Estrogenic activity of parabens by cell proliferation of MCF-7 human breast cancer cells.

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ABSTRACT: Parabens that have estrogenic activity may potentially cause adverse health effects in humans. The estrogenic activities of butylparaben and ethylparaben were assessed by examining estrogenic activity and estrogen-receptor dependent cell proliferation of MCF-7 human breast cancer cells and U2OS cancer cells. Estrogenic activity was measured by performing a luciferase transcriptional assay using U2OS ER-α and ER-β cancer cells; cell proliferation was measured by performing a cell proliferation assay, flow cytometry, real-time PCR, western blot, and ELISA were performed using MCF-7 human breast cancer cells. Butylparaben significantly increased estrogenic activity and estrogen-receptor dependent cell proliferation of MCF-7 human breast cancer cells and U2OS cancer cells. Ethylparaben showed almost no significant estrogenic activity. In conclusion, butylparaben has estrogen-receptor estrogenic activities and cell proliferation of MCF-7 human breast cancer cells.

INTRODUCTION: Parabens are widely used as preservatives and antimicrobial agents found in thousands of products ranging from food and food packaging to cosmetics and pharmaceutical products [1, 2]. Xenoestrogens, chemicals that mimic or increase actions of naturally occurring estrogens like estradiol (E2), are defined as having estrogenic activity (EA); increased EA may produce adverse health effects in humans such as increased rates of breast cancer by acting as endocrine-disrupting chemicals. EA is increased by E2 binding to estrogen-receptors (ER) which increase gene transcription by activating estrogen response elements (EREs) by using two genes: ER-α and ER-β [3]. ERE activates the critical gene, *c-myc*, which produces *myc*-RNA, *myc*-protein, and *myc*-transcription factors that activate phosphatase and phosphatase RNA. Phosphatase RNA removes the cyclin-dependent kinases (CDK) inhibitor, allowing cyclin protein to activate CDK. CDK controls the activity of the retinoblastoma tumor suppressor protein (Rb) by phosphorylating the E2F-Rb complex, removing Rb, and allowing free E2F to activate DNA replication genes. ERE may directly activate the cyclin gene which produces cyclin RNA and cyclin protein [11, 12, 13]. Increased ERE activity is proportional to increased transcriptional gene activity and further increases cell proliferation [4]. Parabens exhibit estrogen-like property *in vivo*, and are mediated by progesterone receptor (PR) and ER-α signaling pathways [5]. Estrogenic activities of *methyl-*, *propyl-*, *butyl-*, *isopropyl-*, and *isobutyl-*parabens were assayed using ER-dependent proliferation of MCF-7 human breast cancer cells. All compounds were shown to stimulate cell proliferation. Parabens with longer and branched side-chains were more potent than parabens with short and linear side chains [6]. Most current studies conclude that butylparabens produce increased estrogenic activities, but debate whether including butylparabens in consumer products may cause adverse human health effects. However, studies have shown that increased estrogen activity is linked to breast cancer. Estrogen promotes the development of mammary cancer in rodents and has direct and indirect proliferative effects on human breast cancer cells. Direct effects may occur by inducing enzymes and proteins involved in nucleic acid synthesis that activates oncogenes. Indirect effects may occur by stimulating the production of growth factors [7]. Long-term use of postmenopausal hormone therapy increases risk of breast cancer. Current evidence indicates that longer duration of use increases risk of breast cancer regardless of formulation with a higher rate of increase

when using combination estrogen plus progestin therapy [9]. Levels of endogenous sex hormones are associated with breast cancer risk in postmenopausal women [10]. The widespread prevalence of parabens poses as a public health concern where long-term effects on human health are still widely debated. Parabens will increase estrogenic activity and cell proliferation of U2OS cancer cells and MCF-7 human breast cancer cells.

METHODS:

Transfection

U2OS cells were transfected with ERE-tk luciferase and CMV-ER-α, and again with CMV-ER-β. The plasmids were transfected into the cells by electrophoresis using 0.25v and 960mp. The plasmids were treated with the following: E2 (10^{-5} M; final concentrations = 10^{-8} M), ethylparaben (EP) (10^{-3} M; final concentrations = 10^{-6} M), butylparaben (BP) (10^{-3} M; final concentrations = 10^{-6} M); incubated for 48 hours. Added lysis buffer to each well of the dish and froze plates at -80C for 30 minutes. Plates were placed into a tissue culture incubator for 15 minutes. The plates were assayed using a luciferase assay and a luminometer. Used T-test significance; data was graphed.

Cell Proliferation Assay

MCF-7 breast cancer cells checked for general health, contamination, and confluency. Cells were trypsinized and centrifuged at 3,000 rpm for 5 minutes. Media was aspirated, and cells resuspended. Cells counted using a hemocytometer, and treated with: ETOH (Control), E2 (10^{-6} M; final concentration = 10^{-9} M), EP (10^{-3} M; final concentration = 10^{-6} M), BP (10^{-3} M; final concentration = 10^{-6} M); incubated for 7 days. Media was aspirated, and trypsinized. Cells incubated for 5 minutes then resuspended. Cells counted using a Coulter Counter. Used T-test significance; data was graphed.

Flow Cytometry

MCF-7 breasts cancer cells were plated and treated with the following: Control, E2 (final concentration = 10^{-9} M), EP (final concentration = 10^{-6} M), BP (final concentration = 10^{-6} M); incubated for 24 hours. Cells harvested by aspirating media, washing cells with PBS, adding trypsin-EDTA, and incubating for 5 minutes. Added complete media to cells and resuspended before centrifuging at 1700 rpm for 5 minutes at room temperature (RT). Aspirated media, added ice-cold PBS, and resuspended pellet before centrifuging again at 1700 rpm for 10 minutes at RT. Aspirated supernatant and froze cell pellet at -80C. Cells were placed into flow cytometer and analyzed with FloJo and graphed.

Real Time PCR

MCF-7 breasts cancer cells were plated and treated with the following: Control, E2 (final concentration = 10^{-8} M), EP (final concentration = 10^{-6} M), BP (final concentration = 10^{-6} M);

incubated for 1 hour. RNA isolated using Aurum total *RNA* mini kit for RNA isolation. Amount of RNA determined in each sample with nanodrop. cDNA was prepared with iScript Kit. Made 1:10 dilution of cDNA by adding RNase/DNase free water. Used Ssofast qPCR supermix for real time PCR kit. Ran real time PCR (RT-PCR).

Western Blot

MCF-7 breasts cancer cells were plated and treated with the following: Control, E2 (10^{-5} M; final concentration = 10^{-8} M), EP (10^{-3} M; final concentration = 10^{-6} M), BP (10^{-3} M; final concentration = 10^{-6} M); incubated for 2 hours. Discarded media and added buffer. Placed plates on ice for 10 minutes, shaking every 2 minutes. Scraped cells off plate, DNA broken up with syringe/needle, and centrifuged at 14,000 RPM for 10 minutes. Took supernatant. Protein concentration determined using the Bradford assay and read in Smart Spec. Generated standard curve and prepared sample for SDS-PAGE. Separated proteins by electrophoresis at 200mv for 35-45 minutes. Transferred proteins to PVDF membrane, prepared by soaking membrane in methanol for 1 minute with gentle shaking. Assembled transferring sandwich. Used roller to remove all bubbles. Electrical transferring performed for 1 hour at 100v; blocked membrane by soaking. Prepared primary antibody solution and incubated with membrane overnight at 4C. Washed membrane with TTBS 3 times. Prepared secondary antibody solution. Added secondary antibody solution to membrane shaking for 1 hour. Discarded secondary antibody solution in sink and washed with TTBS five times. Performed enhanced chemiluminescence (ECL) using ECL Plus reagent. Developed film for 30-60 seconds.

ELISA

MCF-7 breasts cancer cells were plated and treated with the following: Control, E2 (final concentration = 10^{-8} M), EP (final concentration = 10^{-6} M), BP (final concentration = 10^{-6} M); incubated for 30 minutes. Prepared ELISA plate by adding antibody to phosphorylated ER-α to coat ELISA plate. Dumped antibody into sink and added Wash Buffer. Dumped Wash Buffer and repeated washing process two more times. Incubated at 4C. Prepared cell lysates by adding E2 (10^{-5} M), EP (10^{-3} M), BP (10^{-3} M). Incubated for 30 minutes at 37C. Rinsed cells twice with PBS. Added Lysis Buffer and shake plates. Incubated plates on ice for 15 minutes. Centrifuged at 14,000 RPM for 5 minutes and transferred supernatant to a clean microfuge tube. Performed ELISA using Human Phospho-ER alpha/NR3A1 (S118) kit for ELISA.

RESULTS:

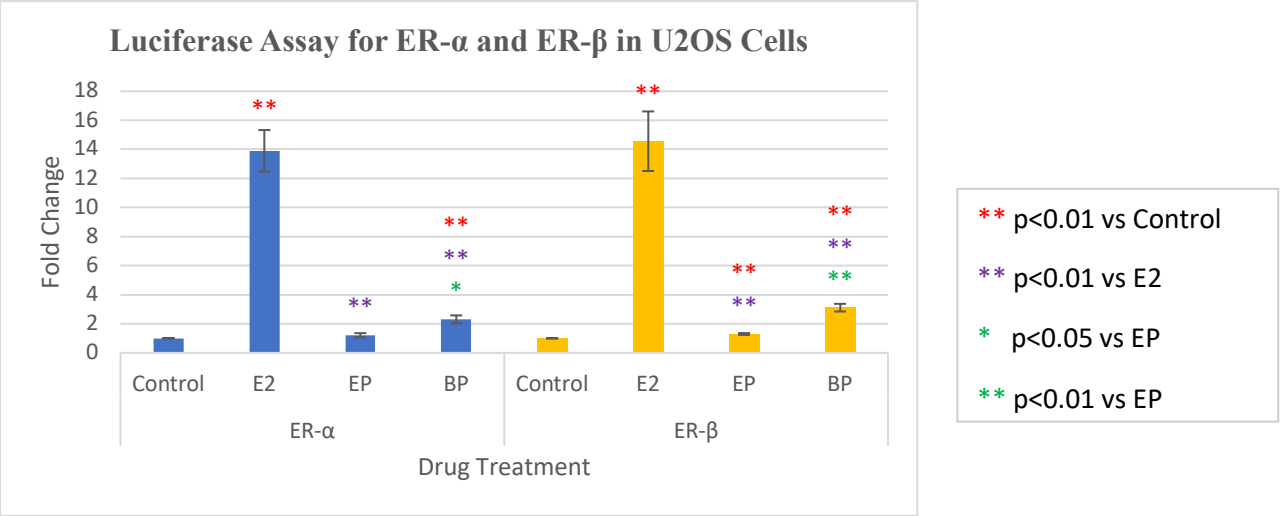

Figure 1. Parabens regulated the expression of ERE-tk-Luc in U2OS cells. U2OS cells were transfected with ERE-tk-Luciferase and CMV-ERα. U2OS cells were then treated with estradiol for 48 hours. Cells were measured with luminometer. Each triplicate was averaged. ER-α: p=0.0008 for E2 vs control, p=0.2082 for EP vs control, p=0.0087 for BP vs control; p=0.0009 for EP vs E2, p=0.0013 for BP vs E2; p=0.0238 for BP vs EP. ER-β: p=0.0027 for E2 vs control, p=0.0080 for EP vs control, p=0.0013 for BP vs control; p=0.0029 for EP vs E2, p=0.0052 for BP vs E2; p=0.0026 for BP vs EP. (*) p-value<0.05; (**) p-value<0.01.

[Figure 1] To determine if parabens regulated gene transcription, we measured their effects on the expression of ERE-tk-Luc in U2OS cells; U2OS cells were selected because they do not express endogenous estrogen receptors. This provided little to no background noise. For ERα: E2 and BP had significant increases compared to the control; EP and BP had significant increases compared to E2; BP had significant increase compared to EP. For ERβ: E2, EP, and BP had significant increases compared to the control; EP and BP had significant increases compared to E2; BP had significant increase compared to EP.

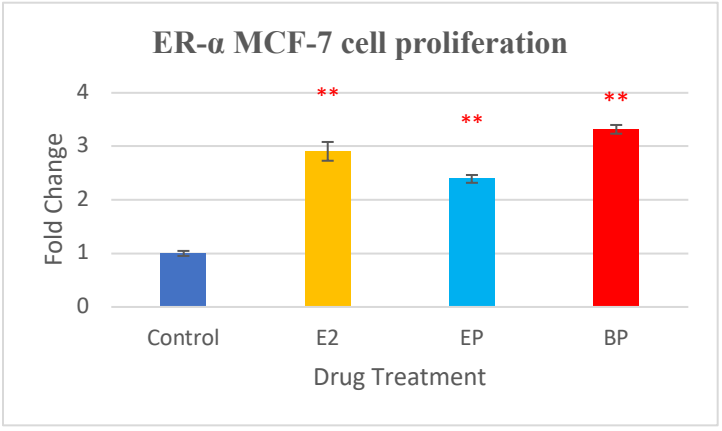

Figure 2. Parabens stimulated cell proliferation of MCF-7 human breast cancer cells by increasing the number of cells as an indicator of EA, treated cells were compared with the positive control, E2. Cells were treated with E2 (10^{-6} M; final concentration = 10^{-9} M), EP (10^{-3} M; final concentration = 10^{-6} M), BP (10^{-3} M; final concentration = 10^{-6} M) for 7 days. Cells were measured with a hemocytometer and Coulter Counter. Each triplicate was averaged. p=0.0033 for E2 vs control, p=0.0004 for EP vs control, p=0.00008 for BP vs control; p=0.1932 for EP vs E2, p=0.6262 for BP vs E2; p=0.0081 for BP vs EP. (**) p-value<0.01.

[Figure 2] To determine if parabens stimulated cell proliferation of MCF-7 human breast cancer cells by increasing the number of cells as an indicator of EA, and measured using a Coulter Counter and hematocytometer following 7 days of treatment. E2, EP, and BP had significantly increased cell count comparing fold change to control. E2 had a ~3 fold-increase; EP had a ~2.5 fold-increase; BP had a ~3.3 fold-increase.

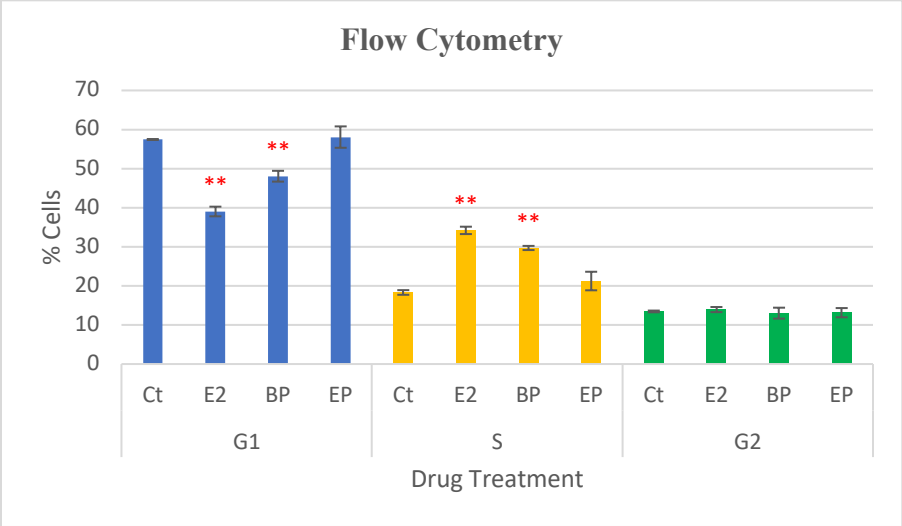

Figure 3. BP increases cell proliferation in MCF-7 cells. MCF-7 human breast cancer cells were treated with E2 (final concentration = 10^{-9} M), EP (final concentration = 10^{-6} M), BP (final concentration = 10^{-6} M) for 24 hours. Cells were measured with flow cytometry and analyzed with FloJo. Each triplicate was averaged. G1: p=0.00001 for E2 vs control, p=0.0003 for BP vs control, p=0.7148 for EP vs control; S: p=0.00001 for E2 vs control, p=0.00001 for BP vs control; p=0.1071 for EP vs control; G2: p=0.2968 for E2 vs control, p=0.6195 for BP vs control, p=0.6707 for EP vs control. (**) p-value<0.01.

[Figure 3] To determine if parabens stimulated cell proliferation of MCF-7 human breast cancer cells by increasing DNA synthesis, we measured the DNA content in MCF-7 cells as they progress through the cell cycle. G1 phase had increased BP and EP % cells compared to E2; S phase had increased E2 and BP % cells compared to control and EP; G2 phase had relatively similar % cells for all treatments. E2 and BP showed significant increase in both G1 and S phases although E2 had a higher magnitude of change compared to BP in the S phase.

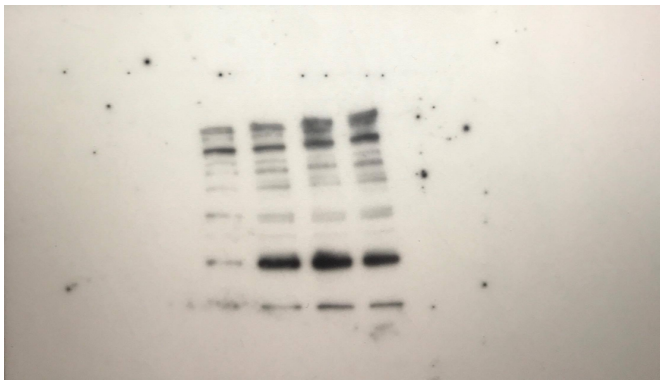

Figure 4. BP and EP increase c-myc protein expression in MCF-7 cells. Cells were plated and treated with the following: Control, E2 (10^{-5} M; final concentration = 10^{-8} M), EP (10^{-3} M; final concentration = 10^{-6} M), BP (10^{-3} M; final concentration = 10^{-6} M), and incubated for 2 hours.

[Figure 4] To determine if parabens increased the amount of c-myc protein, we measured their effects on relative protein levels in MCF-7 human breast cancer cells. Darker bands indicate increased levels of protein expression, and lower bands indicate lower levels of protein expression. The bands are ordered: Control, E2, BP, EP. All bands show increased levels of protein expression.

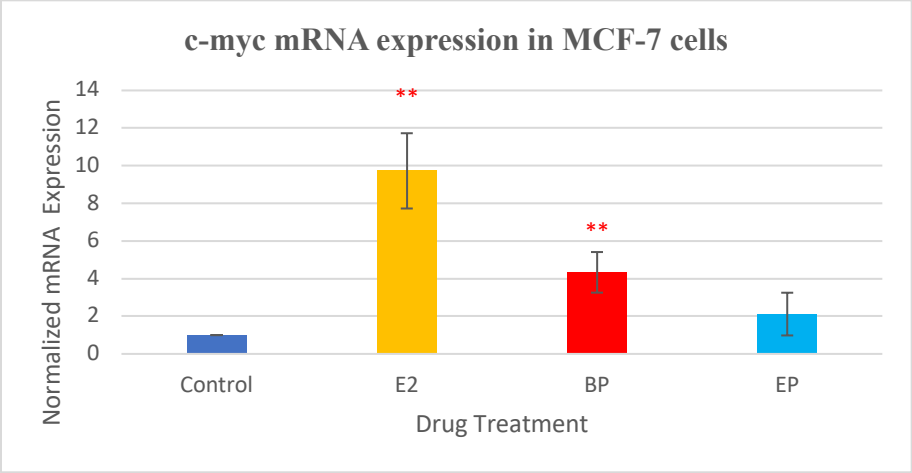

Figure 5. Parabens increased the amount of c-myc mRNA in MCF-7 breast cancer cells. Cells were plated and treated with the following: Control, E2 (final concentration = 10^{-8} M), EP (final concentration = 10^{-6} M), BP (final concentration = 10^{-6} M), and incubated for 1 hour. Cells were measured with RT-PCR. Each triplicate was averaged. p=0.0016 for E2 vs control, p=0.0059 for BP vs control, p=0.1638 for EP vs control. (**) p<0.01.

[Figure 5] RT-PCR determined c-myc mRNA expression induced by parabens and was normalized using GAPDH gene MCF-7 cells. E2 has the greatest fold increase. EP has the least. E2 and BP had statistically significant fold-increases in c-myc mRNA expression. EP showed no statistically significant activity.

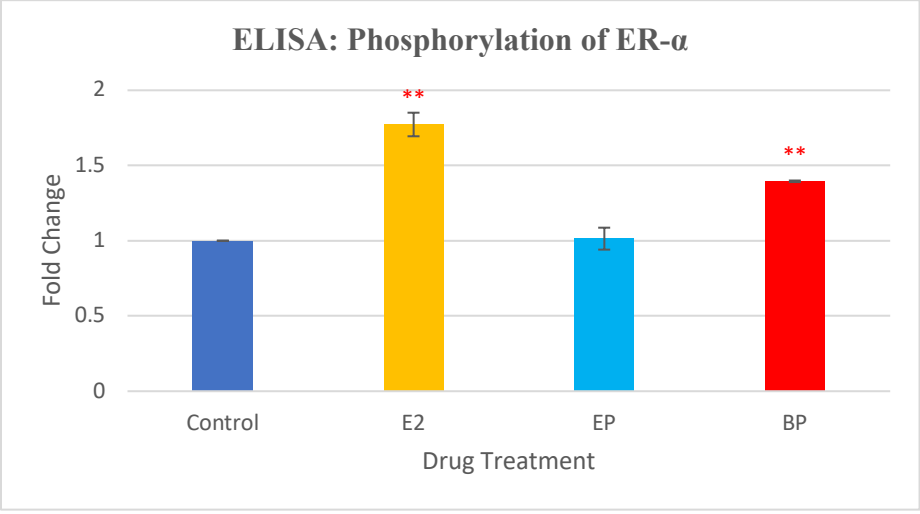

Figure 6. Parabens cause the phosphorylation of ERα. MCF-7 breasts cancer cells were plated and treated with the following: Control, E2 (final concentration = 10^{-8} M), EP (final concentration = 10^{-6} M), BP (final concentration = 10^{-6} M), and incubated for 30 minutes. Cells were measured with ELISA. Each triplicate was averaged. p=0.0783 for E2 vs control, p=0.8121 for EP vs control, p=0.00008 for BP vs control. (**) p<0.01.

[Figure 6] To determine if parabens cause the phosphorylation of ERα, we measured their effects on the transcriptional activity of MCF-7 human breast cancer cells using a sandwich ELISA. We measured the concentration of phosphorylated ERα with the optical detector set to measure absorbance at 450 nm. E2 treated cells exhibited ~1.8 fold-increase; EP treated cells did not exhibit any fold change; BP treated cells exhibited ~1.4 fold-increase and was significantly significant.

DISCUSSION: The results supported the hypothesis that parabens will increase estrogenic activity and cell proliferation of U2OS cancer cells and MCF-7 human breast cancer cells. Several assays were used (transfection, cell counting, flow cytometry, RT PCR, ELISA). BP is a strong estrogen agonist with $p<0.01$ for all assays used. Cell proliferation of MCF-7 cells and transfection of U2OS cancer cells were the only experiments where BP and EP were both statistically significant compared to the control. This is because parabens are selective agonists for ER-β and ER-α; interactions with the estrogen receptors are dependent on the size of the parabens' alkyl groups [6, 14]. EP was only significant compared to the control when transfected with ER-β. This may resulted due to technical error, but EP has shown significant lower binding affinity due to its smaller carbon chain, and parabens with longer alkyl chains have greater binding affinity [6]. BP has a longer alkyl chain compared to EP, and thus has a higher binding affinity. This explains the recurring trend where BP shows significant estrogenic activity while EP has limited estrogenic activity. RT-PCR measures the *c-myc* oncogene; *c-myc* plays an important role in cell development and cell proliferation [11]. EP failing to show significant estrogenic activity may be because of RNAse integration in the RT-PCR solution. A different study testing *c-myc* levels testing the effect of parabens on breast cancer cell lines had similar *c-myc* mRNA and protein levels when compared to our result [6]. The Western Blot also had technical errors. We had to use another lab mates' sample because either our cells died since we did not initially check the dish under the microscope, or we did not scrape the cells well enough. Our cell concentration for EP ended up being too low (0,36 μg/μL); the sample used instead had higher cell concentrations (5.396 μg/μL). Environmental parabens and other chemicals can alter estrogen gene expression. E2 supports the cell growth of cells with mutations, and accumulates, ultimately resulting in cancer. ERE binding is also correlated to increased cell proliferation and our results from ELISA show that BP significantly increases cell phosphorylation [4]. Therefore, BP may increase risk for breast cancer. Parabens, especially compounds with longer alkyl chains like BP, exhibit estrogenic activity and increase risk for breast cancer. Our experiments show that BP has shown significant estrogenic activity compared to the control. However, our experiments, besides cell proliferation using the hemocytometer and Coulter Counter, also show that BP fails to produce activity at the same magnitude as E2. To improve our experiments, we should test parabens at lower concentrations while activating other ligands to produce a synergistic increase in estrogenic activity. A recent study has shown that HER ligands great enhanced the potency of parabens in increasing c-myc mRNA and cell proliferation via ER-alpha in breast cancer cells. Parabens are active at lower exposure levels than currently considered [Pan]. Many toxicological studies test paraben effects in isolation; future studies should focus on testing paraben with other pathways such as HER ligands. Understanding the magnitude of the impact that parabens have on human health may be better understood when testing the synergistic effects of parabens and other pathways. Our results show that BP, and EP in some experiments, stimulate estrogenic effects such as cell proliferation and c-myc expression *in vitro*.

BP and EP's significant estrogenic activity indicate that exposure to everyday parabens may increase risk for breast cancer

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