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Original Article

Parabens Effects on Estrogenic Receptors of C13 Cell Line

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Abstract

Ovarian cancer is one of the major causes of death in women. Parabens are a class of chemicals widely used as preservatives in cosmetic and pharmaceutical products, they mimic estrogens, which are known to play a role in the development of different cancers in women. The Aim of this study was to investigate the proliferative effects of Methyl Paraben (MP) and Propyl Paraben (PP) on human ovarian adenocarcinoma C13 cell line.C13 cell line grown in phenol red-free RPMI 1640, supplemented with 10% NCS, were exposed to either 0-100 µM of estradiol (E)or tamoxifen (T). After 5 days, the number of live cells in each well (12 well plate) was counted using trypan blue assay to obtain the EC_{50} or LC_{50} of E and T using the regression fitness analysis on GraphPad Prism© software. The acquired EC_{50} for E was used for MP and PP exposure, alone or in a co-treatment with LC_{50} of T to investigate their effects on C13 growth curve.LC_{50} and EC_{50} of T and E were 3.125 µg/ml and 12.5 µg/ml, respectively. In a co-administration of these two, T showed to be a good cell growth inhibitor for the first 9 days, when the proliferative effect of estrogenic compounds lead to cell mitosis. Parabens showed estradiol pattern boost in cell growth for the first 8 days, but had more sustained and powerful proliferative effect compared to estradiol with a 300% increase in cell number on day 10. In co-administration with T, MP and E reversed T inhibitory effects from the beginning with MP boosting cell proliferation up to 500% on day 10.Both MP and PP showed delayed proliferative effects much stronger than E on C13 cells. MP was the most potent growth stimulating paraben on C13 cells with a rank order of MP>PP>E. It is concluded that parabens are much toxic in human than thought before, but with a delayed toxicity pattern. More investigation on chronic uses of paraben containing products is required for the safety measurement of these compounds.

Keywords: MethylParaben; PropylParaben; Estradiol; Tamoxifen, Growth Curve; C13 cell line.

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1. Introduction

Ovarian cancer is one of the major causes of death in women. Estrogen induces growth, proliferation and metastasis of ovarian cancers [1-3]. Parabens are a class of chemicals widely used as preservatives in cosmetic, food and pharmaceutical products [4-5]. Parabens, as natural occurring fungicide/bactericides, are believed to be nontoxic in oral and intrapritoneal administrations. Organically known as esters of para-hydroxybenzoic acid, common parabens include: methylparaben, ethylparaben, propylparaben, and butylparaben [6]. Methylparaben can be found in shampoos, moisturizers, shaving gels, personal lubricants, spray tanning solutions and toothpastes. Utilization ofparabens in these products is increasing due to their bactericidal and fungicidal properties, low cost, efficacy as preservatives, and also the inefficacy of other alternatives such as grapefruit seed extract in comparison with these compounds. Although Parabens are rapidly absorbed (particularly dermally), metabolized into their metabolites (such as para-hydroxybenzoic acid, parahydroxyhippuric acid, para hydroxybenzoylglucuronide and para-carboxyphenylsulfate) and excreted, their use remains controversial since low concentrations of parabens have been isolated from breast cancer tumors [7-8]. In a number of *in vitro* studies, parabens have proven to able to bind to the estrogen receptors and activate genes controlled by these receptors and therefore stimulate cell growth and proliferation [9].

The Joint FAO/WHO Food Standard Program announced that the acceptable daily intake (ADI) for parabens is 0–10 mg/kg/day. *In vitro* estrogenic activity has been reported for some Parabens in vitro [10-11]. Adverse effects have been reported in the reproductive system of male rats and mice fed with food containing butyl- and propyl-parabens at the same or lower dose as the ADI [11-12]. In any case, the data on adverse effects of Parabens is controversial and insufficient [13]. On the other hand, about 15% of human couples in developed countries are suffering from infertility, almost half of these cases related to men, through low sperm motility or/and sperm count. It is known that a significant number of cases of male infertility results from defects in testis mitochondria, particularly due to the effects of drug-induced toxicities. It has been reported that the interaction between parabens and mitochondrial function in the testis may be the key in explaining the contribution of parabens in the decrease in reproductive potential [14]. The Aim of this study was to investigate the possible stimulatory effect of MethylParaben (MP) and PropylParaben (PP) on

the estrogenic receptors of a human ovarian adenocarcinoma cell line, C13, that contains estrogenic receptors [15, 16]. An estrogenic receptor inducer and also some blockers have also been used to confirm the interactive nature of parabens with these receptors in C13 cell line.

2. Materials & Method:

2.1. Reagents

Phenolred- free RPMI 1640 cell culture media was obtained from Sigma Chemical Co. Neonatal Calf serum (NCS, heat inactivated), penicillin, streptomycin, and trypsin EDTA were obtained from Gibco Company, Germany.17*β*-Estradiol &Tamoxifenstandards were obtained from Darupakhsh pharmaceutical factory, Iran. Methylparaben&propylparabenwere dissolved in absolute ethanol, before use.

2.2. Cell Culture

C13 human ovarian adenocarcinoma cell line (ATCC, USA) was routinely cultured in phenolred- free RPMI 1640 supplemented with 10% heat-inactivated NCS, 100 IU/ml of penicillin and 100 µg of streptomycin. The cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

2.3. Cell Growth Assay the and Chemical Effects

The C13 cells treated with 17*β*-Estradiol were seeded in 12-well culture plates at a density of 15×10^3 cells per well, while the C13 cells treated with Tamoxifen were seeded at a densityof 25×10^3 cells per well. To obtain the ED_{50} and LC_{50} of these compounds on C13 cell line, the cells were exposed to eight different concentrations (0, 1.56, 3.125, 6.25, 12.5, 25, 50 & 100 µg/ml) of either 17*β*-Estradiol and/or Tamoxifen in the cell culture media for 5 days.Trypan Blue dye assay was used to evaluate the cell proliferation following the exposure to these compounds.

After 24 hours of cell propagation in new media, 17β-Estradiol, Methyl Paraben and Propyl Paraben (at the concentration of 12.5µg/ml equal to the ED_{50} for 17β-Estradiol) were added to the C13 cells media seeded in 12-well culture plates at a density of 15×10^3 cells per well, while Tamoxifen (LC_{50}=3.125 µg/ml) was added to the cells seeded in 12-well culture plates at a density of 25×10^3 cells per well. The exposure time was assigned for 24 hours after which the culture medium was replaced with fresh media and cells were incubated for 12 days. To evaluate the blocking effects of Tamoxifen on this cell line, tamoxifenwas added to the growth media for 24 h after cells incubation.The culture medium was replaced with fresh media without Tamoxifen and cells were incubated for another 12 days before the trypan blue assay. To find out the effects of these compounds in isolated or co-administered models, living cells in 3 wells of every experiment were counted using trypan Blue dye exclusion every day for 12 days after treatments.

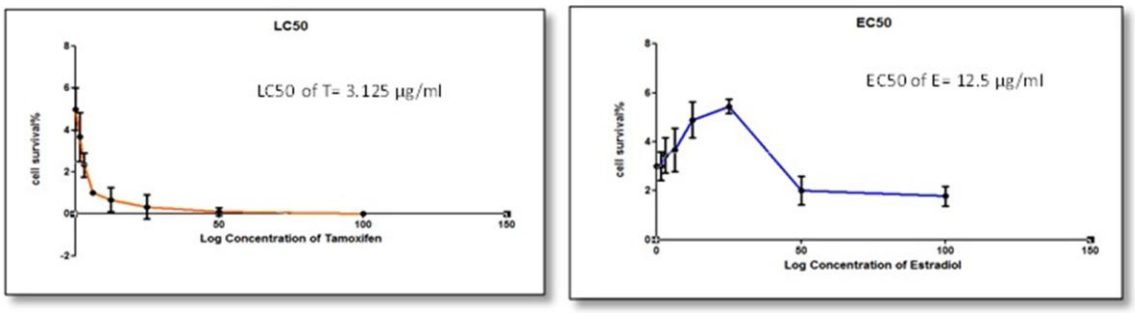

Figure 1. Cell growth curve of human ovarian adenocarcinoma cells after 24h of exposure to Tamoxifen and 17β-Estradiol by Trypan Blue staining assay. Cells were seeded in 12 well plates at a density of 25×10^3 cells for treatment with different concentrations of Tamoxifen and at a density of 15×10^3 cells for treatment with different concentrations of 17β-Estradiol up to 5 days. LD_{50} of Tamoxifen and ED_{50} of 17β-Estradiol were calculated to be 3.125µg/ml and 12.5 µg/ml (T= Tamoxifen; E= 17β-Estradiol.).

2.4. Statistical Analysis

The regression fitness analysis on GraphPad Prism© software was used for the assessment of EC_{50} and LC_{50} of Estradiol and Tamoxifen on C13 cell line. All graphs were plotted using GraphPad Prism© software.

3. Results and Discussion

As is shown in Fig. 1, the LC50 of Tamoxifen on C13 cell line (seeded at a density of 25×103 cells/well) for 5 days was calculated to be 3.125 µg/ml. The EC50 of Estradiol on C13 cell line (seeded at a density of 15×103 cells/well) at the same condition seeded in 12 well plates for 5 days was calculated to be equal to 12.5 µg/ml.

Fig. 2 represents the growth curve of C13 cell line after 24 h of exposure to Estradiol, Tamoxifen, MethylParaben&PropylParaben. The results represent the growth stimulating effects of estradiol, methyparaben and propylparaben compared to the control. It is also demonstrated that methyl paraben has the strongest proliferating effects among these compounds.

The effects of continuous exposure of C13 cells to the Tamoxifen, with or without the three other compounds of estradiol, methyl paraben and propyl paraben have been presented in Fig. 3. The results show the inhibiting inhibory effect of Tamoxifen on stimulation by estradiol, MP and PPwhen the cells were co-treated with tamoxifen.

4. Conclusion

As it has been shown in the figure 2 of the result section estradiol, propyl and methyl parabens do stimulate the C13 cells for a faster growth and proliferation in particular for the first 8 days after exposure. To prove that this stimulatory effect is through the estrogenic receptors, the cells were co-treated with Tamoxifen in which the effect of these compounds was diminished. However, it is

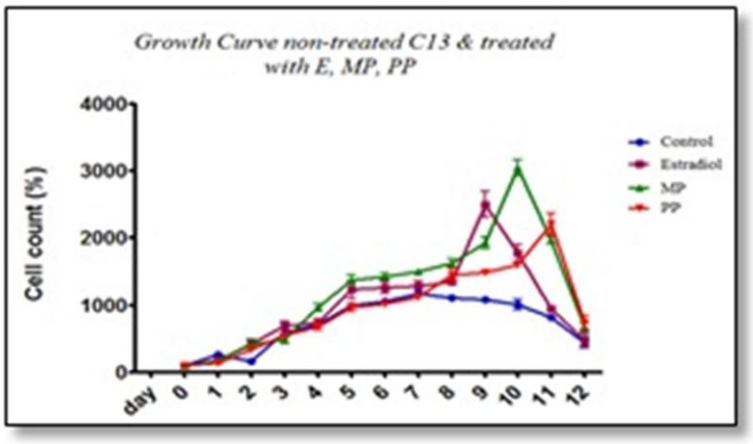

Figure 2. Cell growth curve of human ovarian adenocarcinoma cells after 24h of treatment with different compounds by Trypan Blue staining assay. Cells were seeded in 12 well plates at a density of 25×10^3 cells for treatment with Tamoxifen (LD50= 3.125µg/ml) and at a density of 15×10^3 cells for treatment with 17β-Estradiol, PP and MP (ED50=12.5µg/ml). The cells were counted at different days in triplicates up to 12 days. Results are represented as mean ±SE from 3 wells (Control= Untreated, E= 17β- Estradiol, MP= MethylParaben, PP= PropylParaben).

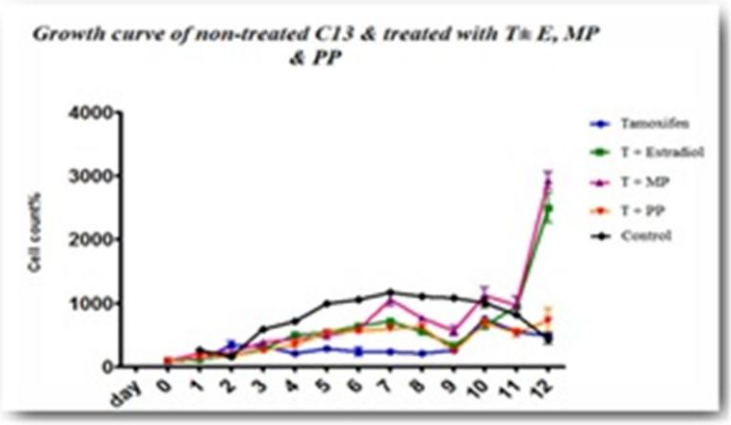

Figure 3. Cell growth curve of human ovarian adenocarcinoma cells after 24h of co-treatment with different compounds by Trypan Blue staining. Cells were seeded in 12 well plates at a density of 15×10^3 cells for co-treatment of Tamoxifen (LD50= 3.125µg/ml) with 17β-Estradiol, PP and MP (ED50=12.5µg/ml). The cells were counted each day in triplicates up to 12 days. Results are represented as mean ±SE from 3 wells (Control= Untreated, E= 17β- Estradiol, MP= MethylParaben, PP= PropylParaben).

important to note that the inhibitory effects of Tamoxifen is very much limited to about first 9 days after which the cells escape and the proliferation rate increases. This escaping behavior from the estrogenic receptor stimulatory effect is especially dramatic and significant when cells are exposed to estradiol and methylparaben. The inhibitory effect of Tamoxifen is seen in all treatments until the 9th day after exposure but this inhibitory effect

remove on cell receptors and as there are the growth stimulatory agents such as Estradiol, PropylParaben and MethylParaben in the cell culture media, the cells activity are faster while if there is only Tamoxifen, the cell growth is lower compared to the present of stimulatory agents. Also comparison between Estradiol plus Tamoxifen, MethylParaben plus Tamoxifen&PropylParaben plus Tamoxifen show that MethylParaben has the most stimulatory effect on cell growth compared to the others. This might further emphasize on the competitive nature of estradiol, parabens and Tamoxifen on the estrogenic receptors which has been shown in previous publications [9-14]. What is unique with the present article is that all of the previous publications have only looked at the parabens' effects in a short period of time (maximum of 194 Hours) [17] to represent the safety of these compounds. However, we have continued the experiment up to about 12 days and we were successful to present a point of dramatic potentially toxic proliferative effect on these cell lines after about 8 days mostly related to the estrogenic stimulatory effects of parabens on this cell line. Further studies on other cell lines and using more paraben compounds is encouraged and will clarify these potential cytotoxic harmful effects on human.

References

[1] Cannistra SA, Gershenson DM, Recht A. Ovarian cancer, peritoneal carcinoma and fallopian tube carcinoma. In: DeVita VT, Hellman S, Rosenberg SA, eds. Cancer: Principles and Practice of Oncology. 8th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2008: 1569-1594.

[2] Collaborative Group on Epidemiological Studies of Ovarian Cancer, Beral V, Gaitskell, Hermon C, Moser K, Reeves G, Peto R. Ovarian cancer and smoking: individual participant meta-analysis including 28,114 women with ovarian cancer from 51 epidemiological studies. Lancet Oncol. 2012; 13(9):946-956. Epub 2012 Aug 3.

[3] Goodman MT, Shvetsov YB. Incidence of ovarian, peritoneal, and fallopian tube carcinomas in the United States, 1995-2004. Cancer Epidemiol Biomarkers Prev. 2009;18(1):132-139.

[4] Steinberg, D.C., 2005 Preservatives Use: Frequency Report and Registration, Cosmetics & Toiletries,121(7), 65-69 (2006).

[5] Elder, R.L. , et al., Final Report on the Safety Assessment of Methylparaben, Ethylparaben, Propylparaben, and Butylparaben, Journal of the American College of Toxicology, 3(5), 147-209 (1984).

[6] Cashman, A. &Warshaw, E. Parabens: structure, formulation and metabolism. Dermatitis, 16 (2), Retrieved from http: //www. medscape. com/ view article/ 508430_2, 2005.

[7] Darbre PD. J ApplToxicol. "Underarm cosmetics and breast cancer," 2003 Mar-Apr;23(2):89-95.

[8] Harvey P.W., Everett D.J. "Significance of the detection of esters of p-hydroxybenzoic acid (parabens)in human breast tumours". Journal of Applied Toxicology 2004, 24 (1): 1–4. doi:10.1002/jat.957).

[9] Crinnion WJ. Toxic effects of the easily avoidable phthalates and parabens. Environmental Medicine, Southwest College of Naturopathic Medicine, Tempe, AZ, USA. Altern Med Rev. 2010 Sep; 15(3):190-6.

[10] Darbre, P. D., Bayford, J. R., Shaw, L. E., Horton, R. A. and Sauer, M. J. (2002) Oestrogenic activity of isobutylparaben in vitro and in vivo. J. Appl. Toxicol., 22, 219–226.

[11] Oishi, S. Effects of butyl paraben on the male productive system in rats. Toxicol. Ind. Health, (2001) 217, 31–39.

[12] Oishi, S. Effects of propyl paraben on the male productive system. Food Chem. Toxicol., (2002) 40,1807–1813.

[13] Mark G et al. the health controversies of Parabens. Skin therapy letter. (2013) 18 (2).

[14] Renata S. T, Fatima C. M, Paulo J. O, João R, Francisco P. P. Parabens in male infertility—Is there a mitochondrial connection? Reproductive Toxicology. 27 (2009) 1–7.

[15] Satoh, K., Nagai, F., Aoki, N. and Nishijima, M. (2000) Competitive binding of some alkyl p-hydroxybenzoates to human estrogen receptor α and β. J. Pharm. Soc. Jpn., 120, 1429–1433.

[16] Bayford, J. R., Shaw, L. E., Drew, M. G. B., Pope,G. S., Sauer, M. J. and Darbre, P. D. (2002) Oestro-genic activity of parabens in MCF7 human breast cancer cells. J. Steroid Biol. Mol. Biol., 80, 49–60.

[17] Anna W, Ewa L.G. Effects of single and repeated in vitro exposure of three forms of parabens, methyl-, butyl- and propylparabens on the proliferation and estradiol secretion in MCF-7 and MCF-10A cells.Pharmacological Reports. 2013, 65, 484-493.