**Assessment of Estrogen-Like Effects of Isobutyl Paraben in Wistar Albino Rats Using the Uterotrophic assay**

**Abstract**

This study aims to evaluate the estrogen-like effects of isobutyl paraben (IBP) using the uterotrophic assay in ovariectomized (OVX) Wistar albino rats. The primary objective was to establish a dose-response relationship between uterine weight and subcutaneous administration of 17-ethinyl estradiol (EE) as a reference estrogen and IBP as a potential endocrine-disrupting chemical (EDC). Parabens, widely used as preservatives in cosmetics, pharmaceuticals, and food, have raised concerns regarding their endocrine-disrupting properties. The study was conducted following the OCSPP guideline 890.1600 established by the U.S. Environmental Protection Agency (EPA) to assess estrogenic activity. Our findings demonstrate that IBP exposure resulted in significant physiological and histological changes in the uterus, comparable to EE, indicating its potential estrogenic effects. These results highlight the implications of IBP in endocrine disruption, raising concerns about its impact on reproductive health. The study underscores the need for stringent regulatory measures and further toxicological investigations to reassess the safety standards for IBP and similar EDCs, given their widespread presence in consumer products.

**Keywords:** uterotrophic assay, endocrine-disrupting chemicals, isobutyl paraben, estrogenic activity, reproductive toxicity.

1. **INTRODUCTION:**

In recent decades, public concern has grown over endocrine-disrupting chemicals (EDCs) (Marx- Stoelting *et al.,* 2011). EDCs can interfere with normal bodily processes by imitating or inhibiting hormones at low levels (Schug *et al.,* 2011). EDC exposure has been linked to numerous illnesses, including obesity in both men and women. Subfertility, poor sperm quality, urogenital birth abnormalities, feminisation of male newborns, delayed or precocious puberty, endometrial hyperplasia, endometriosis, recurrent miscarriage, polycystic ovary Syndrome and menstrual abnormalities (Vom Saal *et al.,* 2012; Diamanti-Kandarakis *et al.,* 2009; Zama and Uzumcu, 2010; Schell and Gallo, 2010; Brouwers et al., 2011; Swan *et al.,* 2005; Grun and Blumberg (2009).

Parabens are used as preservatives in cosmetics, pharmaceuticals, and food goods. However, Research has demonstrated that parabens can behave as endocrine-disrupting chemicals (EDCs) that interfere with hormones by mimicking the female sex hormone, oestrogen. Parabens can bind with oestrogen receptors, causing disturbances in everyday hormonal communication, which may impact reproductive health. Increases the risk of hormone-related malignancies. Parabens are a class of alkyl ester chemicals found in p-hydroxybenzoic acids commonly used as antibacterial agents and preservatives in cosmetics, personal care products, medications and foods. Humans can be exposed to parabens by inhalation, skin contact, and consumption (El Hussein et al., 2007; Soni *et al.,* 2005). Previously, parabens were deemed generally harmless. Compounds of low toxicity and bioaccumulation. However, the literature increasingly indicates that parabens exhibit poor oestrogenic characteristics in invitro and in vivo uterotrophic experiments (Lemini *et al.,* 2003; Routledge *et al.,* 1998). Parabens (methyl-, ethyl-, propyl-, butyl-, benzyl-, isopropyl-, or isobutylparaben) have been shown to exhibit endocrine-disrupting effects in both in vitro and in vivo tests (Hu *et al.* 2013). Parabens resemble oestrogen by binding to oestrogen receptors (ER) on human breast cancer MCF-7 cells (Byford *et al.,* 2002; Charles Darbre 2013; Routledge *et al.*, 1998).

Isobutylparaben, in particular, has caused concern because of its relatively Compared to other parabens such as methylparaben and ethyl paraben, it has higher lipophilicity and a longer alkyl chain. This may improve its ability to cross biological membranes and concentrate in tissues. Studies have IBP has been shown to bind to oestrogen receptors, potentially disrupting endocrine function by interfering with hormonal control of reproductive and developmental processes. These qualities indicate that isobutylparaben, like other parabens, may contribute to adverse health effects, notably through oestrogenic action (P.D. Darbre, *et al*).

The uterotrophic test raised the relative weight of the uterus in a dose-dependent way. Administration of benzyl paraben to female Sprague Dawley rats. Lowest oral observed effect dose (LOED). The daily dose of benzyl paraben was determined to be 0.16 mg/kg body weight (Hu *et al.,* 2013). The LOEDs estimated daily doses of methyl, ethyl, propyl, butylparaben were 16.5, 6, 20, and 7 mg/kg body weight, respectively. When fed orally in a uterotrophic experiment with young CD-1 mice (Lemini *et al.,* 2003). Isobutylparaben also demonstrated estrogen-like properties that the progesterone receptor could mediate. Vo and Jeung (2009) say the signalling route involve the PR and/or ERα receptors. In addition to oestrogens, Parabens are believed to exhibit androgenic activity, implying that some parabens have deleterious effects (Darbre and Harvey (2008) and Oishi (2001, 2002a, 2002b, 2004) discuss the impacts on the male reproductive system. However, a debatable argument is that paraben has minimal toxicity and does not harm humans. (Golden, 2005; Soni, 2005; 1999). The Safety of Parabens in Cosmetics and Personal Care Products. There has been political debate on this issue in the EU in recent years. The Danish Environmental Protection Agency (EPA) has banned propylparaben and butylparaben in cosmetics for children under three since 2011 (Danish MOE, 2011). However, parabens are not on the "Negative List" in Canada's "List of Prohibited and Restricted Cosmetic Ingredients", which also includes the United States (Health Canada, 2014; US FDA, 2000). Many toxicological investigations have explored the four kinds of parabens: methyl, ethyl, propyl, or butylparaben), but isobutylparaben (IBP) (CAS No. 4247-02-3) has not been thoroughly researched. Furthermore, no repeated dose toxicity data were provided to assess the risk of isobutylparaben. The study was designed first to demonstrate a dose-response relationship of uterine weight in adult OVX rats after Subcutaneous injection of the standard oestrogen, 17-ethinyl oestradiol (EE). The uterotrophic assay is frequently used. In vivo assay for measuring the oestrogenic activity of chemical compounds, notably endocrine disruptive. Parabens are one example of an EDC. Parabens are widely used as preservatives in personal care. Products have prompted concerns about their ability to mimic oestrogen and damage the endocrine system. The assay is based on the capacity of oestrogenic substances to cause an increase in uterine weight in Immature or ovariectomised female rodents, making it an appropriate approach for assessing the estrogen-like effects of IBP. The uterotrophic assay, which measures uterine responsiveness, provides valuable insights into the potential dangers presented by IBP, specifically their role in influencing reproductive health and contributing to hormone-dependent Conditions such as breast cancer and fertility problems. As a result, this assay is an essential tool for Understanding IBP toxicity and making informed regulatory judgements about their safe usage.

1. **MATERIALS AND METHODS**

**2.1 Ethical Approval**

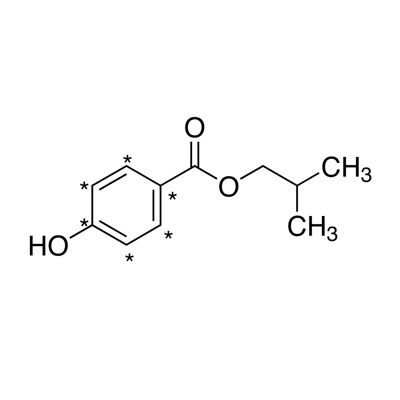
The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the Government of India. Approval was granted with reference number SUK/ZOL/IAEC/01/2024-25 from the Department of Postgraduate Studies and Research in Zoology, Sharnbasva University, Kalaburagi, Karnataka, India (CPCSEA registration no: 2236/PO/ReBiBt/S/23/CPCSEA).

**2.2 Research Framework**

The uterotrophic assay was conducted in alignment with the OCSPP guideline 890.1600, established by the U.S. Environmental Protection Agency (EPA), to assess estrogenic activity in chemicals. Additionally, the study followed the OECD Guideline 440 (2001) for chemical testing, which provides a standardized approach to evaluating compounds with potential estrogen-related effects.

**2.3 Test Compounds**

Isobutylparaben (IBP) (CAS No. 4247-02-3; purity 99.38%) and phenylmercuric acetate (PMA) (CAS No. 62-38-4; purity 99.38%) were purchased from Tokyo Chemical Industry (TCI) India Pvt Ltd, Hyderabad, India. Both compounds were dissolved in corn oil, used as a vehicle. 17α-Ethinyl estradiol (EE) (CAS No. 979-32-8; purity 99.28%) was sourced from Sigma-Aldrich and dissolved in corn oil. All compounds were administered via oral gavage at 5 ml/kg, with EE given subcutaneously.



Picture 1: Chemical structure of Isobutylparaben (IBP)

**2.4 Test Animals**

Female Wistar rats were obtained from the National Institute of Nutrition (ICMR), Hyderabad, India. Upon arrival at 5 weeks of age, all animals were examined for general health and acclimatized to the laboratory environment for one week prior to the ovariectomy. The animals were housed at the Department of Postgraduate Studies and Research in Zoology, Sharnbasva University, Kalaburagi, Karnataka, India.

**2.4 Animal Maintenance**

Rats were housed in polycarbonate cages with solid floors, maintained under a controlled 12-hour light-dark cycle at a temperature of 23 ± 2°C and relative humidity of 50 ± 10%. Bedding consisted of autoclaved rice husk. Animals were randomized into experimental groups based on body weight, ensuring the average weight within each group varied by no more than ± 5 grams. Each cage held three rats, with individual animals identified by tail markings.

**2.5 Feed and Water**

The study followed established guidelines for the care and use of laboratory animals. The rats were fed pelleted rodent chow from Champaka Feeds and Foods, Bangalore, India, and had ad libitum access to purified water in polycarbonate bottles.

**2.6 Ovariectomy Procedure**

Ovariectomy was performed following OECD Test Guideline 440 (OECD Document 4, 1999). Animals were allowed to acclimate for one week before surgery, which was conducted at 8 weeks of age. A dorsolateral incision was made along the midpoint between the ribs and the iliac crest. The ovaries were excised at the juncture of the oviduct and uterine body. The abdominal wall and skin were closed using auto-clips. Post-surgery, the rats were allowed a recovery period of two weeks before treatment.

Picture 2 : Ovariectomy Procedure



UTER INE WEIGHT

**Disconnection line at necropsy**

**2.7 Test Substance Administration**

Fourteen days after ovariectomy, rats were assigned to four treatment groups:

• Control group: Corn oil administered via oral gavage.

• Positive control group: 10 µg/kg EE administered subcutaneously.

• Low-dose IBP group: 10 mg/kg IBP via oral gavage.

• High-dose IBP group: 50 mg/kg IBP via oral gavage.

The doses of 10 and 50 mg/kg/day for IBP were chosen based on prior studies and regulatory standards to assess potential endocrine-disrupting effects. These doses have been commonly used in toxicity assessments for evaluating estrogenic activity in vivo. This range allows for the investigation of both typical human exposure levels and higher exposures for a thorough risk assessment.

**2.8 Observations**

**Clinical Signs:** Animals were observed at least once daily throughout the study, with any abnormalities recorded. Cage inspections occurred twice daily during the working week to monitor for signs of distress or mortality.

**2.9 Body Weight and Food Intake:** Body weight was recorded daily to the nearest 0.1 g, beginning at the start of treatment. Food intake was measured by weighing feeders, with results expressed as grams consumed per rat per day.

**2.10 Uterus and Vagina Weight Measurement**

Twenty-four hours after the final treatment, rats were euthanized by cervical dislocation. The uterus was carefully dissected, trimmed of excess fat, and separated at the junction with the cervix. The vagina was detached from the uterus. The uterus was weighed (wet weight) to the nearest 0.1 mg after removing excess luminal fluid by placing the tissue on damp filter paper. Each uterus was handled individually to avoid dehydration before weighing.

**2.11 Histomorphology**

Uteri were removed and weighed out wet. A piece (4 mm) from the mid-segment, cut perpendicular to the longitudinal axis of the uterus, was dissected out for histological analysis. All uterine tissue were fixed in a 10% formalin solution and stored at room temperature. Finally, the samples were dehydrated in a series of alcohols, embedded in paraffin and cut into serial sections of 10 um in the horizontal plane using a rotary microtome (Senior Rotary Microtome, Weswox MT-1090A). The paraffin from the sections will be removed by placing the slide in toluene and then downgraded them to distilled water from 100% alcohol, then stained with Ehrlich's haematoxylin, washed in running water and placed in distilled water before transferring to 70% and 90% alcohols. Then counter stained with eosin and upgraded to 100% alcohol. After placing in toluene for 5 minutes, the sections mounted in D.P.X. Photomicrographs were taken using Magnus: Microscope Camera, MagCam Series-DC 5. Images were captured at 40 X, 100 x, 200 x, magnifications.

**2.12 Statistical Analysis**

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 10 software, with one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test. A p-value of <0.05 was considered statistically significant.

1. **RESULTS**

**3.1 Clinical signs**

During the study period, there were no signs of mortality, obvious toxicity, or clinical symptoms related to treatment.

**3.2 Body weight and food consumption**

The present study illustrates the effects of ethinyl estradiol (EE) and Isobutyl Paraben (IBP) changes in body weight of female rats that have undergone ovariectomy. In comparison to the untreated control group, the EE group showed a slight increase in overall body weight of 10 µg/kg/day. There were no significant differences in body weights or changes in body weight observed in the treatment groups compared to the untreated control group, (Table- 1 & Fig-1).

Just like changes in body weight, food intake was statistically significant reduced in the group treated with 50 mg/kg/day high dose of IBP compared to the untreated control group. There were no significant changes in food intake in the groups treated with 10 µg/kg/day EE compared to the untreated controls, (Table- 2 & Fig-2).

**3.3 Uterine Weight**

In this experiment, the uterine weight significantly increased at a dosage of 10 µg/kg/day EE compared to the control group, while there was no significant difference in uterine weight at a low dose of 10 mg/kg/day low dose IBP compared to the control group. The animals who received 50 mg/kg/day high dose IBP showed a significant increase in uterine weight compared to the untreated controls vehicle and positive control group 10 µg/kg/day EE (Table- 1 & Fig-2).

**3.4 Histological Findings**

Comparing the normal control group (corn oil) to the other treated groups reveals notable differences in uterine histology:

**1. Ethinyl Estradiol (EE) Group vs. Control Group:** The control group had normal mucosal epithelial cells, some mononuclear cells, and some endometrial gland degeneration. The EE-treated group, on the other hand, had more mucosal epithelial cells, a thicker mucosal layer, edema, cystic degeneration, and less submucosal gland growth. EE causes considerable structural changes and hyperplastic effects not found in the untreated control.

**2. The control group was compared to the Low-Dose IBP group (10 mg/kg/day):** The uterine structure was mostly intact in the control group, but the low-dose group had some vacuolar degeneration and atrophy in mucosal epithelial cells as well as inflammatory cell infiltration (mononuclear and multinuclear). Additionally, the IBP myometrium exhibited excessive growth of connective tissue. This indicates that even low doses of IBP may result in inflammatory and degenerative alterations that were not observed in the control group.

**3. Control vs. High-Dose IBP (50 mg/kg/day):** The normal control group showed low inflammation and epithelial viability. The high-dose IBP group had more inflammatory cells entering the gut, more mucosal epithelial layer loss, and mild submucosal gland degeneration and atrophy. These changes indicate that high-dose IBP causes more severe tissue damage and inflammation, affecting the uterus’s structural integrity to a greater degree than in the control group.

Overall, the normal group's structure stayed the same with few changes. On the other hand, the EE and IBP groups had different levels of hyperplasia, degeneration, and inflammation, with the severity usually rising with the dose of IBP and the estrogenic stimulation from EE.

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| --- | --- | --- | --- |
| **Treatment (mg/kg/day)** | **No. of Rats** | **Body Weight (g)** | |
| **Initial** | **Final** |
| Corn Oil | 6 | 164±14.68 | 167±14.07 |
| EE 10 μg/kg/day | 170.33±14.43 | 167.16±14.68 |
| IBP 10 mg/kg | 171.5±16.56 | 168±16.06 |
| IBP 50 mg/kg | 157.5±8.01 | 154.16±8.15 |

**Table 1.** The impact of 17-ethinyl estradiol and IBP at doses of 10 and 50 mg/kg on the body weight variations in ovariectomized rats treated via subcutaneous injection.

Data are presented as Mean ± SD. Vehicle control received corn oil. Six animals were used per treatment group. All animals were ovariectomized on day 8 week. After 1 week, the test substance was administered subcutaneously once a day for three consecutive days. The animals were weighed and sacrificed by cervical dislocation 24 h after the last treatment. Significantly different from untreated controls at "p<0.05. and "\* p<0.01.

**Table 2**. The effects of 17-ethinyl estradiol and IBP at doses of 10 and 50 mg/kg on the Food consumption variations in ovariectomized rats treated via subcutaneous injection.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Dosing Day** | **Corn oil** | **EE (10 µ/kg)** | **IBP 10 mg/kg** | **IBP 50 mg/kg** |
| Food Consumption (g/day/ animal) | | | | |
| 1-2 | 4.17 | 4.75 | 4.58 | 4.23 |
| 2-3 | 3.98 | 3.62 | 3.53 | 3.26 |
| 3-final | 4.09 | 2.20 | 2.08 | 1.89\*\* |

ANOVA complemented with one tailed Dunnett \*p< 0.05   compared vehicle. E. Estradiol -10 µ/kg;

IBP -10 mg/kg; IBP 50 mg/kg



**Fig-1** The above graphs depict the body weight changes in female ovariectomized rats treated with EE and IBP via orally & subcutaneous injection.



**Fig-2** The above graphs depict the variations in food consumption of female ovariectomized rats treated with EE and IBP via orally & subcutaneous injection.

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| --- | --- | --- | --- | --- | --- |
| **Groups** | **Dosage** | **Wet Uterine, absolute (mg)** | **Wet Uterine, relative (%)** | **Blotted Uterine, absolute (mg)** | **Blotted Uterine, relative (%)** |
|  |
| Control | 0 | 104±27.53 | 63.02±19.56 | 87.16±19.62 | 52.77±14.28 |  |
| EE | 10 μg/kg/day | 158.8±29.45 | 96.32±23.64 | 138.46±29 | 84.09±22.62\*\* |  |
| Low Dose IBP | 10 mg/kg/day | 137.11±21.53 | 81.37±7.42 | 118.95±18.90 | 70.66±7.18 |  |
| High Dose IBP | 50 mg/kg/day | 160.65±33.89 | 104.58±23.07 | 131.95±38.87 | 85.81±25.55\*\* |  |

**Table 3.** The effects of 17-ethinyl estradiol and IBP dose 10, 50 mg/kg on uterus wet and blotted of ovariectomized rats administrated by subcutaneous injection and orally gavage

The data is shown in the format of mean ± standard deviation. The vehicle's control system has been supplied with corn oil. Each treatment group used six animals for the experiment. All animals underwent ovariectomy at the end of the eighth week. After one week, the testing material was given under the skin and by mouth once daily for three days in a row. The animals were weighed and euthanized by cervical dislocation 24 hours after the final treatment. The uteruses were promptly taken out, cut out of any surrounding tissue, and then weighed. The results were found to be significantly different from untreated controls at \*\*p<0.01. Significant level.

|  |  |
| --- | --- |
| C  A | B |
|  | D |

**Fig-3.** The above photographs uterine appearance in response A- Normal Control, B- Positive Control EE - 10µ/kg/day, C-10 mg/kg/day, D- 50 mg/kg/day

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| --- | --- | --- | --- |
|  |  |  |  |
| 40 X | 200 X | 200 X | 200 X |
| * Endometrium – Mucosal epithelial cells are appeared normal – Red arrow * Moderate infiltration mono nuclear cells population [plasma cells, lymphocytes] in sub mucosal region [Green arrow] along with moderate degenerative changes noticed in endometrial gland – Yellow arrow | | | |
| **Fig-4. Representative histological analysis of uterus in VOX (Normal Control -Corn oil)** | | | |

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| --- | --- | --- | --- |
|  |  |  |  |
| 40 X | 200 X | 400 X | 200 X |
| * Endometrium – Moderate hyperplasia of mucosal epithelial cells with increased thickness of mucosal layer – Red arrow * Edema and cystic degeneration of mucosal epithelial cells - Green arrow * Moderate degenerative changes and atrophy of sub mucosal glands –Yellow arrow | | | |
| **Fig-5. Representative histological analysis of uterus in VOX (Positive Control EE 10 μg/kg/day)** | | | |

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| --- | --- | --- | --- |
|  |  |  |  |
| 40 X | 200 X | 200 X | 400 X |
| * Endometrium – Multi focal loss of mucosal epithelial layer mucosal with infiltration of few inflammatory cells – Red arrow * Moderate degenerative changes and atrophy of sub mucosal glands –green arrow | | | |
| **Fig-6. Representative histological analysis of uterus in VOX (High dose IBP 50 mg/kg/day)** | | | |

|  |  |  |  |
| --- | --- | --- | --- |
|  |  |  |  |
| 100 X | 200 X | 200 X | 200 X |
| * Endometrium – Few foci vacuolar degenerative changes and atrophy of mucosal epithelial cells was noticed – Red arrow * Vacuolar degeneration and atrophy of mucosal glands with infiltration of inflammatory cells [mono nuclear and multi nuclear – Neutrophils and plasma cells] – Green arrow * Excessive proliferation of connective tissue in myometrium of uterus – Black arrow | | | |
| **Fig-7. Representative histological analysis of uterus in VOX (Low dose IBP 10 mg/kg/day)** | | | |

1. **DISCUSSION:**

The findings of this study underscore the significant physiological and histological alterations induced by ethinyl estradiol (EE) and isobutyl paraben (IBP) in ovariectomized female rats, highlighting the potential implications for endocrine disruption and reproductive health. The observed increase in body weight in the EE group aligns with established roles of estrogen in regulating body composition and metabolism. Ethinyl estradiol, being a synthetic estrogen, is known to promote adiposity and enhance water retention, contributing to weight gains, as documented in previous studies (Kuhl, 1991; Hopp et al., 2004). Contrariwise, the significant reduction in food consumption seen with high-dose IBP indicates a potential adverse effect of IBP on appetite or gastrointestinal function, suggesting a specific mechanism by which IBP might influence metabolic processes distinct from EE’s effects (Calafat et al., 2009).

Histological analyses revealed profound changes in uterine morphology. The notable hyperplastic effects seen with EE treatment are consistent with its established impact on uterine tissue, where it promotes proliferation and thickening of the endometrial lining (D’Cruz et al., 2020). The increase in mucosal epithelial cells and edema observed in our study aligns with findings by McLachlan et al. (2001), who reported similar estrogenic effects on the uterine tissues of rodents.

In contrast, the low and high doses of IBP presented concerning patterns of degeneration and inflammation in uterine tissues. Even at lower doses, IBP induced vacuolar changes and inflammatory cell infiltration, corroborating results reported by Bhandari et al. (2017), which suggest that IBP may exert significant effects on uterine health, even in the absence of overt toxicity. The increased severity of tissue damage at the higher dose further supports the hypothesis that IBP may function as an endocrine disruptor, capable of altering normal endocrine signaling pathways (Sohoni & Sumpter, 1998). Furthermore, the differential impacts observed between EE and IBP treatment emphasize the complex interaction between synthetic estrogens and environmental endocrine disruptors. The exacerbated histopathological changes observed in the high-dose IBP group illustrate the risk of cumulative exposures to chemical agents that may interfere with hormonal balance (Borg et al., 2002). Overall, this study contributes valuable insights into the endocrine-disrupting potentials of EE and IBP. It raises critical questions regarding the safety of synthetic estrogens and common environmental contaminants, particularly in populations exposed to these compounds through pharmacological or dietary routes. Further research is warranted to explore the mechanisms underlying these alterations and to assess the long-term reproductive consequences of exposure to such agents in both animal models and human populations. These findings necessitate a reevaluation of safety standards for endocrine-disrupting chemicals, advocating for increased regulatory scrutiny to mitigate potential risks associated with exposure to compounds such as IBP in environmentally relevant scenarios.

1. **CONCLUSION:**

The study suggests that ethinyl estradiol has a stimulatory effect on uterine growth and weight, whereas isobutyl paraben demonstrates both estrogenic-like effects and toxicological properties that adversely affect uterine health. Further research is needed to investigate the underlying mechanisms of IBP's effects and its implications for estrogenic activity and potential health risks in context.

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