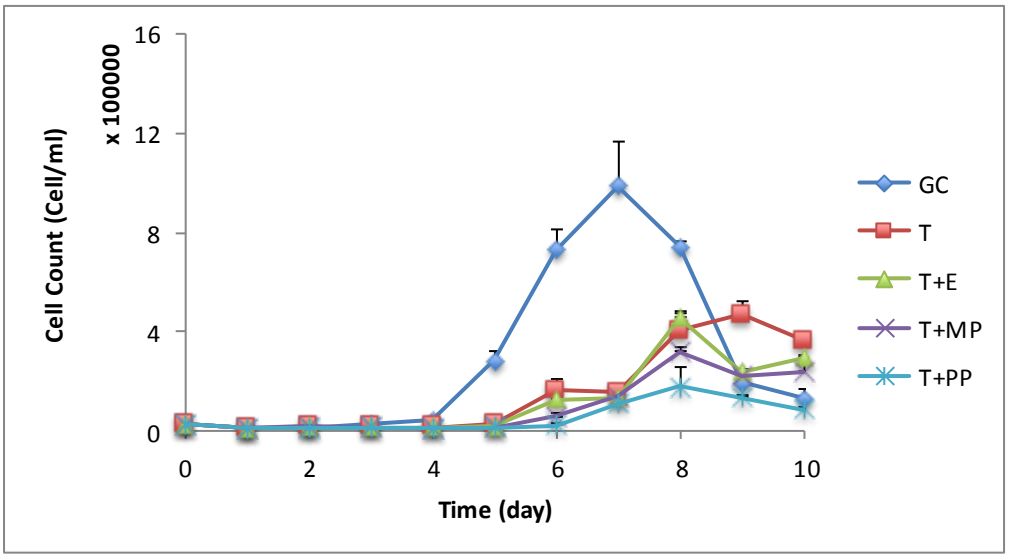

Figure 5: Growth curve comparison of human breast adenocarcinoma cell line (MCF-7) seeded at the density of 2.5×10^4 cells/well. The curves compare the growth curve of the cells in non-treated status (GC) or exposed to 7.017 µg/ml of tamoxifen (T) ± 1.206 of 17-ß-estradiol (E), methylparaben (MP) or propylparaben (PP).

In the absence of TAM or other treatments, the cells start their exponential phase of growth on day 4 and go on till day 7. The cells spend 2 days in the plateau state and after that they enter the death phase of the growth curve ($p<0.0001$) (figure 4).

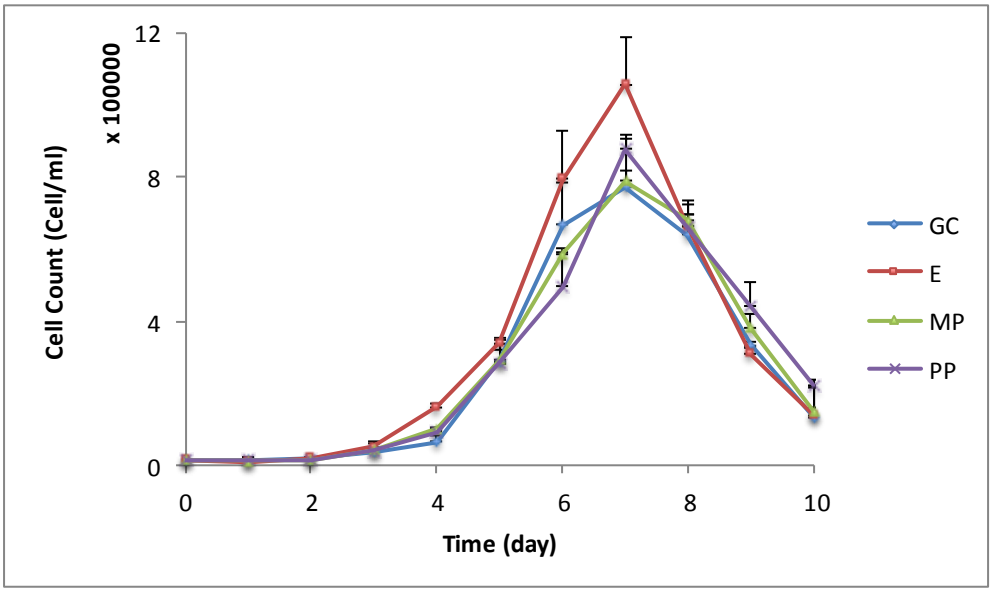

Figure 4: Growth curve comparison of human breast adenocarcinoma cell line (MCF-7) seeded at the density of 1.5×10^4 cells/well. The curves compare the growth curve of the cells in non-treated status (GC) or exposed to 1.206 µg/ml of 17-ß-estradiol (E), Methylparaben (MP) or propylparaben (PP).

Exposure of the cells with TAM alone could cause significant reduced cell growth pattern, exacerbating from day 5. Interestingly, pretreatment of the cells with TAM resulted in reduced growth pattern of the cells for TAM+ EST, MP or PP treatments as well. The presence of EST in the media could overcome the inhibitory effect of TAM only on day 9. The observation showed that the cells treated with TAM caused a late entrance of the cells to the plateau state. The cells reached their maximal count on day 8 and kept their plateau state for 2 days. The inhibitory effect of TAM caused a delayed plateau with less count of cells compared to control (figure 4). As it is already known, phenol red, the pH indicator in culture media, has some weak estrogenic effect [14]. To omit this effect, phenol red free media was used in this study. Furthermore, newborn calf serum (NCS), which is believed to have slight amounts of estrogens, was also applied instead of the routine fetal bovine serum (FBS). However, the results indicate that in the absence of estrogens, or the presence of negligible amounts of these cell growth stimulants, MCF-7 cells keep on their growth pattern and TAM can inhibit this growth well. Addition of EST to TAM treated cells does not cause increased cell count, but the cells enter the death phase on day 9, while the TAM treated cells are in the plateau state. It is possible to estimate that EST, at the point of plateau state, could cause some sort of enhanced proliferation rate, which can further acidify culture medium and damage the cells faster [15, 16]. Slower rate of cell growth in the case of TAM can lead to a plateau phase.

The curves of TAM+MP and TAM+PP are not significantly different, while both curves have statistically significant difference with the curves of TAM and TAM+EST on day 8. These data suggest that MP and PP could not impose as high estrogenic effect as EST to lead to the highest cell count. On the contrary, it seems that these agents could further deduct cell proliferation. The observation can suggest a competitive inhibitory action of TAM on the cells. In fact, although the compounds were used at their half maximal effective concentration, TAM concentration (0.018 M) in the media was more than other compounds (EST: 0.003M, MP: 0.006M, PP: 0.005M). So, besides the fact that parabens have less affinity for ERs [11], TAM can competitively bind the receptor more. It is possible that this binding be reversed in the presence of higher concentrations of parabens.

Comparison of the effects for each compound, alone or co-treated with TAM showed that presence of TAM could significantly decrease the proliferation of MCF-7 cells, indicating a probable role for ERs.

Sum of all, although MP and PP seem to have estrogenic effects, an effect that can be inhibited with TAM, these compounds are not more potent than EST on MCF-7 cell line. At the same time

studies show a more proliferative effect of these compounds on ovarian cell line [13]. Thus, it is also possible to conclude that in comparison between these two cell lines, although ovarian C13 cell line seemed to be less sensitive to the estrogenic effects of EST compared to MCF-7, it might be more sensitive to the proliferative effects of MP and PP. On the other hand, although the length of the side-chain of MP and PP is different, their proliferative effect on MCF-7 was the same in the applied concentrations.

CONCLUSION

We would therefore suggest that parabens interaction with the estrogenic receptors are primarily done through the cyclic side of these compounds which is enough to interfere with the blocking nature of TAM. The side chains of parabens are to determine the estrogenic potency of these compounds which might be different for the corresponding receptors in different cell lines.

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