Food and Chemical Toxicology 131 (2019) 110573

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Oxidative stress in testes of rats exposed to n-butylparaben

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ARTICLE INFO

Keywords:
n-butylparaben (n-ButP)
Peanut oil
Oxidative stress
Testes
Rats

ABSTRACT

This study was aimed at determining if oxidative stress imbalance in testes of rats occurs after n-butylparaben (n-ButP) exposure. Young male Sprague-Dawley rats were subcutaneously treated with n-ButP during one spermatogenic cycle (57 days) at 0 (control-oil), 150, 300 and 600 mg/kg/d with peanut oil as vehicle. A non-vehicle control group was also included. Antioxidant enzyme activities (superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase) and levels of reduced and oxidized glutathione were measured in testes. Lipid peroxidation and H_2O_2 concentrations were also assessed. Results showed an increase of oxidative stress in oil-treated groups, excepting 600 mg/kg/d, suggesting oxidative stress due to peanut oil. A possible antioxidant effect due to n-ButP and its metabolites was suggested at 600 mg/kg/d, the only group not showing oxidative stress. An increase of calcium concentration in testes was also observed. On the other hand, a physiologically-based pharmacokinetic (PBPK) model was developed and the concentrations of n-ButP and its metabolites were simulated in plasma and testes. The peak concentration (C_{max}) in testes was found slightly higher than that in plasma. The current results indicate that peanut oil can cause oxidative stress while high doses of n-ButP can act as antioxidant agent in testes.

1. Introduction

Endocrine disruption chemicals (EDCs) can be found in a number of materials such as pesticides, food, drugs or personal care products, etc. They can mimic or interfere the hormone system (Monneret, 2017), having harmful consequences on the biological systems. Nowadays, a wide range of substances have shown to possess some potential to alter hormone homeostasis. These include dioxin and dioxin-like compounds (PCDD/Fs), polychlorinated biphenyls (PCBs), bisphenol A (BPA) or parabens, among others (Giulivo et al., 2016; Kabir et al., 2015; McKinlay et al., 2008; Monneret, 2017; Sweeney et al., 2015; Waring and Harris, 2005).

Parabens (PBs) are alkyl esters of the p-hydroxybenzoic acid, which have been widely used as preservative in cosmetics, foods, and pharmaceutical products for more than 50 years (FDA, 2016; Moos et al., 2016). PBs are highly used due to their broad spectrum of antimicrobial activity, low reactivity, high chemical stability (for a wide temperature and pH range), odor and colorless, non-volatility and low costs of production (Błedzka et al., 2014; Silva et al., 2018). In 1974, the FAO/WHO Joint Expert Committee on Food Additives (JECFA), fixed the total acceptable daily intake (ADI) at a maximum of 10 mg/kg body weight (bw) for the sum of methylparaben (MetP), ethylparaben (EthP) and propylparaben (ProP) (JECFA, 1974). MetP and EthP are still currently permitted as food additives, but ProP was recommended to be removed due to its estrogenic effects in young male rats (EFSA, 2004; Sun et al., 2016). In pharmaceutical products, MetP and ProP are often used together due to their synergistic effects (Samarasinghe et al., 2018). In cosmetics, the maximum concentration of 0.4% is recommended for one paraben, and 0.8% for a mixture of parabens (Moos et al., 2016). However, the sum of ProP and butylparaben (n-ButP) ester concentrations should not exceed 0.19% in mixtures (Darbre and Harvey, 2014). In 2014, the European Union banned isopropyl-, isobutyl-, benzyl-, pentyl- and phenyl-paraben (Sasseville et al., 2015). Nowadays, the most commonly PBs used are methylparaben (MetP), ethylparaben (EthP), propylparaben (ProP) and butylparaben (n-ButP) (Garcia et al., 2017).

In vitro studies have shown endocrine toxicity of PBs (Byford et al., 2002; Chen et al., 2007; Kjærstad et al., 2010), while *in vivo* studies have reported adverse effects of PBs such as uterotrophic effects (Lemini et al., 2003), estrogenic activity (Boberg et al., 2016; Garcia

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https://doi.org/10.1016/j.fct.2019.110573
Received 4 May 2019; Received in revised form 6 June 2019; Accepted 8 June 2019
Available online 10 June 2019

et al., 2017; Kang et al., 2002; Zhang et al., 2014), and also on the reproductive tract of male and female rats (Harvey and Everett, 2006). Carcinogenic potential for some parabens has been also reported in human breast (Darbre et al., 2004).

A wide variety of EDCs have shown to cause oxidative stress, which targets the endocrine system and can cause reproductive alterations (Sedha et al., 2015). In humans, exposure to PBs through ingestion, inhalation or dermal contact can induce oxidative stress (Kang et al., 2013; Watkins et al., 2015). In rodents (Docea et al., 2018; Shah and Verma, 2011) and in fish models (Ateş et al., 2018; Brown et al., 2018; Silva et al., 2018), it has been found that PBs can induce oxidative stress by altering antioxidants enzymes, the levels of antioxidants molecules, and lipid peroxides.

One of the main factors for male infertility is oxidative stress. Reactive oxygen species (ROS) can induce lipid peroxidation, sperm motility loss, DNA fragmentation at spermatozoa level, disrupt steroidogenesis and membrane stabilization in testes (Aitken et al., 2016). *In vivo* studies have demonstrated a link between abnormalities of male reproductive function and the induction of oxidative stress (Aksu et al., 2017; Khan et al., 2015; Rodríguez-González et al., 2015). Testes are very sensitive to oxidative stress due to the abundance of highly unsaturated fatty acids, and the presence of potential ROS-producing systems such as high rates of cells divisions, corresponding to spermatogenesis process, and also Leydig cells steroidogenesis (Yuksel et al., 2012).

A possible involvement of trace elements in the dysfunction of male reproductive system in terms of endocrine activity, gametes production and gamete quality, has been reported in men and other animal species (Fallah et al., 2018; Kovacik et al., 2018; Slivkova et al., 2009). Associations of trace metals with oxidative status parameters, linked to sperm motility parameters have been also reported (Djuric et al., 2015; Tvrdá et al., 2013). Some trace elements are considered essential for reproduction (Dobrakowski et al., 2018; Türk et al., 2014). However, combined higher levels of these elements in seminal plasma can have adverse effects on sperm motility and oxidative stress production, interfering with physiological processes responsible for successful fertilization (Fallah et al., 2018; Kovacik et al., 2018).

On the other hand, physiologically based pharmacokinetics (PBPK) models have been widely used to describe the distribution and to generate time course concentration profiles of chemicals and their metabolites in different organs and tissues of the body (Sharma et al., 2018a, 2018b). In the current study, a PBPK model has been developed and used to determine the time course of n-ButP target tissue (testes) concentrations. The time course concentrations profile has been used to fit the experimentally observed response for the dose-response analysis.

Recently, we found reproductive abnormalities in young male rats subcutaneously treated with n-ButP during one spermatogenic cycle (Garcia et al., 2017). The aim of the present study was to determine if the reproductive abnormalities could be due to a possible oxidative stress imbalance after n-ButP exposure in testes, as well as if trace elements could be involved in this process. Moreover, we also used a PBPK model to evaluate the time course concentration profile of n-ButP in testes and to relate it to the antioxidant system.

2. Materials and methods

2.1. Chemicals

N-butylparaben (n-ButP) (purity ≥ 99%, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in absolute ethanol and diluted with peanut oil (Sigma-Aldrich, St. Louis, MO, USA) to obtain concentrations of 0 (control-oil), 150, 300 and 600 mg/kg/d. These doses were selected based on data from the scientific literature, as well as the results of previous studies (Garcia et al., 2017; 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).

Rats were randomly divided into five groups (10 animals per group): control (no vehicle), control-oil (peanut oil), 150, 300 and 600 mg/kg/d of n-ButP. The chemical was subcutaneously administered, in a volume of 0.5 mL, in neck and both sides of the inguinal zone, for 3 alternative days per week, in order to ensure a correct penetration of the vehicle, during one spermatogenic cycle (57 days). Clinical signs (abnormal behavior, presence of wounds, infections and mortality) were daily monitored. At the middle of the treatment period, after subcutaneous injection, urine samples were collected for 24 h and stored at −80 °C.

Three days after the exposure period, body weights were recorded, and animals were anesthetized by an intraperitoneal injection of 75 mg/kg of ketamine and 0.5 mg/kg of metedomidine. Subsequently, blood samples were obtained from the portal vein into 10 mL serum collection tubes, and maintained at room temperature (minimum 30 min). Tubes were centrifuged at 1300 g for 10 min at 4 °C in order to obtain serum samples. After centrifugation, serum was immediately separated and stored at −80ªC for subsequent Oxygen Radical Absorbance Capacity (ORAC) assay. Testes were removed, weighed, placed in liquid nitrogen and stored at −80 °C (Garcia et al., 2017).

2.3. Oxidative stress biomarkers analyses

Testes were cut in sagittal plane. Afterwards, 100 mg of each sample were homogenized in 0.2 M cold sodium phosphate buffer at pH 6.25 in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Homogenates were centrifuged at 105000 g for 1 h at 4 °C. The crude soluble fraction was divided into two parts. The first part was precipitated with cold trichloroacetic acid (TCA) 70% (final concentration TCA 10%) for 20 min at 4 °C, being then centrifuged at 2300 g for 10 min at 4 °C. The supernatant was stored at −80 °C for the subsequent reduced glutathione (GSH) and oxidized glutathione (GSSG) determination. The second part was aliquoted and stored at −80 °C to determine superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) activities, hydrogen peroxide (H_2O_2) concentrations and protein content. Total protein concentration was measured by the Bradford's method (1976), using bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) as standard.

2.3.1. Antioxidant enzyme activities

Superoxide dismutase (SOD) and catalase (CAT) were determined according to previously reported methods (Cohen et al., 1970; Misra and Fridovich, 1972). SOD activity determination is based on autoxidation of epinephrine by superoxide anion. Enzyme activity was expressed as the amount of enzyme required to inhibit the epinephrine's autoxidation by 50%. Results are given as U/mg of protein. For CAT activity, the assay measured for 0.5 min at 240 nm, the extinction of H_2O_2 substrate due to the action of catalase. Results are expressed as μmol/min/mg protein.

2.3.2. Glutathione dependent enzymes and reducing power

Glutathione reductase (GR) and glutathione peroxidase (GPx) activities were determined using a Cobas Mira automatic analyzer (Roche Pharmaceuticals, Basel, Switzerland), which reads at 340 nm the consumption of NADPH following the reduction of oxidized glutathione (GSSG) and t-butyl hydroperoxide (t-BuOOH), respectively. GR activity

was measured according to Goldberg and Spooner (1983), while GPx activity was based on the Wheeler's method (1990). The enzyme activity is expressed as mU/mg of protein.

Reduced (GSH) and oxidized (GSSG) glutathione concentrations (Sigma-Aldrich, St. Louis, MO, USA) were measured according to the Hissin and Hilf (1976) method, using a LS50 PerkinElmer fluorimeter (PerkinElmer, Baconfield, UK) at 350 and 420 nm (excitation and emission wavelength, respectively. GSH and GSSG concentrations are given as nmol/mg of protein. Reducing power (GSSG/GSH ratio) was also determined.

2.3.3. Hydrogen peroxide (H_2O_2) concentration

The concentrations of H_2O_2, a biomarker of oxidative stress in tissues, were determined using a commercial kit (Peroxide assay kit, Sigma-Aldrich, St. Louis, MO, USA), based on a colorimetric method, using different concentrations of pure H_2O_2 as standard. Results are given as μM.

2.3.4. Malondialdehyde assay: thiobarbituric acid reactive substances (TBARS)

Malondialdehyde (MDA) concentration, a marker for lipid peroxidation, was also determined. For MDA assay, testes were cut in sagittal plane and 100 mg of each sample were homogenized in a cold solution of 1.15% KCl containing 0.01% butylated hydroxytoluene (Sigma-Aldrich, St. Louis, MO, USA) (Gómez et al., 2004) in a Potter-Elvehjem homogenizer fitted with Teflon pestle. 100 μL of homogenates were incubated in 3 mL of buffer A (constituted for equal volumes of 0.8% thiobarbituric acid and 20% acetic acid at pH 3.5, and supplemented with 200 μL 8.1% SDS and 700 μL H_2O milli-Q) at 95 °C for 1 h. After incubation, homogenates were centrifuged at 3225 g for 15 min at 25 °C. The soluble fraction was read in a standard spectrophotomer at 532 nm (Zingarelli et al., 1999). MDA (Sigma-Aldrich, St. Louis, MO, USA) was used as standard being prepared with buffer B (1% H_2SO_4 and 20% absolute ethanol). Results are given as pmol/mg of protein.

2.3.5. Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay is used to determine the total antioxidant capacity in different matrices. The hydrophilic content of peanut oil (+n-ButP), serum and urine was analysed. Total antioxidant capacity from vehicle (oil) and vehicle + compound (150, 300 and 600 mg/kg/d of n-ButP) was determined. Briefly, vehicle, and vehicle with compound, were diluted 1:1 with 80% of methanol and vortexed for 2 min at room temperature. The solution was centrifuged at 5000 g for 10 min at room temperature and the supernatant was collected. The steps were repeated, being the total supernatant measured and diluted 1:20 with 0.075 M Na–K phosphate buffer at pH 7.4. Serum samples were diluted 1/500 with 0.075 M Na–K phosphate buffer at pH 7.4 and then analysed. Before urine ORAC assay, samples were centrifuged, and the supernatant analysed without dilutions.

This assay is based on a fluorimetric reaction that measures the antioxidant capacity to scavenge peroxide radicals induced by dihydrochloride of 2,2 azobis (2-aminopropane) at 485 nm excitation wave and 538 nm emission wave on a fluorescence plate reader (Fluoroskan Ascent, Labsystems, Helsinki, Finland) (Cao et al., 1998). Results are given as a μmol of TE/mL. TE are equivalents of trolox, which is an analogue of vitamin E, used as standard.

2.4. Protein expression analyses

CAT (MW: 60–65 kDa), GR (MW: 55 kDa) and GPx-4 (MW: 17–22 kDa, the main isoform in testis) expressions were analysed by the Western blot technique. Samples of testes were homogenized in RIPA buffer supplemented with a cocktail of proteases and phosphatases inhibitors. Total protein concentration was determined using DC Protein Assay kit (Bio-Rad, Hercules, CA, USA), which is based on Lowry's method (1951). It was used due to its compatibility with RIPA buffer. BSA was used as standard.

Aliquots containing 30 μg of total protein were mixed in buffer (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% (wt/vol) SDS, 5% (vol/vol) 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95 °C for 10 min. Samples were electrophoresed on SDS-polyacrylamide gel 12.5 (Bio-Rad, Hercules, CA, USA). Proteins were transferred on a PVDF (Bio-Rad, Hercules, CA, USA) membrane using a transblot apparatus (Bio-Rad, Hercules, CA, USA), being subsequently blocked with non-fat milk diluted in TBS-T (50 mM Tris, 1.5% NaCl, 0.05% Tween 20 at pH 7.5) for 1 h. Membranes were incubated with primary antibody solution overnight at 4 °C, being subsequently washed in TBS-T buffer, and incubated with a peroxidase-conjugated immunoglobulin G antibody for 1 h 30 min at room temperature. Proteins were visualized using a chemiluminescent substrate (Western ECL Blotting substrates, Bio-Rad, Hercules, CA, USA). Specific labelled proteins were quantified by measuring the density of the detected bands using Image Lab software (Bio-Rad, Hercules, CA, USA). β-actin (MW: 43 kDa) was used as internal standard. Levels of protein expression were expressed as the relative expression respect to β-actin.

2.5. Analysis of trace elements

Samples of testes were weighed in a Teflon microinsert and 65% nitric acid (Suprapur, Merck, Darmstadt, Germany) was added to digest the samples. Teflon microinserts were introduced in Teflon vessels, which were put into a microwave oven Star D (Milestone, Sorisole, Italy) during 25 min at 200 °C and 1000 W, according to the manufacturer's instructions. The digested solution was diluted at 10 mL with H_2O milli-Q. All materials were previously washed with 10% nitric acid in order to avoid potential contaminations (Gómez et al., 2008). The levels of Cd, Co, Cu, Mn, Pb, Se, and Zn were determined by means of a computer-controlled sequential inductively coupled plasma-mass spectrometer (ICP-MS) (PerkinElmer Elan 6000), while the concentrations of Ca, Fe and Mg were determined by computer-controlled sequential inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer Optima 3200RL), both according to DIN EN ISO 17294-2. Detections limits were the following: Cd, 0.05 ng/g; Co, 0.05 ng/g; Cu, 0.25 ng/g; Mn, 0.05 ng/g; Pb, 0.05 ng/g; Se, 2 ng/g; Zn, 1.5 ng/g; Ca, 0.025 μg/g; Fe, 0.005 μg/g; and Mg, 0.025 μg/g. For quality control, DOLT-5 Standard (Fisher liver, National Research Council Canada, Ottawa, ON, Canada) was used in each assay.

2.6. PBPK model

The rat PBPK model developed for this study comprises six compartments: gut, liver, plasma, kidney, fat, and testes, as well as a compartment representing the rest of the body. The exchange of the chemical between blood and tissue in each organ is described by a perfusion rate-limited PBPK model. A previously developed PBPK model (Campbell et al., 2015) was modified to build the current model by adding the subcutaneous and others several compartments such as testes. Subcutaneous dose was considered to build the model, which was evaluated against the plasma concentrations in the rat, after a dose of 100 mg/kg body weight (Aubert et al., 2012). The partition coefficient was estimated using the QSAR approach of Poulin and Krishnan tissue composition method (Poulin and Krishnan, 1996, 1995; Poulin and Theil, 2000). The clearance of butyl paraben in liver, gut and skin was described using the Michaelis-Menten equation. The PBPK model was developed using ordinary differential equations. The equations were written in the GNU MCSim modelling language (Bois and Maszle, 1997) and solved by numerical integration with the GNU MCSim, using the R platform. Monte Carlo simulations were performed to estimate the impact on model predictions of uncertainty in all of the rate constants and the partition coefficients. The model output simulation was in a range between 2.5th and 97.5th percentiles.

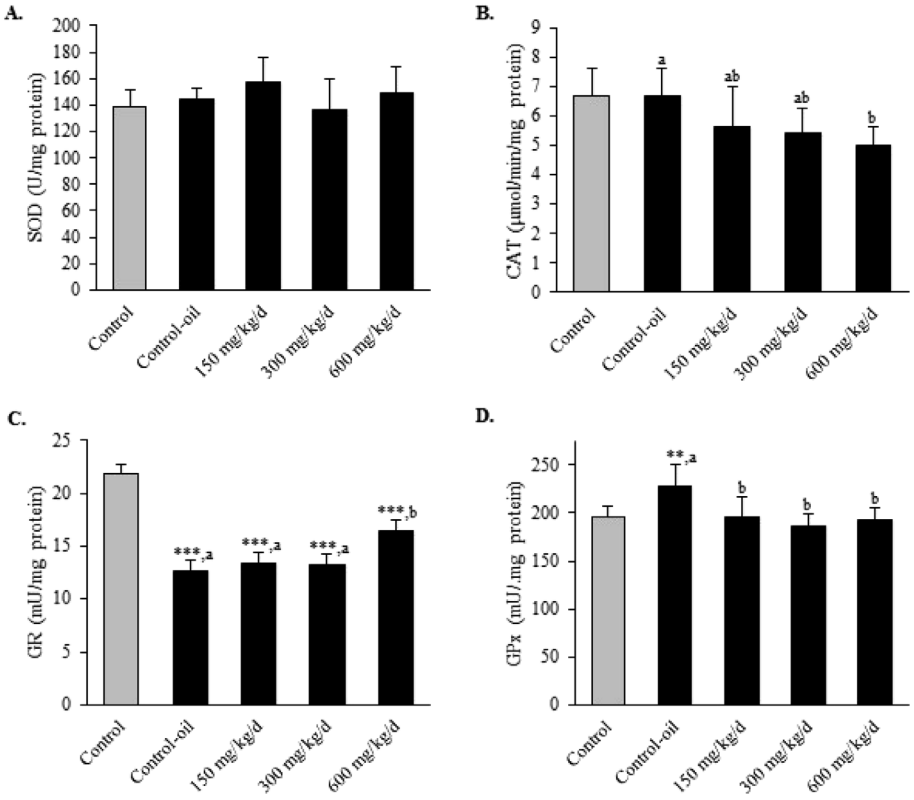

Fig. 1. Antioxidant enzyme activities. A: Superoxid dismutase (SOD) activity (U/mg protein); B: Catalase (CAT) activity (µmol/min/mg protein); C: Glutathione Reductase (GR) activity (mU/mg protein); D: Glutathione Peroxidase (GPx) activity (mU/mg protein). All values are expressed as mean ± SD. Asterisk (*) means significantly different from the control group ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$). Different superscripts (^{ab}) indicate significant differences between groups at $p < 0.05$.

2.7. Statistics

Statistical analysis was performed using ANOVA, followed by the Tukey post-hoc test when variances among treated groups were homogeneous. If variances were not homogeneous, the Kruskal-Wallis test, followed by the Dunns post-hoc test, was used. To determine differences due to vehicle, two-tailed *t*-test between control and control-oil (0 mg/kg/d) groups was performed. If differences between control groups were not found, ANOVA was used between treated groups and control-oil group. If control groups were different, both were then included in the ANOVAs analysis. Statistical analysis was performed using the software Statistical Package for the Social Sciences (SPSS v.25). Significance was set at $p < 0.05$.

3. Results

3.1. Oxidative stress markers

Data on oxidative stress markers in testes are depicted in Fig. 1. SOD activity did not show differences among groups (Fig. 1A) while a dose-related tendency to decrease was noticed in CAT activity, being 600 mg/kg/d group the only group showing differences with respect to the control-oil group (Fig. 1B). Regarding glutathione dependent enzymes activities, differences were noted. An overall effect on the decrease in GR activity due to oil was observed (Fig. 1C), while a significant increase between the control-oil group and the 600 mg/kg/d group was due to n-ButP exposure. According to GPx activity, a significant increase in control-oil group with respect to the control group was noted. Moreover, all n-ButP-treated groups showed a significant decrease with respect to the control-oil group (Fig. 1D).

Glutathione reducing power was established through GSH and GSSG levels and GSSG/GSH ratio. Only concentrations of GSH showed significant differences (Fig. 2). Control-oil and 300 mg/kg/d groups showed a significant decrease in comparison to control group. Although the rest of groups did not show differences with respect to the control group, a tendency to increase their levels in relation to the control-oil group was noted. Moreover, GSH levels at 600 mg/kg/d were significantly different from those in the control-oil group, showing an increase. Fig. 3 shows H_2O_2 concentrations in testes. A significant increase in all oil-treated groups, excepting 600 mg/kg/d, was observed with respect to the control group. The results of the malondialdehyde assay showed a significant increase (in comparison to the control group) for the oil-treated groups, excepting at 600 mg/kg/d (Fig. 4).

As a consequence of the possible oxidizing effects due to peanut oil, the ORAC assay was conducted to determine total antioxidant capacity of the hydrophilic fraction of the peanut oil. Analysis showed some antioxidant capacity in groups treated with n-ButP, while control-oil group did not show antioxidant capacity by itself (Table 1). In relation to serum samples, differences between the control-oil group and the 150 mg/kg/d group were found. Serum samples were collected 3 days after the last subcutaneous injection, being well known that n-ButP is removed after 48 h (Fig. 6B). Finally, urine samples in both control and 600 mg/kg/d groups presented significant high µmol of equivalents of trolox/mL with respect to all oil-treated groups. In addition, the 600 mg/kg/d group showed the same levels that the control group.

3.2. Protein expression of antioxidant enzymes

The relative protein expression levels of antioxidant enzymes CAT, GR and GPx-4 in testes were quantified by Western Blot (Fig. 5). CAT enzyme is minority in testes and, consequently, showed a great variability between the different groups, being 600 mg/kg/d group the only one showing a significant decrease with respect to the control group (Fig. 5A and B). GR expression presented a significant decrease at 300 and 600 mg/kg/d with respect to the control and control-oil groups (Fig. 5A and C). No differences in GPx-4 expression were found (Fig. 5A and D).

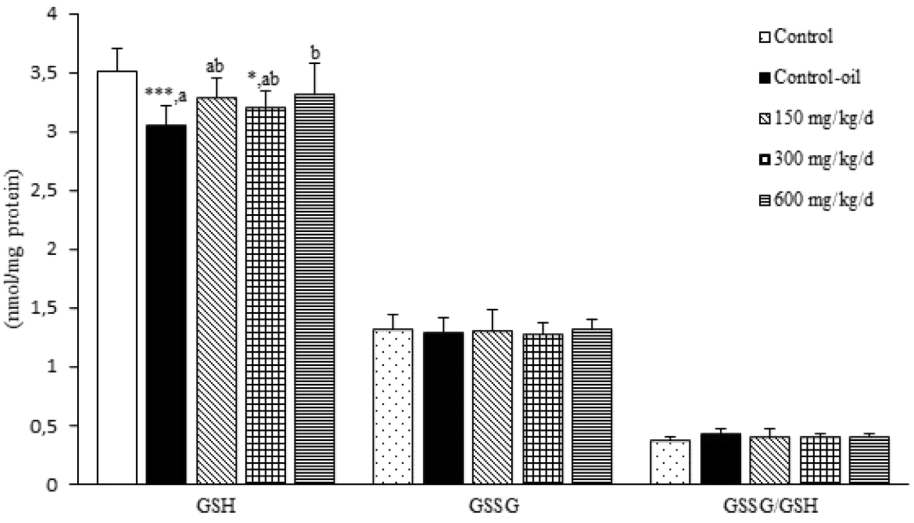

Fig. 2. Glutathione levels (nmol/mg protein). GSH: reduced glutathione; GSSG: oxidized glutathione. GSSG/GSH: ratio between oxidized/reduced glutathione (no units). All values are expressed as mean ± SD. Asterisk (*) means significantly different from the control group (*p < 0.05; **p < 0.01; ***p < 0.005). Different superscripts (ab) indicate significant differences between groups at p < 0.05.

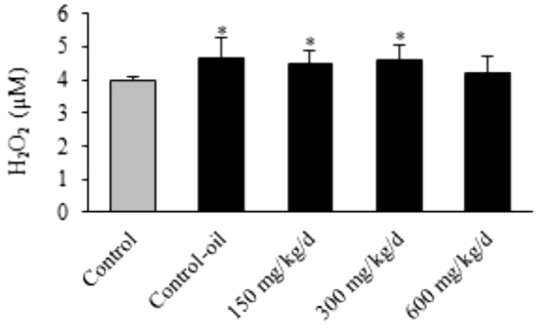

Fig. 3. Hydrogen Peroxide (H_2O_2) concentration (µM). All values are expressed as mean ± SD. Asterisk (*) means significantly different from the control group (*p < 0.05).

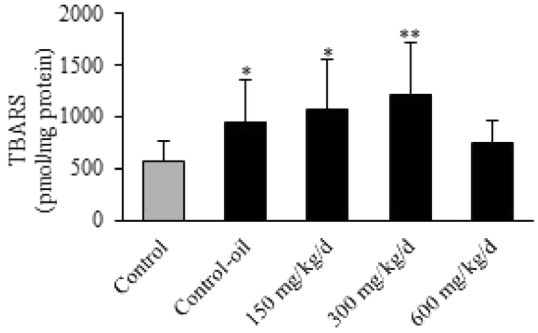

Fig. 4. Thiobarbituric Acid Reactive Substances (TBARS) concentration. All values are expressed as mean ± SD. Asterisk (*) means significantly different from the control group (*p < 0.05; **p < 0.01).

3.3. Concentrations of trace elements

Trace elements levels in testes are summarized in Table 2. Concentrations of Cd, Co and Pb were not detected. Those of Cu, Mn, Se, Zn, Fe and Mg were detected, but no significant differences among groups were found. Only Ca levels showed significant differences, when a significant increase due to oil was found in oil-treated groups with respect to the control group, excepting at 300 mg/kg/d. This group was also the only n-ButP treated group that showed reduced Ca levels compared to the control-oil group.

3.4. PBPK model

The model prediction for ButP plasma concentration is depicted in Fig. 6A with median (red) and their two extremes, corresponding to 2.5 and 97.5 percentiles (blue). The model was evaluated against the experimental *in vivo* data, where the green points represent the experimentally observed mean concentrations and the black bars represents ± SD. The model predicted very adequately the experimental data with the rate of appearance; i.e. initial observed experimental points are at the 50th of the modelling percentile with slight deviation at the tail end 24 h time course curve. The maximum plasma concentration (C_{max}) was reached at approx. 4 h (t_{max}) and decreased within 24 h to a significant lower level, not showing signs of accumulation. In Fig. 6B, the model is simulated for a scenario of the current experimental set-up for the subcutaneous dosing. The simulation of n-ButP concentration in plasma after a subcutaneous dose of 100 mg/kg body weight, at alternative days for 57 days, did not show accumulation (data not shown). The phenomena of no accumulation can be observed in Fig. 6B, which represents the time course of n-ButP plasma concentrations (median) for 72 h for the current experimental dosing scenario.

The model was further simulated for 24 h at the doses used in the

Table 1
ORAC assay (µmol of equivalents of trolox/mL) into three different matrices.

	Control	Control-oil	150 mg/kg/d	300 mg/kg/d	600 mg/kg/d
Hydrophilic fraction[a]	–	ND	D (4.00)	D (3.85)	D (4.49)
Serum *(n = 7)*	14.00 ± 3.70	19.11 ± 7.56[a]	10.56 ± 1.23[b]	18.70 ± 7.18[ab]	14.35 ± 4.18[ab]
Urine *(n = 5)*	0.170 ± 0.003	0.160 ± 0.003**,[a]	0.157 ± 0.003***,[a]	0.158 ± 0.003***,[a]	0.168 ± 0.005[b]

ND: non-detected; D: detected. Hydrophilic extraction values are qualitative.
Values are expressed as mean ± SD.
* Significantly different from the control (**p < 0.01; ***p < 0.005).
[a] Hydrophilic fraction of vehicle (peanut oil) and vehicle with three different doses of n-ButP extracted with 80% methanol.
[ab] Different superscripts in the same row indicate significant differences between groups at p < 0.05.

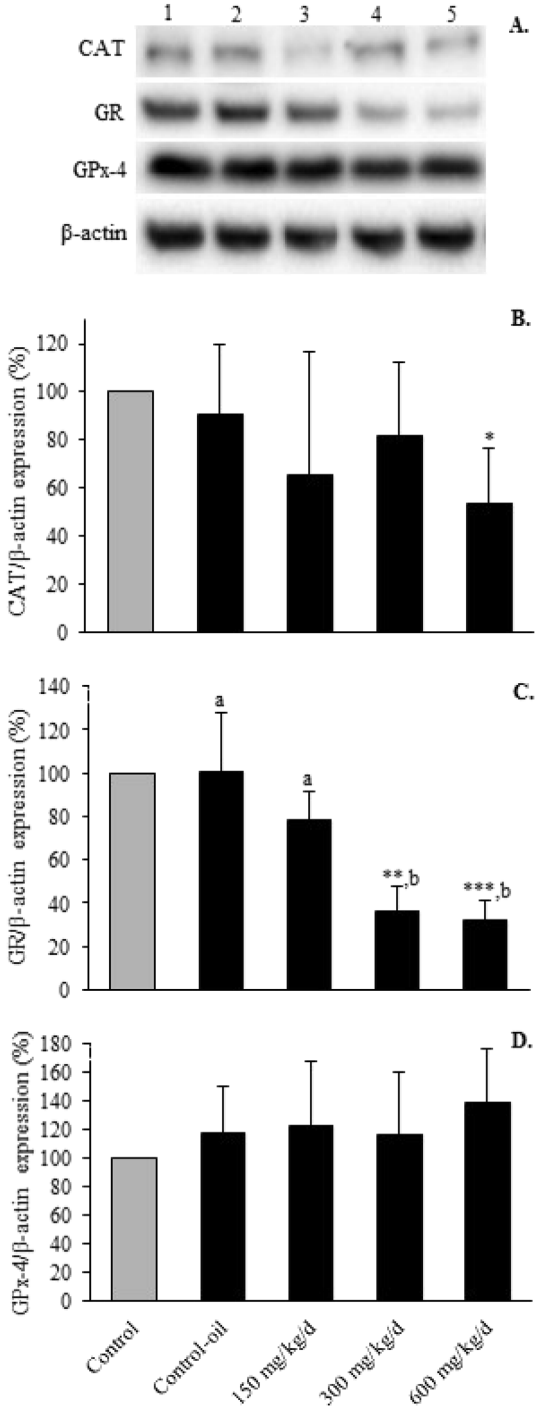

Fig. 5. Protein expression of catalase (CAT, MW: 60–65 kDa), glutathione reductase (GR, MW: 55 kDa) and glutathione peroxidase (GPx-4, MW: 17–22 kDa); β-actin (MW: 43 kDa) as a control. A: detected bands corresponding interested proteins where lane 1: control; lane 2: control-oil; lane 3: 150 mg/kg/d; lane 4: 300 mg/kg/d and lane 5: 600 mg/kg/d. B: CAT/β-actin relative expression. C: GR/β-actin relative expression. D: GPx-4/β-actin relative expression. Data were expressed as a percentage of relative expression respect β-actin (% ± SD). Asterisk (*) means significantly different from the control group ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$). Different superscripts (^{ab}) indicate significant differences between groups at $p < 0.05$.

current study: 150, 300 and 600 mg/kg body weight. The results were recorded for plasma and testis compartments being shown in Figs. 7 and 8. Fig. 7(A, B and C) represents n-ButP plasma concentrations at the different doses. We observed a dose-dependent increase in C_{max} value, with chemical concentrations decreasing within 24 h to a significantly lower level. It indicates a dose-dependent metabolic activity with higher volume of elimination at higher doses, showing no saturation of enzymatic activities (Fig. 7C). PBPK predicted peak plasma concentration to dosing ratio of n-ButP is approximately 10% in case of the administered subcutaneous dose (Fig. 7). The t_{max} (time at which C_{max} occurs) for concentration in the testes was observed at approx. 5 h of treatment, while the plasma t_{max} occurs well before 5 h, which might be due to the distribution delay. The corresponding C_{max} values in testes were slightly higher than those in the plasma at the given subcutaneous doses, suggesting that testes are exposed to a higher concentration than the plasma (Fig. 8). However, the elimination profile in testes followed the similar pattern to that of plasma, being almost eliminated within 24 h (Fig. 8).

4. Discussion

In the present study, rats were subcutaneously treated with peanut oil during one spermatogenic cycle. The present results show that TBARS and H_2O_2 levels increased with respect to these in the control group, excepting at 600 mg/kg/d, which indicates oxidative stress in testes. It may be due to the peanut oil effect and not to the administration route. In the current investigation, oil-treated rats started to show little depots under the skin and Levoe et al. (2014) found depots and skin abnormalities after subcutaneous treatment with peanut oil during 21 days in rats. They suggested that an activation of immune system was due to the peanut oil and no to the own injection. It is well known that immune response is highly related to oxidative stress (Marri and Richner, 2015) and that subcutaneous route is painless (Nebendahl, 2000). In addition, peanut oil has a high proportion of unsaturated fatty acids: 35–58% of oleic (18:1, n-9) and 20–43% of linoleic (18:2, n-6,9), being the rest saturated fatty acids (Carrín and Carelli, 2010). Unsaturated fatty acids can be oxidized faster and are related with elevated TBARS levels (Samarasinghe et al., 2018; Verma and Asnani, 2007; Yun and Surh, 2012). We found that oxidative stress in testes was mainly caused by peanut oil administration, but the 600 mg/kg/d group seemed to act independently on the other oil-treated groups. The high dose of n-ButP showed an antioxidant effect with a tendency to restore the normal TBARS levels. This effect might be due to exposure to n-ButP.

P-hydroxybenzoic acid and p-hydroxyhippuric acid are the metabolites of n-ButP hydrolysis. Both are widely known as antioxidants being established that phenolic compounds have the property to stabilize some oils (Gómez-Alonso et al., 2002). It can suggest the possibility that the antioxidant effects due to n-ButP (or its metabolites) are higher than the oxidant effects due to peanut oil at 600 mg/kg/d and, as result, no oxidative stress was found. Popa et al. (2014) treated rats orally with BPA + MetP, reporting that p-hydroxybenzoic acid had the capacity to trapping hydroxyl radicals and, in consequence, can inhibit lipid peroxidation in plasma. In turn, Kopalli et al. (2013) reported that MetP could inhibit LPO in mice neuronal cells. These studies would support our suggestion on the role of n-ButP and its metabolites to help to keep a non-oxidative state at 600 mg/kg/d. In contrast, Riad et al. (2018) found that butylparaben can cause reproductive abnormalities and oxidative stress in testis of weanling rats treated orally with a dose of n-ButP lower than 150 mg/kg/d. Some studies have also reported oxidative stress due to n-ButP (Brown et al., 2018; Hegazy et al., 2015; Shah and Verma, 2011; Yang et al., 2018). These apparently contradictory results could be due to the different animal species, the analysed tissues, the duration of the treatment, as well as the route of administration, among other experimental conditions.

In relation to ORAC analyses, the equivalents trolox/mL were found at all doses of n-ButP, whereas the vehicle group did not show this antioxidant capacity. This is in accordance with our previous suggestion that n-ButP could have antioxidant properties. However, ORAC in serum did not show differences in antioxidant capacity, which can be

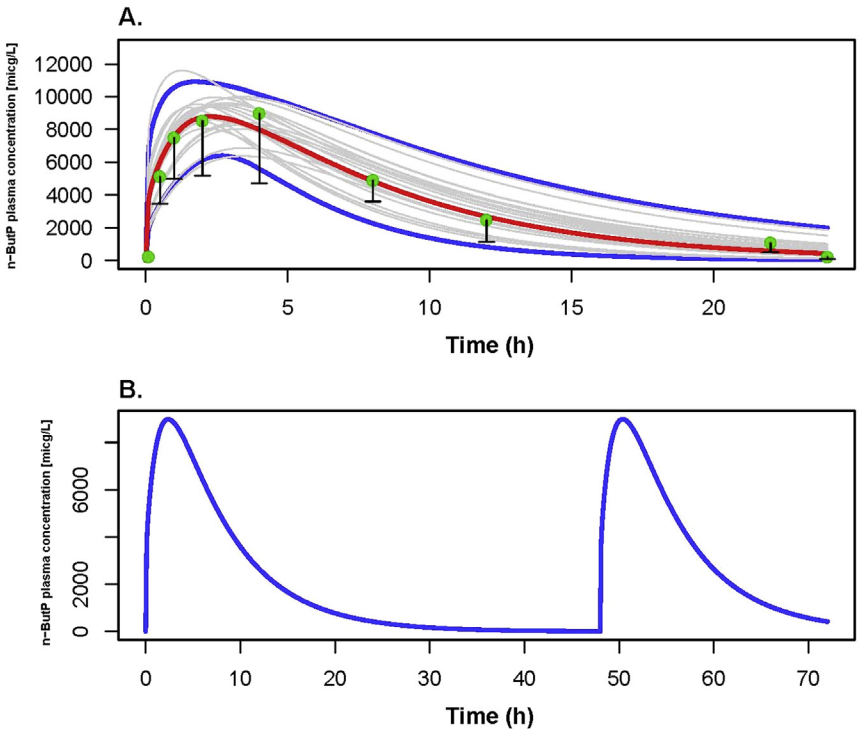

Fig. 6. PBPK model. A: Simulation of butylparaben (n-ButP) plasma concentrations in the rat after a subcutaneous dosing of 100 mg/kg body weight. Blue lines: 2.5 and 97.5 percentiles; Gray lines: 20 simulations chosen at random from the 15000 iterations; Red lines: median prediction; the green dots indicate the mean concentrations and black lines indicate the mean ± SD reported in (Aubert et al., 2012). B: Simulation of n-ButP plasma concentrations in the rat after a subcutaneous dosing of 100 mg/kg body weight at alternative days. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Levels of trace elements in testes of male rats treated with n-ButP (n = 8).

		Control	Control-oil	150 mg/kg/d	300 mg/kg/d	600 mg/kg/d
Cd	ng/g	ND	ND	ND	ND	ND
Co	ng/g	ND	ND	ND	ND	ND
Cu	ng/g	2654 ± 656	2389 ± 249	2463 ± 497	2520 ± 497	2388 ± 395
Mn	ng/g	409.0 ± 95.1	368.4 ± 47.4	409.0 ± 98.3	374.4 ± 71.8	361.3 ± 69.3
Pb	ng/g	ND	ND	ND	ND	ND
Se	ng/g	1513 ± 164	1571 ± 228	1422 ± 138	1725 ± 244	1475 ± 255
Zn	ng/g	27276 ± 12492	35655 ± 6891	32063 ± 8925	34878 ± 13267	31602 ± 6931
Ca	μg/g	22.28 ± 9.77	61.13 ± 20.00^{***,a}	50.8 ± 14.94^{**,ab}	35.1 ± 7.93^{b}	51.67 ± 14.53^{**,ab}
Fe	μg/g	26.80 ± 6.75	25.14 ± 3.20	25.34 ± 1.87	27.52 ± 4.32	24.04 ± 3.45
Mg	μg/g	190.8 ± 49.5	179.8 ± 17.5	192.1 ± 50.5	183.0 ± 36.6	161.7 ± 19.4

ND: non-detected.
Values are expressed as mean ± SD.
* Significantly different from the control ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$).
^{ab} Different superscripts in the same row indicate significant differences between groups at $p < 0.05$.

due to the fact that n-ButP is almost removed after 48 h, just like PBPK model shows (Aubert et al., 2012). Regarding urine samples, all groups with an increase of ROS had less antioxidant capacity with respect to the control group. These results would support our hypothesis, which suggests a little antioxidant capacity due to n-ButP and their metabolites, being this effect only effective at the highest dose of the chemical.

On the other hand, SOD, CAT and GPx are the first defence line against oxygen radicals. In the present study, SOD did not show activity differences. Animals were sacrificed 3 days after the last exposure, being n-ButP completely metabolized and eliminated after 48 h. Therefore, we suggested that SOD activity could be restored after the treatment. By contrast, CAT and GPx levels were altered, suggesting that their concentrations were not restored. CAT and GPx are implied in H_2O_2 degradation, while GPx also acts removing all kind of peroxides. Increasing levels of TBARS and H_2O_2 can generate an activation of CAT and GPx, being GPx predominant in testes (Bauché et al., 1994). The current results show an increase of GPx activity in the control-oil group, which suggests a reactivation of GPx activity as a consequence of oxidative stress. However, all n-ButP treated groups showed a significant decrease in this activity with respect to the control-oil group. We suggest on scenario in which GPx activity of control-oil group increases due to H_2O_2 levels, while possible n-ButP antioxidant effects would help to restore normal activity values. Only the highest n-ButP dose showed a decrease of CAT activity in comparison to that in the control-oil group. At the highest dose, no differences in TBARS and H_2O_2 levels were found, which suggests that the lower activity of CAT respecting that in the control-oil group, might be due to the antioxidant n-ButP effects.

With respect to GR, it is an enzyme implied in a second line of antioxidant defence. All n-ButP treated groups showed a decrease in GR activity (regarding the control group) related with an oxidative state. GR depletion was observed in all groups with elevated levels of TBARS and H_2O_2. Moreover, a decrease in GR activity was also observed at 600 mg/kg/d. However, this group showed a significant increase in GR activity with respect to the rest of oil-treated groups. This increase could be related with a previous oxidative stress, which is possibly being restored. Shah and Verma (2011) reported a decrease in antioxidant enzymes activities, depletion of GSH, and an increase of TBARS levels due to exposure to n-ButP in mice liver respecting a control-oil group. In the current investigation, similar results due to peanut oil

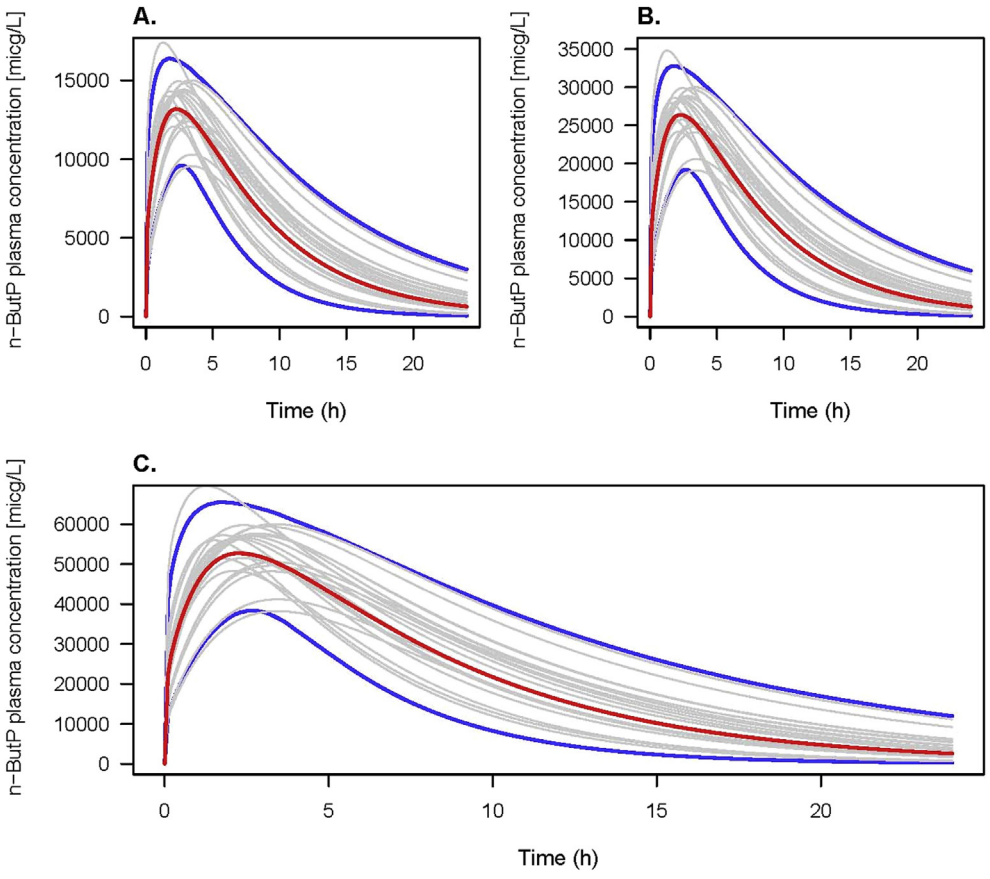

Fig. 7. Simulation of n-ButP plasma concentrations according to PBPK model in the rats after a subcutaneous dosing of different scenarios: A. 150 mg/kg; B. 300 mg/kg; C. 600 mg/kg. Blue lines: 2.5 and 97.5 percentiles; Gray lines: 20 simulations selected at random from the 15000 iterations; Red lines: median prediction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

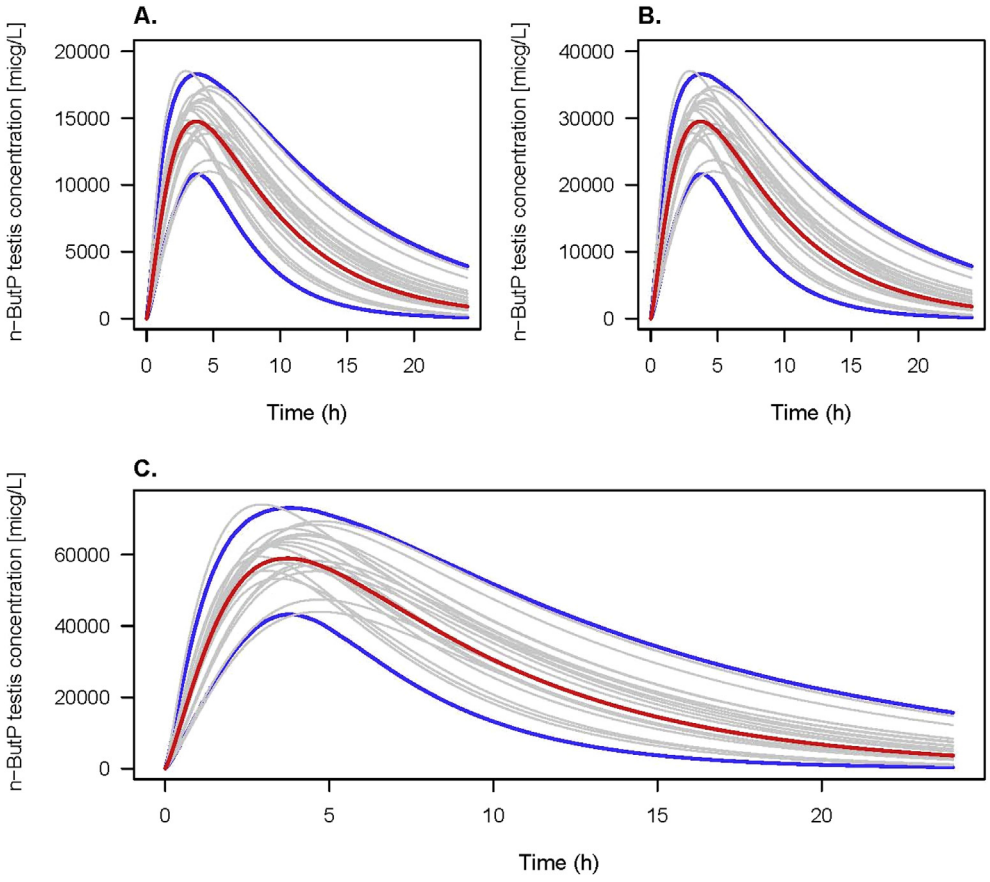

Fig. 8. Simulation of n-ButP testes concentrations according to PBPK model in the rats after a subcutaneous dosing of different scenarios: A. 150 mg/kg; B. 300 mg/kg; C. 600 mg/kg. Blue lines: 2.5 and 97.5 percentiles; Gray lines: 20 simulations selected at random from the 15000 iterations; Red lines: median prediction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were found in testes.

According to glutathione concentrations, a depletion in GSH levels were found in the control-oil and 300 mg/kg/d groups, which indicates oxidative stress in testes. In contrast, the 600 mg/kg/d group showed an increase in GSH concentrations, with a tendency to restore normal levels. These results are in accordance with our hypothesis that peanut oil can generate ROS. In contrast, Nakagawa and Moldeus (1998) reported GSH depletion due to n-ButP in isolated hepatocytes. These authors suggested that a suppression of GR could be related to GSH depletion, and the significant decreased enzyme activities might be due to protein oxidation induced by n-ButP.

Protein expression was analysed using Western blot technique. It is important to remark that GPx-4 is the major isoform in testes (Liang et al., 2009). GPx-4 expression did not show differences among n-ButP treated groups with respect to control-oil group. It could mean that there is only an enhancement in control-oil activity due to oxidative stress, but this effect was not enough to affect GPx-4 expression. Whit respect to CAT and GR expressions, all results would suggest that ROS levels due to peanut oil can affect protein activity, but were not sufficient to damage protein expression. Probably, the lower expression of CAT and GR at the highest doses was due to an elevated levels of n-ButP in testes for 3 alternative days per week during 57 days (Fig. 8C), which would affect other metabolic pathways as response to its endocrine disruptor potential. It is known that in some cases, the activity and expression of a same protein does not change in the same way. Shah and Verma (2011) suggested that protein expression reduction might be due to n-ButP.

Trace elements have an important role in cell homeostasis. In this study, Cu, Mn, Se, Zn Fe and Mg concentrations did not show significant differences between groups. Only Ca showed a significant increase with respect to the control group, with the exception of the 300 mg/kg/d group. Yang et al. (2018) treated trophoblast cells with parabens reporting that ROS induced a cytoplasmatic Ca increase while Popa et al. (2014) also reported disruption of cytosolic Ca homeostasis in liver and kidney due to MetP. In contrast, in the present study, peanut oil induced ROS, and consequently, Ca in testes could increase in the oil-treated groups with respect to the control group.

On the other hand, delivery, distribution and residence time of n-ButP was simulated with a PBPK model for all the three dose scenarios. The C_{max} was slightly higher in testes than in plasma at the corresponding doses, suggesting a higher exposure in testes of n-ButP than in the circulatory fluid of the rat. However, the elimination profile of n-ButP in both plasma and testes was similar with no accumulation. Time course concentration profile in the testes was directly proportional to the animal dose, validating the delivery, distribution and elimination of n-ButP, as also reported in a recent previous (Aubert et al., 2012).

5. Conclusion

To the best of our knowledge, this is the first study evaluating alterations in oxidant-antioxidant status, as well as the concentrations of trace elements in rat testes subcutaneously treated with n-ButP. Rats subcutaneously treated with peanut oil + n-ButP can develop oxidative stress due to oil, but not to n-ButP. These results suggest that either peanut oil can mask the effects of n-ButP, or the chemical can have not sufficient antioxidant effects. High doses of n-ButP are metabolized in high quantities of p-hydroxybenzoic acid and p-hydroxyhippuric acid. These metabolites can then act as antioxidant compounds. In addition, they can stabilize peanut oil, reducing its oxidant effects.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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