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Effects on the reproductive system of young male rats of subcutaneous exposure to n-butylparaben

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ABSTRACT

This study was aimed at determining whether an *in vivo* subcutaneous exposure to n-butylparaben (n-ButP) during one complete spermatogenic cycle could be harmful to the reproductive system of young male rats. Animals were subcutaneously given 0, 150, 300 and 600 mg/kg/day of n-ButP with vehicle (peanut oil). Body and organ weights, n-ButP excretion, biochemical parameters, sperm and spermatid count, sperm motility, viability, maturity and morphology were examined. Results showed that after a completed spermatogenic cycle, although n-ButP did not induce dose-related changes in the different biochemical parameters, a significant decrease of triacylglicerides (TAG) −due to the vehicle-was found. Furthermore, no effects of n-ButP on body weight gain and relative organ weight changes were noted. Regarding sexual organs, prostate relative weight was significantly increased at the high dose of n-ButP. On the other hand, a significant increase of abnormal sperm morphology due to n-ButP exposure, accompanied by different alterations in sexual organs histopathology, was found. The current results indicate that subcutaneous exposure of n-ButP in young male rats induced toxic effects on the reproductive system, which could affect the capacity of fertilization of animals.

1. Introduction

In recent years, there has been growing interest about xenobiotics that can act as endocrine disruptors. They alter endocrine system functions and can have harmful consequences for the reproductive system and the progenies. A wide range of substances has shown to cause endocrine disruption. Among others, these include dioxin and dioxin-like compounds, PCBs, bisphenol A, as well as DDT and various pesticides (Giulivo et al., 2016; McKinlay et al., 2008; Sweeney et al., 2015; Waring and Harris, 2005). Parabens (PBs) are a kind of chemicals used in a significant variety of products, to which people are exposed in the daily life (e.g., cosmetics and pharmaceutical products) (Błędzka et al., 2014; FDA, 2016). PBs have had an important appearance in media because of their endocrine-disruption potential (Golden et al., 2005; Soni et al., 2005). Common PBs are methylparaben (MetP), ethylparaben (EthP), propylparaben (ProP), isobutylparaben (iBuP), benzylparaben (BnP) and butylparaben (ButP). MetP, EthP and ProP are permitted in the European Union as food additives in four categories of processed foods (EC, 1995; Larsson et al., 2014). Various *in vitro* screening tests have shown endocrine toxicity of PBs (Byford et al., 2002; Darbre and Harvey, 2008; Harvey and Everett, 2006; Okubo et al., 2001). *In vivo* toxic effects on the reproductive tract of females and males have been also reported after exposure to PBs (Harvey and Everett, 2006). Some studies suggest that PBs and their metabolites are taken up on oral and dermally exposures at considerable amounts. However, they are rapidly metabolized and excreted, being the concentration of compounds expected to be very low at the active sites of target organs (Boberg et al., 2010). Dermally administered PBs can penetrate the stratum corneum. Once inside, keratinocytes carboxylesterases hydrolyze parabens, and the conjugated metabolites,

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are excreted through bile and urine (Anderson, 2008; Darbre and Harvey, 2008; Kirchhof and Gannes, 2013; Pedersen et al., 2007).

Nowadays, it is a well established fact that the reproductive health of males is declining, being male fertility affected. Human exposure to environmental contaminants such as endocrine dirusptors chemicals (EDCs) has been proposed as one of the factors related to this kind of effects (Anderson et al., 2003; Luccio-Camelo and Prins, 2011; Noorimotlagh et al., 2016). Until recently, most studies focused on the effects of androgens in males, and estrogens in females. However, an increasing number of investigations have remarked the important role of estrogens on the reproductive system of males (Chianese et al., 2016; Hess, 2003; Schulster et al., 2016). Estrogenic endocrine-disrupting chemicals (e-EDCs) are a class of EDCs that modulate estrogen-like responses in the organism (Swart and Pool, 2013; Yoon et al., 2014). A number of studies have shown that PBs are weakly estrogenic, increasing the harmful effects with the side chain length of the chemical, and resulting in the following potency order: methyl-, ethyl-, propyl-, butyl- and isobutylparaben (Boberg et al., 2010; Darbre and Harvey, 2008; Golden et al., 2005; Hu et al., 2013). Therefore, ButP is one of the PBs with longer alkyl chains, for which a number of studies have shown that it exerts harmful effects on the male reproductive system due its estrogenic activity.

On the other hand, it has been reported how a single oral dose of 1000 mg/kg of ButP increases the number of apoptotic spermatogenic cells and altered Sertoli cells. Epigenetic changes in DNA methylation of epididymal rat spermatozoa have been also reported following oral ButP exposure of adult male rats. (Alam et al., 2014; Alam and Kurohmaru, 2014; Boberg et al., 2016; Park et al., 2012). Likewise, *in vivo* studies have shown ButP effects on testicular structure and sperm quality of exposed animals (Guerra et al., 2016; Hoberman et al., 2008; Oishi, 2001). Furthermore, recently Zhang et al. (2016) suggested that increased aromatase (CYP19) and decreased estrogen sulfotransferase (SULT1E1) expression were responsible for the upregulation of estrogen receptor α (ERα), showing a possible mechanism of the n-ButP endocrine and reproductive alterations.

Although there is an increasing number of investigations aimed at assessing the endocrine toxicity of the PBs in the male reproductive tract, most of them have been conducted through oral exposure of pregnant animals. To the best of our knowledge, data on the effects of subcutaneous exposure in young peripubertal male rats are not currently available. The purpose of the present study was to assess the potential toxic effects of n-ButP on the male reproductive system. To investigate the n-ButP effects on testis and epididymal sperm parameters of young Sprague-Dawley male rats, we here examined the effect of various subcutaneous doses of n-ButP during one complete spermatogenic cycle.

2. Materials and methods

2.1. Chemicals

N-butylparaben (n-ButP) (purity $\geq$ 99%, Sigma-Aldrich, St. Louis, MO, USA) was first dissolved in absolute ethanol, followed by dilution with peanut oil (Sigma-Aldrich) to obtain final concentrations of 0, 150, 300 and 600 mg/kg bw/day. Doses were selected based in previous literature (Kim et al., 2015).

2.2. Animals and experimental design

Young male Sprague-Dawley rats (6-week old) (Charles River Laboratories, Sant Germain-L'Arbresle, France) were housed in a room equipped with automatic light cycles (12 h light/dark cycle) and maintained at 22 ± 2 °C and 40–60% humidity. Food (Panlab rodent chow, Barcelona, Spain) and tap water were offered *ad libitum*. The experimental design was approved by the Ethics Committee of Animal Research, "Rovira i Virgili" University (Tarragona, Spain).

At the beginning of treatment, rats were randomly divided into the following five groups (10 animals per group): control (no vehicle), control-oil (peanut oil), 150, 300 and 600 mg/kg bw/day. *N*-ButP was subcutaneously administered in a 0.5 mL of volume, in neck and both sides of the inguinal zone, for 3 alternating days per week to ensure a correct penetration of the vehicle, during 57 days. Clinical signs (abnormal behavior, presence of wounds, infections and mortality) were daily monitored. In tur, body weight, food intake and water consumption were recorded once a week. At the middle of the period of treatment, urine samples were collected to analyze the excretion of n-ButP and its metabolites.

At the end of the treatment, body weight was recorded and rats were then anesthetized by an intraperitoneal injection of 75 mg/kg of ketamine and 0.5 mg/kg of metedomidine. Blood samples were collected -from the portal vein-for hematological, biochemical and hormonal analyses. Liver and sexual organs (testes, epididymis, seminal vesicle and prostate) were immediately removed and weighed. The cauda of the right epididymis was used to obtain the diffusion of sperm, which was used to determine motility, maturity, viability and morphology. On the other hand, a part of the left testis and the left epididymis were immediately stored at −20 °C for a subsequent count of the number of spermatids and spermatozoa. Moreover, samples of liver and kidney were placed in liquid nitrogen and stored at −80 °C for subsequent exam. For histopathological exam, samples of liver and sexual organs were cleaned and putted into formaldehyde (4%) for 24 h.

2.3. n-ButP and its metabolites in urine

On day 30 of n-ButP exposure, animals were housed into individual metabolic cages for the collection of 24 h urines. Samples were stored at −80 °C until analysis. An aliquot of 300 μL of each sample was mixed with 600 μL of ammonium acetate (0.5 M pH 5), 6 μL of β-glucuronidase and 6 μL of sulfatase (both from *Helix Pomatia*). It was incubated at 37 °C and −20 °C for 3.5 h and overnight, respectively. Subsequently, samples were thawed and centrifuged for 5 min at 15 000 rpm and 4 °C. A volume of 50 μL of the supernatant was collected and 50 ng of internal standard (butyl 4-hydroxybenzoate-ring-$^{13}C_6$ solution, Sigma-Aldrich, St. Louis, MO) were added. The sample was then diluted with 44 μL of MeOH:acetonitrile (1:1).

Analysis of n-ButP and metabolites (*p*-hydroxybenzoic acid and *p*-hippuric acid) were performed using a 1290 UHPLC Series Liquid Chromatograph coupled to a 6490 QqQ/MS (Agilent Technologies, Palo Alto, CA, USA). Ionization by electrospray ion source (ESI) and acquisition in MRM mode were conducted. Chromatographic separation was done in a ZORBAX Eclipse XDB-C18, 2.1 × 150 mm, 1.8 μm (Agilent, Palo Alto, USA.), at a flow rate of 0.4 mL/min, using 0.2% acetic acid in water (solvent A) and MeOH (solvent B). Method detection limits (MDL), defined as the concentrations corresponding to three times the signal/noise ratio, were the following: 2.35 μg/L for *p*-hydroxybenzoic acid, 1.86 μg/L for *p*-hippuric acid, and 0.11 μg/L for n-ButP.

2.4. Hematology and serum biochemistry

Whole blood samples were collected into a 500 μL EDTA blood collection tubes. An automatic hematology analyzer ADVIA 120 (Siemens Diagnostics, NY, USA) was used to measure the following parameters: total white blood cells, red blood cells, hemoglobin, platelet, hematocrit, mean corpuscular volume, mean corpuscular