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Pancreatic beta cells are a sensitive target of embryonic exposure to butylparaben in zebrafish (*Danio rerio*)

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Abstract

BACKGROUND—Butylparaben (butyl p-hydroxybenzoic acid) is a common cosmetic and pharmaceutical preservative reported to induce oxidative stress and endocrine disruption. Embryonic development is sensitive to oxidative stress, with redox potentials playing critical roles in progenitor cell fate decisions. Because pancreatic beta cells have been reported to have low antioxidant gene expression, they may be sensitive targets of oxidative stress. We tested the hypotheses that butylparaben causes oxidative stress in the developing embryo, and that pancreatic beta cells are a sensitive target of butylparaben embryotoxicity.

METHODS—Transgenic *insulin:GFP* zebrafish embryos (*Danio rerio*) were treated daily with 0, 250, 500, 1,000 and 3,000 nM butylparaben. Pancreatic islet and whole embryo development were examined through 7 days post fertilization, and gene expression was measured by quantitative real-time PCR. Glutathione (GSH) and cysteine redox content were measured at 28 hours post fertilization using HPLC.

RESULTS—Butylparaben exposure caused intestinal effusion, pericardial edema, and accelerated yolk utilization. At 250 nM, beta cell area increased by as much as 55%, and increased incidence of two aberrant morphologies were observed- fragmentation of the islet cluster and ectopic beta cells. Butylparaben concentrations of 500 and 1,000 nM increased GSH by 10 and 40%, respectively. Butylparaben exposure downregulated transcription factor *pdx1*, as well as genes involved in GSH synthesis, while upregulating GSH-disulfide reductase (*gsr*).

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¹Regarding the formatting of gene and protein names, we adhere to the nomenclature guidelines established by the Zebrafish Model Organism Database (<https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines>). Human genes and proteins are labeled using all capitals (*INSA* and *INSA* respectively), and rodent genes and proteins are labeled *Insa* and *INSA* respectively. Zebrafish genes and proteins are characterized as *insa* and *Insa* respectively.

Conflicts of Interest

The authors have nothing to declare.

CONCLUSIONS—The endocrine pancreas is a sensitive target of embryonic exposure to butylparaben, which also causes developmental deformities and perturbs redox conditions in the embryo.

Keywords

paraben; endocrine; glutathione; oxidative stress; redox; developmental toxicology; developmental defects

1. Introduction

Parabens are alkyl esters of the p-hydroxybenzoic acid family, widely used as preservatives in personal care products, pharmaceuticals, and food products (Aubert et al., 2012). It is estimated that the United States population is exposed to an average of 76 mg of parabens per day, and butylparaben is the third most abundant exposure (Cashman and Warshaw, 2005). Butylparaben exposure estimates vary widely; they can be as low as 0.26 mg/day (Matsen, 2005) or as high as 17,760 mg/day for adults and 378 mg/day for infants (Anderson, 2008). Parabens and their metabolites have been found in human skin, intestine, urine, serum, and breast milk samples (reviewed in (Bledzka et al., 2014)). Butylparaben is a lipophilic paraben, with a log K_{ow} of 3.24 (Golden et al., 2005). Studies have identified butylparaben in cord blood (Towers et al., 2015), in placental tissues (Jimenez-Diaz et al., 2011), and in amniotic fluid (Philippat et al., 2013), demonstrating an ability to cross the placenta and a likely risk for direct fetal exposure to butylparaben. However, little is known about the developmental effects of butylparaben, or which embryonic stages are the most susceptible windows of exposure.

Butylparaben has been found to act as an endocrine disruptor in numerous studies. Developmental exposures to butylparaben have been shown to affect expression of the estradiol receptor β in fetal ovaries in rats (Boberg et al., 2008), expression of estrogen receptors α and β and the progesterone receptor in breast cancer cells *in vitro* (Wrobel and Gregoraszczyk, 2014), and have also been shown to have anti-androgenic effects including reduction of sperm counts (Alam et al., 2014; Boberg et al., 2016; Zhang et al., 2016; Zhang et al., 2014). There are also findings of butylparaben impacting epigenetic mechanisms of gene regulation, including hypermethylation of DNA in rat sperm (Park et al., 2012) and decreased methylation of the ER α promoter in male rats (Zhang et al., 2016). Butylparaben is also estrogenic in rainbow trout (Alslev et al., 2005; Pedersen et al., 2000), brown trout (Bjerregaard et al., 2008), and medaka fish (Yamamoto et al., 2011). Butylparaben also activates PPAR γ and promotes adipogenesis *in vitro* (Hu et al., 2013; Hu et al., 2017; Taxvig et al., 2012). Exposure *in utero* have been shown to affect plasma leptin levels (Boberg et al., 2008), suggesting dysregulation of metabolic function.

One mechanism by which butylparaben has been shown to act is via oxidative stress. For example, butylparaben decreased glutathione (GSH) (43.87%) along with the enzymatic activities of GSH peroxidases and GSH-S-transferases in the liver tissues of mice (Shah and Verma, 2012; Shah and Verma, 2011). Also, mice that were exposed *in utero* showed reduced GSH in the brain and increased oxidized glutathione concentrations (glutathione

disulfide; GSSG) (Hegazy et al., 2015). Urinary butylparaben levels have been shown in epidemiology studies to be associated with biomarkers of oxidative stress, such as isoprostane and the biomarker of oxidative DNA damage, 8-hydroxydeoxyguanosine (Kang et al., 2013; Watkins et al., 2015).

The most prevalent endogenous antioxidant that defends against oxidative stress is glutathione (GSH), a tripeptide of cysteine, glutamate and glycine. GSH is synthesized via two ATP-dependent steps: first, the enzyme glutamate-cysteine ligase (Gcl) combines glutamate and cysteine, and second, a glycine is added to the glutamate-cysteine molecule in a reaction catalyzed by glutathione synthase (Gss). One way that GSH modulates redox state is by scavenging reactive oxygen species (ROS), which oxidize the cysteine moiety, resulting in dimerization of two GSH molecules into glutathione disulfide (GSSG); GSSG can be recycled back into reduced glutathione (GSH) in a NADPH-dependent reaction, catalyzed by the enzyme glutathione disulfide reductase (Gsr). Measured concentrations of GSH and GSSG can be used in the Nernst equation to calculate redox potentials (E_h) (Jones, 2002). Fluctuations in redox potentials play an important role in normal embryonic development by guiding cell signaling, cell-fate decisions and apoptosis, and left-right asymmetry (Coffman et al., 2009; Coffman and Denegre, 2007; Covarrubias et al., 2008; Garcia-Gimenez et al.; Hernandez-Garcia et al., 2010; Markovic et al., 2007; Ufer et al., 2010; Winkler et al., 2011). Cell fate decisions are closely related to E_h , with more oxidized E_h associated with differentiation, and more reduced E_h with proliferation (Hansen et al., 2001; Jones, 2002; Kirlin et al., 1999). Therefore, cell populations that have low levels of antioxidant capacity may be particularly susceptible to oxidative stress and butylparaben exposure.

Pancreatic beta cells are reported to have a low antioxidant capacity and be particularly vulnerable to oxidative stress (Lenzen et al., 1996; Thompson and Al-Hasan, 2012; Tiedge et al., 1997). The Islets of Langerhans are comprised of a central core of beta cells that produce and secrete insulin, surrounded by alpha, delta and epsilon cells that produce the hormones glucagon, somatostatin and ghrelin, respectively (Kinkel and Prince, 2009). These hormones collectively comprise a feedback system that maintains glucose homeostasis: insulin inhibits the secretion of glucagon from alpha cells, while glucagon activates the secretion of insulin and somatostatin from beta and delta cells, whereas somatostatin and ghrelin inhibit insulin secretion. We have previously shown that exposure to chemicals that cause oxidative stress during embryonic development can result in aberrant islet morphology and perturbed expression of genes involved in the glucose homeostasis hormone axis in the zebrafish model (*Danio rerio*) (Sant et al., 2017; Sant et al., 2016b; Timme-Laragy et al., 2015).

The zebrafish pancreas is structurally similar to the mammalian pancreas, and shares the same cellular makeup (Kinkel and Prince, 2009). During development, the pancreas arises from the endoderm germ layer and differentiates into the exocrine and endocrine tissues. As reviewed by Tiso *et al* (2009), during the 24 somite stage of the developing zebrafish, pancreatic islet precursor cells arise from endodermal progenitor cells and bud off dorsally from the gut, giving rise to endocrine tissues that express insulin, along with other pancreatic hormones (Tiso et al., 2009). Between 24 and 48 hours post fertilization (hpf), the rotating gut dislocates this dorsal bud on the right side of the gut, forming a primary islet that is

organized similarly to the mammalian islet by 48 hpf (Biemar et al., 2001; Tiso et al., 2009). The mature zebrafish islet consists of an organized mass of beta cells, surrounded by alpha and delta cells; around 7 days post fertilization (dpf) smaller secondary islets that begin to emerge and are nested in the exocrine tissue matrix (Tiso et al., 2009). Because zebrafish embryos are transparent and develop externally, development of pancreatic beta cells can be observed *in vivo*, in real time, using transgenic zebrafish that express green fluorescent protein (GFP) under control of the *preproinsulin* promoter (*Tg(ins:GFP)*) (diIorio et al., 2002).

To date, no studies have been conducted that examine the effects of butylparaben in the zebrafish model, and the effects of butylparaben on the developing pancreatic beta cells are unknown. A preliminary study in 1956 found butylparaben esters were recovered in the pancreata of dogs that were intravenously administered 100 mg/kg butylparaben per day (Jones et al., 1956). However, no further studies have been conducted to investigate whether butylparaben affects the developing pancreas.

Here, we use the zebrafish embryo model to test the hypotheses that 1) butylparaben causes oxidative stress in the developing embryo, and 2) pancreatic beta cells are a sensitive target of butylparaben embryotoxicity.

2. Materials and Methods

2.1. Chemicals

All chemicals were acquired from Thermo Fisher Scientific (Pittsburgh, PA, USA), and were of the highest available purity. Butylparaben was dissolved in 100% dimethyl sulfoxide (DMSO). Solutions were stored at -20°C , and were fully thawed and vortexed before each use.

2.2. Zebrafish

Tg(ins:GFP) embryos were obtained from Dr. Phillip diIorio at the University of Massachusetts Medical School Zebrafish Facility (Worcester, MA) (diIorio et al., 2002). This strain was chosen due to its utility in identifying beta cells with GFP. Wildtype AB fish were obtained from Boston Children's Hospital (Boston, MA). All fish used in these experiments were maintained at 28.5°C under a 14 hr light: 10 hr dark cycle, in a recirculating Aquaneering system (San Diego, CA). Adults were fed a daily diet of Gemma Micro granule fish food (Skretting, Westbrook, ME) per manufacturer feeding instructions.

Embryos were collected from group matings of approximately 30 fish with a 1:2 male to female ratio. Embryos were washed, screened, and were maintained at low densities (approximately 30 embryos) in 20 mL 0.3× Danieau's solution (17 mM NaCl, 2 mM KCl, 0.12 mM MgSO_4 , 1.8 mM $\text{Ca}(\text{NO}_3)_2$, 1.5 mM HEPES, pH 7.6) prior to dosing (Westerfield, 2007). All procedures were approved by the University of Massachusetts Amherst IACUC committee (Animal Welfare Assurance Number A3551-01).

2.3. Chemical Exposures

Complete water changes and dose renewals were performed every 24 hr. Embryos were maintained in an incubator at 28.5°C with a 14 hr light: 10 hr dark cycle. All embryo exposures began at the mid-blastula transition, approximately 3 hpf, in order to confirm fertilization prior to commencing experiments. In each experiment, DMSO was used as the vehicle solvent with a final concentration of 0.01% v/v in 0.3× Danieau's embryo media. We have previously determined that this concentration of DMSO was appropriate and does not perturb beta cell development (Sant et al., 2016b). To identify the high range of butylparaben exposures tolerated by zebrafish embryos, wildtype (AB) zebrafish were exposed to 1,000, 3,000 and 5,000 nM butylparaben, suspended in 0.01% (v/v) DMSO. Newly fertilized embryos were placed (1 embryo per well) into 96-well plates (Fisher Scientific) with 200 µl volume per well via pipette. Embryos (n= 27–30 embryos per treatment) were examined at 24, 48 and 72 hpf under transmitted light microscopy, and scored as either “alive” or “dead,” according to the OECD guidelines for the Fish Embryo Acute Toxicity (FET) Test (www.oecd.org).

To understand the range of butylparaben concentrations that affect the endocrine pancreas, embryos were exposed to 0, 250, 500, 1,000, or 3,000 nM butylparaben in 200 µL of 0.3× Danieau's medium. Exposures began at 3 hpf and were renewed daily with water changes until 7 dpf by manually removing water with a pipette. Embryos in all experiments were manually dechorionated at 1 dpf using Watchmakers forceps. This experiment was repeated at least four independent times with a minimum of five fish per exposure group.

To determine the No Observed Effect Concentration (NOEC) of butylparaben exposure on the pancreatic islet, *Tg(ins:GFP)* embryos were exposed daily to concentrations of 62.5, 125 and 250 nM butylparaben. Embryos were imaged and islet size and morphology were analyzed at 4 dpf as described below. This procedure was repeated twice with 10 embryos per treatment.

2.4. Microscopy and Image Analysis

Embryos (*Tg(ins:GFP)*) were imaged live at 3, 4, 5 and 7 dpf, with an EVOS FL Auto inverted microscope (Life Technologies, Pittsburgh, PA). Embryos were briefly anesthetized with MS-222 (tricaine) in 0.3× Danieau's solution and then placed into individual drops of 3% methylcellulose in a right lateral orientation. Transmitted light microscopy images were taken with 2× and 4× objectives to observe whole-embryo development, and 10× and 20× objectives were used to obtain a transmitted light and GFP overlay to observe pancreatic beta cell development. After imaging, embryos were thoroughly washed in 0.3× Danieau's, re-dosed with their respective treatment, and maintained at 28.5 °C.

To analyze images, the acquisition files were independently blinded, and islet areas and morphologies were assessed using EVOS software. Islets that were not in focus or that were occluded by pigmentation were excluded (1–4 embryos per concentration per trial). Islet areas were obtained by tracing the outline of the beta cell cluster on GFP fluorescence images. Islet areas were analyzed for distribution in JMP-Pro Software (version 13, SAS, Cary, NC) to create violin plots and to identify the 90th and 10th percentiles of the control

groups. Islet areas that exceeded the 90th percentile of the control group for a given timepoint were identified as hypermorphic; islet areas that were below the 10th percentile of the control group were identified as hypomorphic. In addition to islet area, two categories of islet morphology variants were observed: islet fragmentation (beta cells of the islet are dispersed) and ectopic beta cells (a beta cell away from the primary islet). To ensure objectivity, islet morphology variant analysis was carried out on the blinded images by two individuals. To quantify whether butylparaben affects other embryo growth parameters, yolk sac area (a reflection of the embryo's nutrient utilization and metabolic needs) and larval lengths (rostral-to-caudal to measure growth) were measured. Because the swim bladder is derived from the same endoderm progenitor cells as pancreatic beta cells, swim bladder inflation was also evaluated.

A total deformity index was used to quantify gross developmental deformities (Harbeitner et al., 2013; Wassenberg and Di Giulio, 2004). Five deformities were evaluated: pericardial edema, yolk sac utilization, intestinal effusion (fluid retention in the intestinal lumen), craniofacial malformations, and spinal malformations. Briefly, each deformity was scored on a severity scale from 0–3, with 0 as normal, 1 for a mild deformity, 2 for a moderate deformity, and 3 for a severe deformity. The deformity index was then calculated as the sum of scores for each individual embryo divided by the maximum score possible, and multiplied by 100.

2.5. HPLC Analysis of Soluble Thiols

Triplicate pools of 20 embryos were exposed starting at 3 hpf to 0, 500 or 1,000 nM butylparaben as described above. Embryos were collected at 24 hpf, or re-exposed to butylparaben at 24 hpf and collected at 28 hpf. All embryos were placed in thiol preservation buffer (5% perchloric acid, 0.2 M boric acid, 10 μ M γ -glutamylglutamate) and stored at -80°C . Immediately prior to analysis, samples were thawed and processed as previously described (Timme-Laragy et al., 2013). Quantification of the soluble thiol redox couples [GSH and GSSG; Cysteine (Cys) and Cystine (CySS)] was performed at the University of Michigan (Ann Arbor, MI) using High-Performance Liquid Chromatography (HPLC). Briefly, a Waters 2695 Alliance Separations module (Milford, MA) equipped with a Supelcosi LC-NH₂ column (Sigma, St. Louis, MO) was coupled with a Waters 2475 Fluorescence Detector. Reverse phase chromatography measured reduced glutathione (GSH), oxidized glutathione (GSSG), reduced cysteine (Cys), and oxidized cystine (CySS). The flow was set to 1 mL/min using mobile phase A (80 % methanol) and mobile phase B (62.5 % methanol, 12.5 glacial acetic acid and 214 mg/mL sodium acetate trihydrate). Fluorescent detection (excitation 335 nm, emission 518 nm) allowed peaks to be visualized, which were then processed with Waters Empower software (Sant et al., 2016a). The Nernst equation (pH 7.4) was used to calculate the GSH/GSSG $E_h = -264 + 30 \cdot \log([GSSG]/[GSH]^2)$, and Cys/CySS redox potentials $E_h = -250 + 30 \cdot \log([CySS]/[Cys]^2)$ as per (Harris and Hansen, 2012). Protein concentrations were determined by the BCA assay and used to normalize thiol concentrations. Total glutathione (tGSH) and total cysteine (tCys) were calculated by doubling the oxidized component and adding that to the reduced component (e.g. $2 \cdot \text{GSSG} + \text{GSH} = \text{tGSH}$).

2.6. RNA Extraction and Reverse Transcription

To measure changes in gene expression, whole-embryo RNA was first isolated. Pools of 10–12 embryos were exposed to butylparaben as described above, fixed at 3 dpf (due to the significant pancreatic morphological variants observed at this time point; Figure 1 and 2, Table 1) in 100 μ L RNA-Later (Thermo Fisher Scientific), and stored at -80°C . Prior to RNA extraction, all materials and surfaces were sprayed with RNase Away® (Molecular BioProducts, Thermo Fisher Scientific). RNA extractions were carried out according to the GeneJET RNA Purification Kit Total RNA Purification Protocol (Thermo Fisher Scientific Inc., Waltham, MA). Samples were sonicated for one second at 15 % amplification with a Branson Digital Sonifier® (Danbury, CT) that was sterilized with 75 % ethanol between each sonication. RNA concentration and quality was analyzed using a BioDrop μ LITE (BioDrop, Cambridge, UK). Reverse transcription to cDNA was carried out according to the manufacturer's protocol using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) with an Eppendorf Mastercycler® nexus gradient thermal cycler (Eppendorf, Hauppauge, NY) according to the manufacturer's protocol. A total of 5 biological replicates per exposure concentration were collected from three independent experiments.

2.7. Quantitative Real-Time PCR

To assess whether butylparaben exposures may disrupt the function of beta cells, gene expression of the pancreatic endocrine hormone axis was measured. Gene expression of *preproinsulin a (insa)*, *glucagon a (gcga)*, *ghrelin (ghrl)*, *somatostatin 2 (sst2)* and the transcription factor *pancreatic duodenal homeobox 1 (pdx1)* were quantified. The expression of glutathione-related genes: the catalytic and modifier subunits of Gcl (*gclc* and *gclm*), *glutathione synthetase (gss)*, *glutathione disulfide reductase (gsr)*, and two glutathione-s-transferase genes (*gstp1* and *gsta1*) were also measured to understand butylparaben's effects on glutathione redox dynamics. β -actin (*actb*) was used as a housekeeping gene, and these data confirmed that expression did not change due to chemical exposures.

Commercially available PrimePCR™ primers for *sst2*, *ghrl*, *gclm*, *gss*, and *gsr* were purchased from Bio-Rad. Primers for *gcga*, *insa*, *pdx1*, *gclc*, *gstp*, *gsta1* and housekeeping gene *actb* were exon-spanning to avoid DNA contamination (Supplemental Table 1). cDNA was diluted to a working concentration of 0.125 ng/ μ L (Rousseau et al., 2015). A 20 μ L PCR reaction was carried out that contained 0.5 μ L of each forward and reverse primer, 10 μ L of 2 $\times$ iQ™ SYBR® Green Supermix (Bio-Rad), 5 μ L of nuclease-free water, and 4 μ L of diluted cDNA. Quantitative real-time PCR (qPCR) was performed in a Bio-Rad CFX Connect™ real-time system under the conditions of 2 minutes at 95°C followed by 10 seconds at 95°C , and then 25 seconds at 60 – 68°C depending on the melting temperature of each primer. Data obtained from qPCR were analyzed using the Bio-Rad CFX Connect Manager™ software, version 3.0 (Bio-Rad). The $\Delta\Delta\text{CT}$ method was used to calculate fold change (Livak and Schmittgen, 2001).

2.8. Statistical Analysis

Data were analyzed using Microsoft Excel, JMP Pro version 13, and Stata statistical software, version 14.1. One-factor or two-factor ANOVAs with a Tukey's or Wald's post hoc test were used to compare exposed groups and controls. Logistic regression analysis was

performed to determine significance for swim bladder data and a Mann-Whitney U test was used to analyze the deformity index. Probit analysis was performed to analyze survival. A McNemar's test for significance was used to determine independence between islet variants and gross morphological deformities. Data are presented as the mean and standard error of the mean (SEM). N is defined as the number of embryos for morphometric endpoints, and the number of pooled embryos for gene expression and thiol measurements.

3. Results

3.1. Butylparaben Disturbs Pancreatic Islet Development

To determine the low-range toxicity of butylparaben on islet development, the NOEC was calculated for islet area and defects at 96 hpf. Islet area increased slightly in a dose-dependent manner (Suppl. Fig. 2), but was only statically significant at 250 nM ($p < 0.05$). Butylparaben also increased the incidence of previously observed variant islet morphologies (Sant et al., 2016b) at 250 nM, with 70% of islets displaying variant morphology compared to 20% of embryos exposed to 125 nM, and 0% of embryos exposed to 62.5 nM (Suppl. Table 2). Based upon these findings, we determined the Lowest Observed Effect Concentration (LOEC) for islet variant morphology was 250 nM, and the No Observed Effect Concentration (NOEC) was 125 nM.

Area of beta cell fluorescence was measured at 3, 4, 5 and 7 dpf. Islet area increased significantly for all concentrations of butylparaben when compared to controls at 3 dpf ($p < 0.05$) by as much as 55% (Figure 1). Embryos exposed to 250 nM butylparaben had significantly larger islet areas than controls at all time points (3, 4, 5 and 7 dpf). At 4 dpf, islet area was significantly greater in embryos exposed to 250 and 500 nM butylparaben ($p = 0.04$ and 0.01 respectively) and at 5 dpf, only embryos exposed to the lowest concentration of 250 nM saw a significant increase in islet area ($p = 0.01$). At 7 dpf, islet area was significantly greater in embryos exposed to the lowest (250 nM) and highest (3,000 nM) concentrations of butylparaben ($p < 0.05$) (Figure 1A). These data also show that in control embryos the islet area consolidates as the larvae mature. To identify hypermorphic (greater than 90th percentile of control islet areas) and hypomorphs (less than 10th percentile of control islet areas), we created violin plots (Figure 1B) that show the distribution of the islet areas in relation to these percentile markers, and also calculated the percent of each exposure group that fell into either a hyper or hypomorph islet category (Figure 1C). At 3 dpf only hypermorphic islets were observed, with over 50% of fish in each butylparaben exposed group falling into this category. Incidence of hypomorphs was rare, with the 1000 nM exposure group at the 4 dpf timepoint having the only increase (35% of fish). Over the course of the 7-day exposure, most islet areas of exposed fish recovered to fall within the 90th and 10th percentiles of the controls, although some fish islets remained in either the hypermorphic or hypomorph categories (notably 50% of the 250 nM exposure group).

Exposure to butylparaben increased the prevalence of variant beta cell architecture in the pancreatic islet. The most prevalent variant morphologies observed were: fragmentation of the beta cells, where the beta cells of the islet appear more dispersed, and ectopic beta cells, in which a beta cell emerges away from the primary islet (Figure 2). The incidence of these variants changed with butylparaben exposure. Fragmented islets were most prevalent at the

250 nM concentration (44%), whereas ectopic beta cells were most prevalent at 500 nM (16%) (Table 1). Between 3 and 7 dpf, 90% of control embryos displayed normally developed islets compared to 53% of the 250 nM group, 75% of the 500 nM group, 71% of the 1,000 nM group, and 67% of embryos exposed to 3,000 nM groups. Three dpf embryos that were exposed to 250 nM and 3,000 nM butylparaben had the greatest prevalence of total islet variants (56% and 58% respectively) (Table 1). Islet fragmentation decreased over time (Table 1). There are no obvious trends relating to the prevalence of ectopic beta cells.

3.2. Developmental Toxicity of Butylparaben

To determine the effects of butylparaben on embryonic development, eleutheroembryos were analyzed for developmental deformities. *Tg(ins:GFP)* embryos were exposed daily to concentrations of 250, 500, 1,000 and 3,000 nM butylparaben, and were imaged at 3, 4, 5 and 7 dpf. Five developmental deformities were used in a deformity index: pericardial edema, yolk sac utilization, intestinal effusion, craniofacial malformations and spinal malformations. Butylparaben increased total deformities in a dose-dependent manner. Embryos exposed to the lowest concentration, 250 nM, did not significantly differ from controls at any time point. The highest concentration, 3,000 nM, increased total deformities at all time points ($p < 0.01$). Embryos exposed to 500 nM butylparaben had increased deformities only at 3 and 7 dpf ($p = 0.01$ and $p = 0.03$ respectively), and those exposed to 1,000 nM saw significantly more deformities at 3, 4, and 7 dpf ($p < 0.01$, $p = 0.01$, and $p = 0.02$ respectively) (Figure 3). At 3 dpf, butylparaben exposures greater than 250 nM increased total deformities ($p < 0.05$) (Figure 3, Supplemental Figure 3), and some embryos displayed multiple deformities. However, most embryos exposed to the 250 nM concentration were considered normal.

To observe the effects of butylparaben on growth, yolk sac utilization and fish length were quantified. Embryonic yolk sac areas were measured at 3, 4 and 5 dpf to measure yolk utilization. No significant relationship was observed between butylparaben concentration and yolk sac area. Although a trend in smaller yolk sac area was seen throughout all time points at the 250 nM exposure, this was not significant (Suppl. Fig. 4). Embryonic growth was quantified by measuring rostral-to-caudal linear length. Similarly to yolk sac area, embryonic body length was not significantly affected by butylparaben at any concentration from 3–7 dpf (Suppl. Fig. 4).

Impaired swim bladder inflation in response to chemical exposures is an endpoint that is often observed in zebrafish embryotoxicity tests. Because the teleost swim bladder is derived from the same progenitor endoderm layer as the pancreas, the effect of butylparaben on swim bladder inflation at 4, 5, and 7 dpf was examined. At 4 dpf there was a trend in accelerated swim bladder inflation at 250 nM when compared to controls, ($p < 0.05$). Swim bladder inflation was significantly decreased at the highest concentration (3,000 nM) at 4, 5 and 7 dpf ($p < 0.05$) (Suppl. Fig. 5).

To further investigate whether these developmental morphologies contribute to the occurrence of islet variants, the relationship between the specific morphologic deformities (swim bladder inflation, intestinal effusion, spinal malformations, yolk sac utilization, pericardial edema, and craniofacial malformations) and islet architecture was observed using

a McNemar's test for independence. Here, the relationship between specific deformities and the occurrence of pancreatic islet variant morphologies was measured in control groups and embryos that were exposed to 250 and 3,000 nM butylparaben at 4 dpf. No clear relationship was observed between the occurrence of developmental deformities and islet architecture, suggesting that these are independent events.

3.3. Analysis of Glutathione and Cysteine Redox Potentials

To analyze redox status in the embryos, GSH, Cys, GSSG, CySS, tGSH, tCys and the GSH and Cys E_h were measured using High-Performance Liquid Chromatography (HPLC), utilizing the Nernst equation to calculate the GSH and Cys redox potentials. There were no significant changes in the redox status of embryos that were exposed to butylparaben at 3 hpf and collected at 24 hpf (data not shown). However, significant changes in redox status were observed in 28 hpf embryos that were exposed to butylparaben at 3 hpf and again at 24 hpf. Butylparaben concentrations of 500 and 1,000 nM increased GSH by 10% and 40%, respectively. GSH redox potentials became more reduced with 500 nM and 1,000 nM butylparaben exposures, increasing redox potentials by 7 and 18 mV, respectively ($p < 0.05$). Cysteine redox potentials also became more reduced by 17 and 28 mV ($p < 0.05$); however, tGSH and tCys did not significantly change (Figure 4).

3.4. Expression of Pancreas-related Genes

To assess the effects of butylparaben on beta cell function, the gene expression of the pancreatic endocrine hormone axis was measured. Gene expression of the pancreatic hormone index: *preproinsulin a (insa)*, *glucagon a (gcga)*, *ghrelin (ghrl)*, and *somatostatin 2 (sst2)* was measured using qPCR. The transcription factor pancreatic duodenal homeobox 1 (*pdx1*) was also measured, and β -actin (*actb*) was used as a housekeeping gene. There was no significant relationship between butylparaben exposure and the gene expression of the pancreatic hormone index (data not shown). However, the expression of *pdx1* was significantly downregulated at the highest concentration (3,000 nM; $p < 0.05$) (Figure 5).

3.5. Expression of Glutathione-related Genes

Expression of glutathione-related genes was measured to show the induction of phase II detoxification enzymes to potentially indicate redox compensatory mechanisms. Expression of genes encoding for the two subunits of the enzyme glutamate-cysteine ligase (*gclc* and *gclm*) and glutathione synthase (*gss*) were measured in addition to glutathione disulfide reductase (*gsr*), which recycles oxidized glutathione (GSSG) back into reduced glutathione (GSH). The expressions of two genes that encode for glutathione-S-transferases (*gstp* and *gstal*) were also measured. No significant relationship was found between butylparaben exposure and the expression of *gclm*. However, *gclc*, and *gstal* were modestly downregulated 10–20% and *gss* was downregulated by 60% at the highest concentration (3,000 nM), whereas *gsr* was significantly upregulated at all concentrations in a dose-dependent manner ($p < 0.05$) (Figure 5). The expression of *gstp* was slightly upregulated at the 500 nM concentration ($p < 0.05$) (Figure 5).

4. Discussion

This exploratory study examined the effects of developmental exposure to butylparaben on the developing embryo and the concurrent effects on the glutathione and cysteine redox systems. We hypothesized that butylparaben would cause oxidative stress in the developing embryo and that pancreatic beta cells would be a sensitive target of butylparaben embryotoxicity. We used the zebrafish embryo model to characterize the impact of butylparaben on both the endocrine pancreas and overall gross development in zebrafish embryos, in addition to butylparaben's effects on redox dynamics. This study was prompted by the wide-spread use of butylparaben in personal care products and the potential for fetal exposures. The NHANES study (2005–2006) detected butylparaben in 47% of samples analyzed (Calafat et al., 2010), and butylparaben has also been found in human amniotic fluid at concentrations of 0.3 µg/L (Philippat et al., 2013). The Islets of Langerhans are highly vascularized, and parabens in the blood stream have also been deposited in pancreatic tissues (Jones et al., 1956), suggesting this may be an important but understudied target tissue.

Developmental exposure to some concentrations of butylparaben increased islet area and the prevalence of aberrant beta cell architecture in the principal pancreatic islet throughout development (Figures 1 and 2, Table 1). Exposure concentrations of 250, 500, 1000, and 3000 nM butylparaben all resulted in larger islet areas when measured at 3 dpf, in a U-shaped dose response curve. However, the lowest concentration (250 nM) was the only exposure to result in persistent hypermorphic islets at all timepoints measured (3, 4, 5, and 7 dpf; Figure 1); these measurements were driven by the high occurrence of islet fragmentation at the earlier timepoints (Figure 2, Table 1). Surprisingly, the higher concentrations (500, 1000, and 3000 nM) resulted in embryos with islet sizes that recovered into a normal range after the 3 dpf timepoint, while the highest concentration (3000 nM) resulted in a reemergence of the hypermorphic phenotype at the latest timepoint, 7 dpf, a finding that did not co-occur with fragmented islet morphology. Additional experiments are required to fully understand the mechanisms underlying this complex response, but non-linear dose-response curves are commonly observed with endocrine disrupting compounds including butylparaben (e.g. (Hu et al., 2013; Wrobel and Gregoraszczyk, 2013)). As islets are an endocrine tissue, it is not unexpected to see a non-linear dose response curve in this structure (e.g. (Sant et al., 2017)). This may also indicate different mechanisms occurring at different ends of the dose-response range. For instance, the islet measurements of control embryos show consolidation of this structure as the larvae mature (this paper and (Jacobs et al., 2018)), and it may be that low concentrations of butylparaben interfere with this process, whereas at high concentrations compensatory responses may mitigate this impact.

The fragmented islet phenotype mimicked observations in other cell types. For example, butylparaben-induced cell dispersion has also been shown in rodent models; Alam *et al* found butylparaben to cause the collapse of rat Sertoli cell vimentin filaments, allowing spermatogenic cells to separate from the Sertoli cells (Alam and Kurohmaru, 2014). Vimentin filaments are found in pancreatic precursor cells and in beta-cell neogenesis, (Ko et al., 2004), and an increase is a marker of pancreatic cancer progression in zebrafish

(Schiavone et al., 2014). Studies are under-way to determine whether vimentin disruption contributes to the aberrant beta cell architecture in the developing islet.

To further characterize the effects of butylparaben on islet development, expression of genes in the pancreatic hormone index (*insa*, *gcga*, *sst2* and *ghrl*) and the transcription factor *pdx1* were measured. Exposure to butylparaben significantly decreased the expression of *pdx1* only at the highest concentration (3,000 nM), but did not significantly affect the expression of genes in the pancreatic hormone index. Pdx1 plays a role in regulating endocrine differentiation in the pancreas and in modulating beta cell function via regulating the expression of insulin. Pdx1 is redox sensitive and *in vitro*, and its activity has been found to decrease with the induction of oxidative stress (Matsuoka et al., 1997). Downregulation of *pdx1* without downregulation of *preproinsulin* suggests the presence of a feedback loop responding to the enlarged beta cell mass present at the highest concentration of butylparaben.

Toxicological assessments in this study identified the LOEC for aberrant islet development as 250 nM. While lower concentrations of 62.5 nM and 125 nM appeared to slightly increase islet area in a dose-dependent manner, this trend was not significant; the NOEC for effects on islet development was 125 nM. Other studies have identified NOEC and LOECs in fish species, but few have examined toxicity in the embryo. Fathead minnow (*Pimephales promelas*) larvae were exposed to butylparaben daily for 7 days, the larval growth LOEC was 1.0 mg/L butylparaben (5.15 μ M) (Dobbins et al., 2009). Our study did not identify a significant effect on growth in any of the concentrations used (Supplemental Figure 4), although these were much lower than the fathead minnow study, with our highest concentration being 3,000 nM.

To determine whether the effects on the beta cells from butylparaben exposure are specific or associated with a generalized toxicity, the incidence of morphologic deformities was also examined. Yolk sac area and fish length were measured to determine whether butylparaben affects growth, and the incidence and severity of morphologic deformities was accounted for in a deformity index. Our data did not show any significant changes in yolk sac area or fish length (Supplemental Figure 4), although we did note a mild, qualitative increase in yolk sac utilization (Figure 3, Supplemental Figure 3). Overall, growth and nutrient utilization in butylparaben-exposed embryos was normal at these concentrations. With respect to morphologic deformities, there were some that occurred with butylparaben exposure, namely intestinal effusion and pericardial edema at the higher concentrations in the study; however, the majority of exposed embryos at the 250 nM concentration did not have any deformities beyond aberrant islet size and morphology, and no relationship was observed between these deformities and islet architecture in any of the exposure groups. This finding contrasts what has been reported by in rats, where developmental exposure to butylparaben did not result in significant effects on gross fetal development, soft tissue alterations, or skeletal alterations at all oral doses tested, with a maximum concentration of 1,000 mg/kg/day (Daston, 2004). Our data suggest that islet development is a more sensitive endpoint than deformities such as pericardial edema and craniofacial malformations, but this has not been assessed in other models.

Butylparaben has been shown to cause oxidative stress in rodent models (Hegazy et al., 2015; Shah and Verma, 2012; Shah and Verma, 2011). Since pancreatic beta cells are especially sensitive to oxidative stress (Lenzen et al., 1996; Thompson and Al-Hasan, 2012; Tiedge et al., 1997), changes in redox status may contribute to aberrant development of the islets. We previously reported that a single exposure to the model oxidant *tert* butylhydroperoxide at 72 hpf resulted in hypomorphic islets (Sant et al., 2016b) and our subsequent studies show an increased incidence of fragmented islets (paper in preparation). It was therefore a contradictory finding to see that butylparaben, a chemical reported to cause oxidative stress, produced primarily hypermorphic islets while still mirroring the islet morphology (fragmentation) produced with tBOOH. To better understand this, we measured redox potentials of the GSH/GSSG and Cys/CySS couples in response to butylparaben exposure in 24 hpf and 28 hpf zebrafish embryos, a time after beta cell specification but still early in islet morphogenesis. Significant changes in redox status were only seen in embryos that underwent an acute exposure before collection (exposed at 24 hpf and collected at 28 hpf), which significantly affected both the GSH and Cys redox potentials (Figure 4). Cys is regulated independently of GSH and sits at a more reduced state (Jones, 2006). If butylparaben were to cause oxidative stress, it would be expected that the GSH and Cys redox potentials would both become more oxidized with increasing butylparaben concentration. Rather, GSH and Cys increased, while oxidized GSSG and CySS decreased with increasing concentrations of butylparaben, resulting in a more reduced redox state. Because these measures were taken in whole-embryos, and the amount of cell-sorted beta cells that would be required to directly measure these parameters is currently prohibitive, it is difficult to link the altered redox state directly with the beta cell morphology. But the congruence with fragmentation observed in our previously published positive controls suggests that the daily administration of butylparaben was sufficient to disrupt islet morphology and development, and likely involves redox-dependent mechanisms.

Expression of genes involved in the synthesis and recycling of glutathione was then measured at a later developmental stage (78 hpf). Embryos exposed to the highest butylparaben concentration (3,000 nM) had significantly decreased expression of synthesis genes *gclc* and *gss*, while expression of *gsr*, the gene encoding for the enzyme that reduces GSSG to GSH, significantly increased at all concentrations analyzed (250, 500 and 3,000 nM). Because *gsr* expression was upregulated at concentrations that were also analyzed for redox status, it is possible that Gsr enzymatic activity is also increasing. Increased Gsr activity would recycle greater amounts of GSSG back into GSH, possibly resulting in less GSSG and more GSH. This increase in Gsr activity may be causing the increased levels of reduced GSH and decreased levels GSSG that are seen in the redox analysis data. These results also further suggest a reduced environment with butylparaben exposure, and because gene expression was measured two days after redox potentials were measured, it appears these reductive effects are lasting. These data suggest that the glutathione dynamics within the embryo have been disturbed, but do not necessarily indicate oxidative stress.

It is important to note that all butylparaben concentrations used in these experiments represent low-dose and environmentally relevant exposures. Estimates of butylparaben exposure vary widely and are generally tied to long-term cosmetic use; they can be as low as 0.26 mg/day (Matsen, 2005) or as high as 17,760 mg/day for adults and 378 mg/day for

infants (Anderson, 2008). Multiple studies have observed post-exposure concentrations of un-metabolized butylparaben in human urine (0.2–1,240 µg/L), blood (135 µg/L), amniotic fluid (0.3 µg/L) (Calafat et al., 2010; Janjua et al., 2007; Philippat et al., 2013), and human placental tissues (0.4 ng/g) (Jimenez-Diaz et al., 2011). In these studies, zebrafish embryos were exposed to butylparaben concentrations that range from 48.5–582.66 µg/L (250–3,000 nM), and are within the range found in human samples.

This study identifies pancreatic beta cells as a highly sensitive target of embryonic exposure to butylparaben. Butylparaben can also cause other deformities at higher concentrations including intestinal edema, accelerated yolk utilization, pericardial edema, and craniofacial malformations. These effects occur alongside disruptions in the glutathione and cysteine redox systems in the embryo, but the relationship between redox stress and occurrence of these deformities requires further investigation. This study, along with others (e.g. (Jacobs et al., 2018; Sant et al., 2016b; Timme-Laragy et al., 2015)), also highlights the utility of the *Tg(ins:GFP)* zebrafish embryo to screen for toxicants that pose potential hazards to the developing pancreas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GSH	Glutathione
GSSG	Glutathione disulfide
dpf	Days post fertilization
hpf	Hours post fertilization
ROS	Reactive oxygen species
GFP	Green fluorescent protein
E_h	Redox potential
DMSO	Dimethyl sulfoxide

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Figure 1. Developmental exposure to butylparaben increases islet area

Tg(ins:GFP) zebrafish embryos were exposed daily to 250, 500, 1,000, and 3,000 nM butylparaben beginning at 3 hpf. The area of the beta cell cluster in the primary islet was measured at 3, 4, 5, and 7 dpf. A) Data are presented as the mean + SEM, and the letters represent treatments that are statistically different from one another. Results of a one-factor ANOVA followed by a Tukey's post hoc test are represented by letters; $p < 0.05$; $n = 10-22$ individual embryos. B) Islet areas plotted as violin plots to demonstrate the spread of the data. Horizontal lines on the plots represent the 90th percentile and 10th percentile of the control group for each timepoint. C) The percent of each exposure group that fell above the 90th percentile (defined as hypermorphic islet size; black bars) and below the 10th percentile of the controls (defined as hypomorphic islet size; white bars). Data cumulative of four independent experiments.

A

B

Figure 2. Examples of islet deformities and occurrence with dose and age

A) Images of normal and aberrant beta cell architecture in the principal islet in zebrafish embryos at 4 dpf exposed daily to butylparaben in *Tg(ins:GFP)* embryos. The image on the left-hand side is a control (DMSO) embryo with a characteristically spherical islet. The center image shows fragmentation of the islet in a butylparaben-exposed embryo. The right-hand image shows an ectopic beta cell (arrow) in a butylparaben-exposed embryo. Images are overlaid trans and GFP images taken with a 20× objective, and are oriented in a left to right posterior-anterior direction. B) Occurrence of these islet morphologies over dose and age as a percent of total islet morphology; numbers and percentages can be found in Table 1.

Figure 3. Gross deformities resulting from butylparaben exposure

Tg(ins:GFP) zebrafish embryos were exposed to 0, 250, 500, 1,000 and 3,000 nM butylparaben daily, and evaluated for morphology at 3, 4, 5, and 7 dpf. A) A deformity index was calculated incorporating the incidence and severity of all noted developmental deformities including pericardial edema, accelerated yolk sac utilization, intestinal effusion, and craniofacial malformations ($n = 17$ – 26 embryos cumulative from four independent experiments); $* = p < 0.05$, $** = p < 0.01$. B) The percent of embryos with each morphological deformity and the severity of the malformation was compared for the 4 dpf

exposure groups. Representative images of morphological deformities in 4 dpf eleutheroembryos were taken with light microscopy on a 4× objective.

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Figure 4. Redox analysis of glutathione and cysteine at 28 hpf

A) Reduced and oxidized glutathione (GSH, GSSG) and cysteine: cysteine was quantified at 28 hpf after exposure to DMSO, 500 nM or 1,000 nM butylparaben at 3 hpf and 24 hpf using HPLC to quantify soluble thiols. The Nernst equation was used to calculate cellular redox potentials (E_h). One factor ANOVAs were used to determine differences between exposed and control groups followed by a Tukey's post hoc test ($p < 0.05$, $n = 3$ samples of 20 pooled embryos). B) Two-dimensional plots of redox potentials and total concentrations for glutathione and cysteine. The dot at the center of each ellipse represents the mean and the boundaries of the ellipses represent the standard error.

Figure 5. Gene Expression of pancreas and glutathione genes

Wildtype AB embryos were exposed daily to DMSO, 250, 500 or 3,000 nM butylparaben, and were collected at 78 hpf. Expression of *pdx1*, *gsta1*, *gclc*, *gss* and *gsr* was measured and the ddCt method was used to calculate fold change, followed by a one factor ANOVA and Tukey's post hoc test ($p < 0.05$); $n = 5$ biological replicates per group with each replicate containing pools of 10–13 embryos.

Table 1

Incidence of pancreatic islet morphology variants in zebrafish embryos and larvae exposed to butylparaben.

Butylparaben	Variant Islets	Age				
		3 dpf	4 dpf	5 dpf	7 dpf	
0 mM	Total	16% (7/14)	17% (7/42)	14% (6/42)	5% (2/40)	
	Fragmented	6/44	6/42	4/42	2/40	
	Ectopic	1/44	1/42	2/42	0/40	
250 mM	Total	52% (14/27)	44% (12/27)	41% (11/27)	26% (7/27)	
	Fragmented	13/27	11/27	9/27	6/27	
	Ectopic	1/27	1/27	2/27	1/27	
500 mM	Total	28% (7/25)	17% (4/14)	8% (2/24)	13% (3/24)	
	Fragmented	3/25	2/24	0/24	2/24	
	Ectopic	4/25	2/24	2/24	1/24	
1000 mM	Total	20% (5/25)	32% (8/25)	8% (2/25)	16% (4/25)	
	Fragmented	4/25	5/25	0/25	2/25	
	Ectopic	1/25	3/25	2/25	2/25	
3000 mM	Total	2% (1/45)	9% (4/45)	16% (7/45)	13% (6/45)	
	Fragmented	1/45	2/45	3/45	4/45	
	Ectopic	0/45	2/45	4/45	2/45	