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Original Article

The effects on growth and reproductive function by parabens in *C. elegans*

Yuko Sakaguchi[1], Nana Hirota[2], Satoshi Fukushima[2], Nobuhiro Ichikawa[1] and Koji Arizono[3]

[1]College of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1, Nojihigashi, Kusatsu, Shiga 525-8577, Japan
[2]Faculty of Environment and Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100 Tsukide, Higashi-Ku, Kumamoto 862-8502, Japan
[3]Kumamoto University Graduate School of Pharmaceutical Sciences, 5-1, Oehonmachi, Chuo-ku, Kumamoto 862-0973, Japan

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ABSTRACT — In recent years, paraoxybenzoic acid esters (parabens) have been used in pharmaceuticals, cosmetics, and food additives. Parabens have been reported to have a weak estrogenic effect in *in vitro* test systems, and it is presumed that the longer the alkyl chain of the paraben, the greater its endocrine-disrupting and reproductive function effects. However, the effects of parabens on human health are still unclear. In this study, we evaluated the effects of six parabens (methyl p-hydroxybenzoate [MP], ethyl p-hydroxybenzoate [EP], propyl p-hydroxybenzoate [PP], isopropyl p-hydroxybenzoate [IPP], butyl p-hydroxybenzoate [BP], and isobutyl p-hydroxybenzoate [IBP]) on the reproductive function of the model organism *Caenorhabditis elegans*. We used 25, 50, and 100 µg/mL solutions of parabens in 0.1% dimethyl sulfoxide (DMSO). Bioassays (growth and maturation effect tests and reproduction effect tests) were performed on L1 larvae of wild-type *C. elegans*. In the growth effects test, all parabens were observed to have no effect. In the maturation effects test, there was a significant decrease in maturity at each concentration of five of the six parabens, with the exception being MP. In the reproduction effects test, a significant decrease in the number of lifetime offspring was observed at each concentration of five of the six parabens, with the exception being EP. This decrease was remarkable with PP, which has been reported to adversely affect reproductive function in rats. It is necessary to continue to focus on the estrogen-like action of parabens, including PP, and perform genetic analyses, such as RNA sequencing.

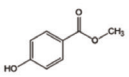
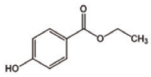
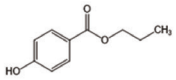
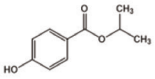
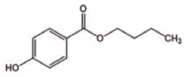
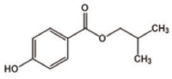
Key words: *C. elegans*, Growth, Maturation, Parabens, Reproduction

INTRODUCTION

Paraoxybenzoic acid esters, commonly abbreviated as parabens, are phenolic preservatives that are commonly used in pharmaceuticals, cosmetics, children's products, and food additives. Parabens include methyl p-hydroxybenzoate (MP), ethyl p-hydroxybenzoate (EP), propyl p-hydroxybenzoate (PP), isopropyl p-hydroxybenzoate (IPP), butyl p-hydroxybenzoate (BP), and isobutyl p-hydroxybenzoate (IBP). The descriptions and structures of these parabens are listed in Table 1. EP, BP, and PP were designated as food additives in 1948, while IBP and IPP were designated as food additives in 1963. These five parabens have set usage standards in soy sauce, fruit sauce, vinegar, soft drinks, and syrups, and on the epidermis of fruits and fruit vegetables. They cannot be used for oth-

Correspondence: Koji Arizono (E-mail: arizono@kumamoto-u.ac.jp)

Table 1. Description of parabens used in this study.

Abbreviation	MP	EP	PP	IPP	BP	IBP
name	Methyl 4-Hydroxybenzoate	Ethyl 4-Hydroxybenzoate	Propyl 4-Hydroxybenzoate	Isopropyl 4-Hydroxybenzoate	Butyl 4-Hydroxybenzoate	Isobutyl 4-Hydroxybenzoate
CAS. No.	99-76-3	120-47-8	94-13-3	4191-73-5	94-26-8	4247/2/3
Molecular weight	152.15	166.18	180.2	180.2	194.23	194.23
Side chain carbon number	2	3	4	4	5	5
Permit as a food additive in Japan (year)	×	1948	1948	1963	1948	1963
Permit as a food additive in the EU and US	○	○	○	×	×	×
ADI (JECFA)	group ADI: 0–10ng/kg	group ADI: 0–10ng/kg	Excluded from group ADI	-	-	-
Structure						

er foods. Additionally, these parabens, along with MP, are also used in pharmaceuticals, cosmetics, and children's products. The preservative effect of parabens is a result of impaired membrane transport and mitochondrial function in microorganisms. Parabens have a strong bacteriostatic effect and are effective against a wide range of microorganisms (Jackson, 1992). In addition, a synergistic effect appears when they are used in combination, rather than alone, and antiseptic capability can be enhanced by using a smaller amount of the paraben (Gottfried, 1962).

Owing to these properties, parabens are widely used as preservatives. However, at the 67th Joint FAO/WHO Expert Meeting on Food Additives (JECFA), parabens were reported to have a weak estrogenic effect in *in vitro* test systems. It is estimated that the longer the alkyl chain of the paraben, the greater its endocrine-disrupting effect as well as its effect on reproductive function (Harvey and Darbre, 2004; Oishi, 2001; Okubo *et al*., 2001; Routledge *et al*., 1998). Moreover, parabens are percutaneously absorbed; therefore, personal care products (PCPs) must be considered in addition to food products (Soni *et al*., 2005). Animal experiments have shown that MP and EP reduce the mass of the reproductive organs of rats, and the male testis decreases in a dose-dependent manner (Oishi, 2004), especially when exposed to BP (Oishi, 2001). Additionally, exposure of immature CD1 mice to EP resulted in an increase in uterine weight (Lemini *et al*., 2003). In contrast, administration of MP and EP to immature B6D2F1 mice by gastric tube feeding did not result in an estrogen response (Hossaini *et al*., 2000). In humans, the relationship between paraben exposure and breast cancer has been a matter of concern, and the involvement of parabens as a causal factor in diseases caused by hormonal disorders has been discussed (Byford *et al*., 2002; Darbre, 2006; Darbre and Harvey, 2008; Giulivo *et al*., 2016). Thus, although attention is being paid to the estrogen-like action of parabens, their effect on human health is still unclear.

In recent years, investigation on the effects of PP on the reproductive system of male rodents showed that PP causes a decrease in stored sperm volume, sperm concentration, sperm production, and blood testosterone concentration in the same amount as the EP group acceptable daily intake (ADI). Therefore, JECFA excluded PP from the group ADI of parabens based on toxicity data in male rats. The ADI has not been established for BP, IBP, and IPP, which are not recognized in the EU and the US. Thus, there is a lack of research on parabens. As multiple types of parabens are often used in combination, it is essential to ensure the overall safety of parabens.

It is important to consider the safety of chemical substances and correctly evaluate their toxicity in humans. Since the toxicity of chemical substances cannot be tested in humans, rodents such as mice and rats, and non-rodents such as dogs and monkeys have mainly been used for testing. However, testing on these animals requires large-scale equipment, time, effort, and high costs. Moreover, it is difficult to perform several tests from the viewpoint of animal welfare. Therefore, in this study, we evaluated the soil nematode *Caenorhabditis elegans* (*C. elegans*), a new model organism that can be easily tested over multiple generations.

C. elegans has been established as a model organism since the late 1980s (Mitani, 2008). In recent years, toxicity evaluation of chemical substances as well as food functionality and safety evaluation tests have been

carried out using nematodes (Sakaguchi *et al.*, 2022a; Sakaguchi *et al.*, 2022b). *C. elegans* is a hermaphroditic and self-fertilizing model organism with excellent fertility; they lay approximately 300 offspring eggs. *C. elegans* worms have extremely small individual differences. Their life cycle, from a fertilized egg to a mature individual, is as short as 3–4 days, and the next generation hatches in 4–5 days. Owing to these characteristics, it is possible to evaluate the biological effects of several types of parabens in *C. elegans* in a short period of time. Furthermore, the entire genome sequence of *C. elegans* has been published, and there is a high homology between *C. elegans* and human genes (Consortium, 1998; Culetto and Sattelle, 2000). In the future, *C. elegans* is expected to be used as a model organism to elucidate not only the mechanism of action of chemical substances but also the functions of human genes. In this study, we focused on the estrogen-like action of parabens and conducted growth, maturation, and reproductive effect tests on *C. elegans*.

MATERIALS AND METHODS

Experimental animals

The wild-type nematode *C. elegans* (Bristol strain N2) was used. Live *Escherichia coli* (*E. coli*) DH5α was used as food.

Reagents

Methyl p-hydroxybenzoate (MW:152.15, CAS:99-76-3, purity: 99.0%), ethyl p-hydroxybenzoate (MW:166.17, CAS:120-47-8, purity: 99.0%), propyl p-hydroxybenzoate (MW:180.20, CAS:94-13-3, purity: 98–102%), isopropyl p-hydroxybenzoate (MW:180.20, CAS:4191-73-5, purity: 98%), butyl p-hydroxybenzoate (MW:194.23, CAS:94-26-8, purity: 98.0%), and isobutyl p-hydroxybenzoate (MW:194.23, CAS:4247-02-3, purity: 97.0%) were purchased from FUJIFILM Wako Pure Chemical Industries, Co., Ltd., Japan. Each sample was dissolved in dimethyl sulfoxide (DMSO) manufactured by Nacalai Tesque and used as test solution.

NaCl, KH_2PO_4, agar powder, KOH, cholesterol, ethanol, Bacto Tryptone, yeast extract, $MgSO_4$, $CaCl_2$, Na_2HPO_4, and KCl were purchased from FUJIFILM Wako Pure Chemical Industries, Co. Ltd., Japan. All reagents were of special grade.

Breeding of the nematode *C. elegans*

Mature nematodes were placed on nematode growth medium (NGM) plates and left for 3–4 days in a thermostatic chamber (20°C) to propagate by self-fertilization. Once the next generation of nematodes were confirmed to have reached maturity under a stereomicroscope, they were transferred to new NGM plates.

Preparation and exposure of nematode suspensions

After culturing on NGM plates, the propagated worms were collected and the adults were soaked in MilliQ water. After the adults were washed, they were dissolved in 0.25 M KOH and 5% sodium hypochlorite aqueous solution to obtain fertilized eggs. Immediately after the eggs were obtained, they were thoroughly washed, placed on unfed NGM plates and left for 15 hr for hatching and tuning. After 15 hr, the L1 larvae were washed with S basal, transferred to a test tube, and used as an L1 larval suspension.

A complete S medium containing *E. coli* was used as a breeding medium. Exposure solutions containing parabens were prepared in breeding medium at concentrations of 100, 125, 150, and 200 μg/mL with 0.1% DMSO solution. Breeding medium with DMSO adjusted to 0.1% was used as a control. The exposure solutions were dispensed into 24-well plates at a volume of 0.5 mL per well. Ten L1 larvae were added to each well for the growth and maturation effect tests, and one worm was added to each well for the reproduction effect test.

Growth and maturation effect test

The L1 larvae were incubated in the exposure solutions for approximately 60 hr at 20°C while shielded from light. After incubation, sodium azide was added. The body lengths of the nematodes were then measured and the percentage of nematodes with fertilized eggs was calculated. Body length was used as an index to evaluate the effect on growth, and the percentage of nematodes with fertilized eggs was used as an index to evaluate the effect on maturation.

Reproductive effects test

The L1 larvae were incubated in the exposure solutions under shaded conditions at 20°C. The day after the first egg was laid in each exposure group, the parent worms were transferred to a new exposure solution. This process was repeated every three days until a total of 140 hr of exposure was reached. The number of lifetime offspring for each worm was counted.

Statistical analysis

All results obtained for the growth, maturation, and reproductive effect tests are presented as mean ± standard deviation. All statistical analyses were performed using the statistical software R-3.5.2. Significant differences were determined through multiple comparative analyses

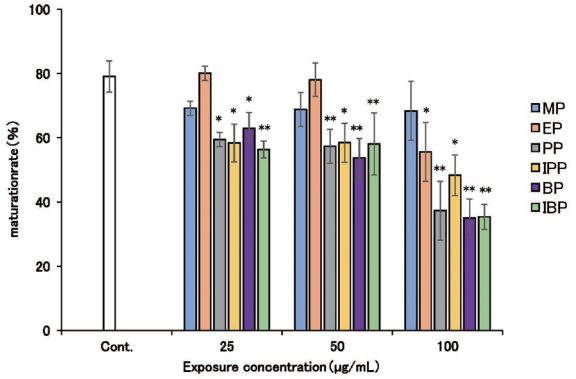


Fig. 1. Effect of parabens on the maturation rate (%) of *Caenorhabditis elegans*. 1 group: n = 10, 3 groups for each paraben. Dunnett test: *p < 0.05, **p < 0.01.

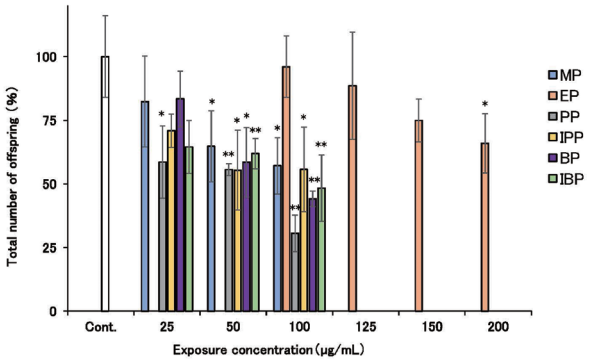


Fig. 2. Effect of parabens on the total number of *C.elegans* offspring. n = 3. Dunnett test: *p < 0.05, **p < 0.01. No effect was observed at EP concentrations of 25 and 50 μg/mL.

using Dunnett's test for the growth and maturation effect tests and reproductive tests. Comparisons were made between solvent control groups and exposure groups in the bioassays. The level of statistical significance was set at a risk rate < 5%.

RESULTS

Growth and maturation effect tests

Parabens were dissolved in DMSO and tested at concentrations of 0 (control), 25, 50, and 100 μg/mL. The results are shown in Fig. 1. In the growth effects test, no effect was observed by any of the parabens at any concentration after 60 hr of exposure. On the other hand, in the maturity effects test, the maturity rate was significantly reduced by all parabens except by MP (Fig. 1). This suggested that exposure to parabens, except MP, affects the maturation of *C. elegans*. In addition, exposure to PP, BP, IPP, and IBP resulted in a significant decrease in maturity rate at the minimum concentration of 25 μg/mL, which is lower than the concentration required for MP and EP to produce an effect. At 100 μg/mL, a significant decrease in maturity was observed for almost all parabens except MP, and especially in those exposed to PP, BP, and IBP. It has been reported that the longer the alkyl chain of the paraben, the stronger its estrogenic effect. The maturation effect test performed in this study showed that the effects of PP, BP, IPP, and IBP were observed at lower concentrations than those of MP and EP; this tendency is similar to results observed in previous studies.

Reproduction effects test

The results of the reproduction effects test are shown in Fig. 2. EP did not produce an effect within the test concentration range; therefore, additional tests were performed at 125, 150, and 200 μg/mL. The number of lifetime offspring was significantly reduced at 200 μg/mL. When exposed to MP, IPP, BP, and IBP, a significant reduction in the number of lifetime offspring was observed at ≥ 50 μg/mL, and PP caused a significant decrease at the set minimum concentration of 25 μg/mL. Thus, in the reproduction effects test, the relationship between alkyl chain length and strength of effect was not as apparent as in the maturation effect test. The effect on reproduction was remarkable upon exposure to PP.

DISCUSSION

In this study, *C. elegans* exposed to parabens did not significantly differ in body length when compared to *C. elegans* exposed to control solution, but their maturity rate and number of lifetime offspring decreased. Parabens have been shown to affect the maturation of *C. elegans* and cause a decline in fertility. It has been reported that for parabens, a longer alkyl chain correlates with a stronger estrogenic effect (Watanabe *et al*., 2013). In the maturation effects test, results similar to those of previous studies were obtained. Moreover, in the reproduction effects test, the effect of PP was remarkable.

Among the parabens studied, the length of the side chain of PP ranks in the middle. Multiple types of parabens have been used in combination. In the safety evaluation by JECFA, the group ADI, which is the sum of MP, EP, and PP, was set to 10 mg/kg body weight/day. However, upon review, PP was excluded from the group ADI (WHO, 2007). In this study, PP showed the strong-

est effect on maturation and reproduction among the parabens, although length of its alkyl chain is not the longest. Parabens are used for various purposes other than as food additives, including as preservatives in PCPs. In cosmetics, in addition to the five paraben types used as food additives, MP, benzyl paraoxybenzoate, methyl sodium paraoxybenzoate, and other parabens, can be blended at amounts of up to 1.0 g of paraben per 100 g of product. For UV absorbers, such as sunscreen, parabens can be mixed at amounts up to 4.0 g or more are allowed to be mixed (Ministry of Health, Labour and Welfare, 2000). On the other hand, for pharmaceuticals, only MP, EP, BP, and IBP are approved for use, and the allowable amount is specified for each application. MP has the widest range of use, and IBP has the most limited range; PP is not approved for use. MP has not been recognized as a food additive in Japan. This is because the methyl group portion of MP is thought to be converted to formaldehyde and formic acid by alcohol dehydrogenase in the body (Takenaka, 1967). However, since this enzyme is not found in *C. elegans*, no such effect was observed.

According to a 2008 survey conducted in Japan as part of the Ministry of Health, Labor, and Welfare's market basket-based daily intake survey of food additives, the total daily intake of parabens is 0.036 mg/day for IPP, 0.024 mg/day for IBP, and 0.025 mg/day for BP. The daily intake of parabens has been reported to be 0.0017 mg/kg bw and up to 0.010 mg/kg bw (Miyakawa *et al*., 2010). Furthermore, based on FDA data, the intake of parabens from different products was 0.833 mg/kg/day from PCPs, 0.017 mg/kg/day from food, and 0.417 mg/kg/day from pharmaceuticals. Thus, it is important to consider exposure to parabens not only from food sources but also from PCPs. Considering that the ADI was set at 10 mg/kg body weight/day and the amount of parabens intake are low, the risk of routine exposure is considered low. According to a survey by Makino *et al*. (2000), parabens are added to nutritional drinks that are not classified as foods at an average concentration of about 50 ppm. Nutritional drinks have been suggested to be a relatively large source of parabens. In a survey by Yamamoto and Ishii (2018), half of university student respondents reported using energy drinks to prevent drowsiness and improve concentration. Fetuses can also be exposed to parabens via maternal blood. This exposure has been reported to cause overweight in childhood (Leppert *et al*., 2020). Therefore, the toxicity of parabens needs attention.

In this study, we simultaneously compared the toxicity of six types of parabens using *C. elegans* as a model organism. The results showed that the strength of the effect on maturity and reproduction did not correlate with the length of the alkyl chain of the paraben, and that PP had the strongest effect. It is necessary to continue to focus on the estrogen-like action of parabens, including PP, and perform genetic analyses such as RNA sequencing.

Conflict of interest---- The authors declare that there is no conflict of interest.

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