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# Estrogenicity of parabens revisited: Impact of parabens on early pregnancy and an uterotrophic assay in mice

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ABSTRACT

Parabens, a class of preservatives routinely added to cosmetics, pharmaceuticals, and foods, have estrogenic properties. Given that intrauterine implantation of fertilized ova in inseminated females can be disrupted by minute levels of exogenous estrogens, we assessed the impact of parabens upon early gestation. In Experiment 1, butylparaben was administered subcutaneously in several doses ranging from 0.05 to 35 mg/animal/day to inseminated CF-1 mice on days 1–4 of pregnancy. Butylparaben exposure did not affect litter size, the number of pups born, postnatal day 3 litter weights, or the number of pups surviving to postnatal day 5. In contrast, administration of 500 ng/animal/day 17β-estradiol terminated all pregnancies. In Experiment 2, propylparaben was subcutaneously administered to inseminated CF-1 mice on gestational days 1–4. Dams were sacrificed on gestation day 6 and the number of implantation sites was counted. Propylparaben had no impact on the number of implantation sites observed. Since Experiments 1 and 2 did not yield the anticipated results, an uterotrophic assay was conducted in Experiment 3 to re-evaluate the *in vivo* estrogenicity of parabens. Ovariectomized CF-1 and CD-1 mice were administered butylparaben in doses ranging from 0.735 to 35 mg per animal for three consecutive days. Mice were sacrificed on the fourth day, and uterine mass was recorded. There was no effect of butylparaben on uterine wet or dry mass at any dose in either strain. In contrast, administration of 17β-estradiol consistently increased uterine mass in both strains. These data indicate that the estrogen-sensitive period of implantation is not vulnerable to paraben exposure, and that the *in vivo* estrogenicity of parabens may not be as potent as previously reported.



## 1. Introduction

There is an increasing concern in the public health and scientific communities over the use of synthetic chemicals that mimic the actions of endogenous hormones [1]. Such chemicals include the esters of para-hydroxybenzoate (parabens), which have been used as antimicrobial preservatives in foods, pharmaceuticals, and cosmetics at increasing rates for over 50 years [2]. A growing literature suggests that parabens are weakly estrogenic. *In vitro* studies show that parabens bind to estrogen receptors α and β, and that estrogenic potency increases with linear length and/or branching of the alkyl side chain [3–6]. Parabens have also been shown to increase proliferation of estrogen-dependent MCF-7 human breast cancer cells [5], regulate expression of the reporter gene ERE-CAT [3,4,7], and compete with 17β-estradiol for estrogen receptors [6–8]. These actions can be inhibited by anti-estrogens such as tamoxifen or ICI 182,780 [5,6]. Like 17β-estradiol, butylparaben and isobutylparaben can down-regulate estrogen receptors and up-regulate progesterone receptors [5].

Parabens have also been shown to be estrogenic *in vivo*, especially through uterotrophic assays [3–6,9–11]. Estrogens regulate production of growth hormone [12] and activation of the insulin-like growth factor 1 pathway [13], which cause growth and cellular proliferation of uterine tissue [14]. Therefore, an increase in uterine mass is a reliable indicator of estrogenic activity [15]. Subcutaneous butylparaben administration has been reported to increase uterine mass in ovariectomized and immature rats [6] and mice [10,11]. Isobutylparaben and benzylparaben have also been found to increase uterine mass in immature mice [3,4]. In fish, parabens increase another index of estrogenic activity, plasma vitellogenin, following dietary supplementation, spiking of tank water, or intraperitoneal administration [16,17].

Human exposure to parabens is pervasive and has been estimated to average 76 mg/day [2]. Most human exposure occurs through the use of cosmetics and toiletries, including but not limited to, face make-up preparations, deodorants, sun-care products, and body lotions [2,18,19]. An analysis of 215 cosmetics found that 93% contained parabens including 77% of rinse-off and 99%

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of leave-on products [20]. It was once assumed that such exposure was safe because the skin contains esterases that metabolize parabens to PHBA [21]. However, PHBA itself may be estrogenic [9,11] and paraben metabolism in the skin may be incomplete [22]. In humans, whole-body application of a cream containing 2% butylparaben increased free serum butylparaben to peak levels (135 μg/l) within 3 h of application, while after 24 h levels had declined but remained above baseline (18 μg/l) [23]. Parabens have been detected in human breast tumor tissue and have been posed as possibly contributing to rising breast cancer rates [24,25]. Parabens have been detected in nearly 100% of urine samples tested, and PHBA has been found in the plasma of pregnant women, umbilical cord blood, and breast milk at concentrations of 73, 96, and 108 ppb respectively [26–28].

While some evidence indicates adverse effects of paraben exposure upon male reproductive parameters [29,30], effects of parabens on female fertility are largely unexplored. Of particular interest is the potential for parabens to disrupt intrauterine implantation of fertilized ova. It is well established that minute exogenous doses of 17β-estradiol (120 ng/animal) terminate early pregnancy in mice, while other estrogens and androgens also have this effect at somewhat higher doses [31–33]. Oral or subcutaneous exposure to another weak xenoestrogen, bisphenol-A, can prevent blastocyst implantation when administered to inseminated mice on days 1–4 of gestation [34,35]. In addition, just one administration of 6.75 mg bisphenol-A per animal on the day following insemination significantly reduces the number of implantation sites counted on day 6 of gestation [35]. Since bisphenol-A and the more estrogenic parabens have similar potencies [6,8,17,36], we predicted that butylparaben and propylparaben, the most estrogenic of the commonly used parabens, could also disrupt intrauterine implantation.

Accordingly, we administered doses of butylparaben and propylparaben to inseminated female CF-1 mice and observed subsequent litter size and intrauterine implantation sites. Several doses were examined, ranging from those approximating human exposure levels to the maximal dose that could be administered within constraints of solubility and immediate health of animals. Subsequently, we re-examined whether paraben exposure affects uterine growth in ovariectomized adult mice. Since several studies report *in vivo* estrogenic effects in CD-1 mice [3,4,9–11] and there are known strain differences in sensitivity to endocrine disruption [37], we examined sensitivity to butylparaben exposure in this uterotrophic assay in both CF-1 and CD-1 mice.

## 2. Materials and methods

### 2.1. Animals and housing

This research was approved by the Animal Research Ethics Board of McMaster University, in compliance with the guidelines of the Canadian Council on Animal Care. CF-1 mice (*Mus musculus*) were bred in this laboratory from stock originally obtained from Charles River Breeding Farms of Canada (La Prairie, Quebec). CD-1 mice were directly obtained from that supplier and given at least 10 days adaptation to this laboratory before use. Colony rooms were maintained at 21 °C on a reversed 14:10 light:dark cycle. Cages consisted of 28 cm × 16 cm × 11 cm (height) polypropylene with wire-bar tops allowing *ad libitum* access to food (8640 Teklad Certified Rodent chow; Harlan Teklad, Madison, Wisconsin) and water. The mean (±SE) mass of CF-1 and CD-1 female mice was 36.9 g (±0.26 g) and 35.0 g (±0.45 g) respectively. All female mice were sexually naïve and 100–180 days old, while breeding males were 150–420 days old.

### 2.2. Experiment 1

CF-1 females were weighed and isolated at the onset of the experiment. Four days later, each female was paired with a male of proven fertility in a clean cage with fresh bedding. Females' hindquarters were inspected for the presence of sperm plugs three times daily thereafter. The day of sperm plug detection was considered day 0 of pregnancy. Subcutaneous injections of parabens occurred on each of days 1–4 of pregnancy, commencing 3–6 h into the dark cycle. Injections occurred at four different sites to minimize any irritation resulting from paraben administration: right and left flank, rear middle area, and scruff of the neck. Age and weight were counterbalanced across conditions. Inseminating males were removed from the female's cage prior to the first injection.

Butylparaben (Sigma–Aldrich H9503) was administered in doses of 0.00, 0.05, 0.5, 5, 10, 15, 20, 30, and 35 mg per animal. Other females were each administered 500 ng 17β-estradiol (Sigma–Aldrich). Due to solubility constraints of butylparaben, doses were dissolved in different amounts of peanut oil: 0.05 and 0.5 mg and estradiol in 0.05 ml; 5 mg in 0.1 ml; 10, 15, and 20 mg in 0.3 ml; and 30 and 35 mg in 0.45 ml. In vehicle control subjects, injection volumes and sites were matched to those of experimental conditions. Females were placed into a cage with fresh bedding 14–16 days following mating, but were otherwise left undisturbed until parturition. The number of pups born was recorded at parturition. Litter mass was measured on postnatal day 3 and the number of pups remaining on postnatal day 5 was recorded in all conditions, with the exception of 5 and 10 mg butylparaben which did not have data from postnatal day 3 recorded due to experimental error.

An additional replication at the highest dose was conducted in order to confirm the results of this experiment using a distinct source of butylparaben. Females were administered 35 mg butylparaben (Sigma–Aldrich W220302; ≥99% purity) in 0.45 ml peanut oil with concurrent control females receiving this volume of oil alone.

### 2.3. Experiment 2

This experiment was conducted to observe whether high doses of propylparaben would impact pregnancy and employed direct measures of uterine implantation sites. CF-1 females were prepared and inseminated as in Experiment 1. Due to solubility constraints of propylparaben, doses were dissolved in 0.05 ml dimethylsulfoxide (DMSO). Females each received DMSO vehicle alone or either 35 or 40 mg propylparaben (Sigma–Aldrich P5835) on each of days 1–4 of gestation. Two days following the last injection, dams were sacrificed via $CO_2$ asphyxiation and their uteri excised via abdominal incision. The number of visible intrauterine implantation sites was counted as described previously [35]. Briefly, an implantation site was defined as a small, round swelling in an otherwise smooth, uninterrupted uterine horn.

### 2.4. Experiment 3

Adult CF-1 and CD-1 female mice were bilaterally ovariectomized under sodium pentobarbital anaesthesia. These females were housed in groups of five for a 24-h recovery period and individually caged thereafter. They were randomly assigned to receive subcutaneous injections of peanut oil containing varied amounts of butylparaben or estradiol, with these injections repeated on each of days 27–29 following ovariectomy. A vehicle control group received peanut oil alone. A positive control group received 500 ng 17β-estradiol. Three doses of butylparaben were examined, 0.735, 7.35, and 35 mg. Age and weight were counterbalanced across conditions. The range of butylparaben doses was selected to include concentrations reported to elicit an uterotrophic response [10] and to include the highest concentration from Experiment 1. All chemicals were dissolved in 0.45 ml peanut oil and injections occurred 3–6 h into the dark cycle. Three different injection sites were used to minimize irritation resulting from paraben administration: right and left flank, and scruff of the neck. At 24 h following the final injection, animals were reweighed and sacrificed via $CO_2$ asphyxiation. Hysterectomies were performed via abdominal incision. Sutures that were around the uterine horn at ovariectomy to prevent excessive bleeding were removed by excising the uterus from the base of the suture. Uteri were trimmed free of fat and mesentery and placed into pre-weighed 1.5 mL micro-tubes to obtain uterine wet mass. Uterine dry mass was obtained following 21 days of tissue storage in the presence of calcium sulphate crystals.

### 2.5. Statistical analysis

Comparisons were made between conditions using one-way analysis of variance for Experiments 1 and 2, and factorial analysis of variance (strain by dose) for Experiment 3. Where a significant factor was found in the analysis of variance, pairwise comparisons were made among conditions using the Newman-Keuls procedure. For all analyses, results were considered to be significant at the $p < 0.05$ level.

## 3. Results

### 3.1. Experiment 1

Most animals receiving butylparaben at doses exceeding 0.5 mg developed skin irritation at one or more injection sites, particularly in the scruff of the neck. Irritation typically increased in size and severity with increasing butylparaben concentration. One animal receiving 35 mg and another receiving 20 mg butylparaben were removed from the experiment due to lesions and poor health in the initial days following injections. There were no differences among