MJFAS
Malaysian Journal of Fundamental and Applied Sciences

Zulkifli *et al*. | Malaysian Journal of Fundamental and Applied Sciences, Vol. 18 (2022) 227-236

RESEARCH ARTICLE

General Health of Pregnant Sprague-Dawley Rats and Neonates' Small Intestine Morphology Upon Maternal Bisphenol A Exposure: A Preliminary Study

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Abstract Associations between xenoestrogen bisphenol A (BPA) and multiple types of diseases, including metabolic syndrome, have been recorded in various studies. However, certain subsets of the human population are particularly more vulnerable to BPA repercussions, such as pregnant women, neonates, and children. This study was conducted to investigate the effects of BPA exposure during pregnancy on the general health of mothers and the histopathology of neonates' small intestines. Eighteen Sprague-Dawley rats were divided into three groups: control, vehicle Tween-80, and 5mg/kg/day BPA after positive mating was confirmed. Physiological parameters consisted of body weight, waist circumference, water, and food intake, and blood pressures were measured at pregnancy day -1 or 2, 7, and 14 to see whether BPA exposure could exert obesogenic impacts on pregnant rats. Newborns were sacrificed to collect blood plasma for BPA analysis and intestinal samples for histopathological examination. Maternal BPA exposure did not affect the physiological parameters of pregnant rats. The number of pups delivered per litter and the sex ratio of BPA offsprings was not significantly different to those of control and vehicle groups ($p>0.05$). Likewise, the small intestine morphology of BPA neonates was comparable to those of controls and vehicles (preserved structure and absence of inflammatory cells infiltration). The nonsignificant difference in plasma BPA levels of control and BPA-exposed mothers and neonates may explain these findings. Future longitudinal studies which include the dose-dependent impacts of BPA on pregnant mothers' health and neonates' small intestine would be more beneficial.

Keywords: Bisphenol A, maternal exposure, pregnancy's health, small intestine, histopathology.

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Received: 14 August 2021
Accepted: 21 April 2022

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Introduction

Bisphenol A (BPA) is regarded as one of the essential industrial chemicals in the world due to its extensive usage in polycarbonate plastics (such as reusable bottles and medical devices) and epoxy resins (beverage and food cans lining) manufacturing [1-3]. The reason for this is that this compound effectively exhibits good mechanical properties (durability and flexibility) and is stable at high temperatures. Despite that, BPA presence in the environment, for example river water, indoor dust, and outdoor air [4, 5], as well as leaching into food and water, increases daily human exposure to this contaminant [6-9]. Consequently, human fluids such as blood and breast milk, and tissues including placenta and adipose tissue show detectable levels of BPA at nM concentration [10-12]. Different BPA levels have been associated with various human medical conditions including obesity and infertility, and similar results were reflected even in animal and *in vitro* studies [13-16].

BPA direct contact with the gut after ingestion brings researchers to study the effect of BPA on the small intestine, a site where most digestion and absorption of nutrients occurs and plays a pivotal role in the health and growth of mammals [17, 18]. On top of that, effortless transfer of BPA from mother to offspring during pregnancy is possible due to the oestrogen-like structure and endocrine-disrupting activity that the substance exhibits, in addition to the decreased hepatic expression of multidrug resistance-associated protein 2 (Mrp2) in pregnant mothers [19, 20]. A lot of research has revealed that multiple adult-onset diseases such as heart diseases originated from early life exposure to BPA, which corroborates with David Barker's hypothesis, whereby late-onset diseases increase due to poor prenatal nutrition [21-23]. The window of susceptibility to chemical exposure is not limited to foetal development only but also the period of endocrine systems maturation that persists throughout childhood and adolescence. It is suggested that BPA permanently alters the structural and functional property of the systemic organs [18, 24-26].

To the best of our knowledge, studies on the repercussions of developmental BPA exposure on neonate's small intestine and the general health of pregnant mothers are relatively sparse, and each demonstrates different outcomes. Therefore, the rationale of this study is to explore the impacts of BPA exposure on the physiological parameters of pregnant mothers and how this exposure affects the intestinal morphology of the offspring, which may impact its development and health later in life.

Materials and methods

Experimental protocol

All experimental procedures were approved by the Committee on Animal Research and Ethics of Universiti Teknologi MARA (UiTM) (approval number UiTM CARE: 294/2020 (7/2/2020)). A total of 18 pregnant SD rats (10 to 12 weeks of age) were randomly divided into three groups: Group 1 (control group, without Tween-80 (CTL)), Group 2 (vehicle control group, with Tween-80 (P80)) and Group 3 (water spiked with 0.05mg/ml BPA (Sigma)). Pregnant rats were housed individually under standard conditions (22°C, 12-hr light-dark cycle), with access to food (standard chow diet) and water ad libitum. Pregnant females were administered with BPA water via drinking water (to mimic the most common route of human exposure) with dosage of 5mg/kg body weight/day beginning of pregnancy day 2 (PD2) until end of pregnancy as previously described [27]. Tween-80 (P80) was prepared in 0.25% total volume before further dilution with water. BPA free water bottles were used to avoid potential contaminations from sources other than administered drinking water. Maternal food intake, volume of water consumed, body weight gain and blood pressure (BP) were measured weekly to reduce stress.

Pregnant rats delivered the pups normally then 0.5 ml blood sample was collected from orbital sinus. Newborn rats were collected. The number of newborn rats per litter and the sex ratio were recorded. Six newborns (day 0 to day 2) from each group were sacrificed (by decapitation) and blood samples were collected to ensure that the BPA consumed by the mothers were transferred to the pups. Intestinal samples were collected for histopathological evaluation.

Solid phase extraction liquid chromatography-mass spectrometry (LC-MS)

BPA levels in mothers' and offsprings' blood plasma were measured by using LC-MS technique. Samples (500µL) were treated with 1M H_3PO_4, vortex mixed and spiked with 10µL of ^{13}C-labelled internal standard 1µg/mL. Deglucuronidation of BPA was performed by incubating spiked samples with 500µL of 1 M ammonium acetate and 10 µL of β-glucuronidase enzyme at 37℃ for 120 minutes. Samples were then diluted with 2ml of 0.1M formic acid prior to being passed through cartridges conditioned with 2ml methanol and 2ml water at the flow rate of 1ml/minute. Cartridges were washed with 1ml of water and 1ml of 40% methanol in water. BPA was eluted with 3ml of acetonitrile, concentrated under gentle stream of nitrogen gas at 25℃, and dissolved in 100µL of 70% methanol in water (solid phase extraction). Samples were sealed with laboratory film and stored at -20℃. BPA quantification by LC-MS method was outsourced and conducted by the Faculty of Pharmacy, UiTM.

Histopathological evaluation

Small intestine morphology was observed by haematoxylin and eosin (H&E) staining. Intestinal tissues of 1cm in length collected from offsprings were fixed in 10% neutral buffered formalin for 72 hours. Tissues were then processed in Tissue-Tek VIP 6 AI and embedded in paraffin wax. Embedded tissue was then trimmed and sectioned at 3 µm thickness and placed in water bath at 40℃ before being mounted onto slides and left air dry. Tissue sections were then left in the oven for 15 minutes, followed by placing them in three different xylene solutions (3 minutes each) for dewaxing, followed by a series of decreasing concentrations of alcohol. Haematoxylin and eosin staining was then performed. After air dried, tissue sections were mounted with DPX mountant, covered with coverslips and then observed under light microscope.

Statistical analysis

For comparison between study groups, Kruskal-Wallis test was performed. Post HOC analysis (Dunn's multiple comparisons) was performed when differences were statistically significant ($p<0.05$). All calculations were performed on GraphPad Prism Version 8.4.0 and data was expressed as mean values ± standard error of the mean.

Results

BPA exposure does not change the general health parameters of pregnant mothers exposed to BPA

No significant difference in body weight, waist circumference, food and water intake had been observed between control, vehicle and BPA exposed mothers (N=18) throughout pregnancy (Figure 1a–d), except for vehicle group which significantly consumed more food than BPA (23.5±0.8 versus 16.3±1.6 g; $p<0.05$) and control (23.5±0.8 versus 17.7±0.9 g; $p<0.05$) group on PD2. Systolic, diastolic and mean arterial pressure in BPA group were not significantly different than the other control groups (Figure 1e–f). However, vehicle group showed significantly lower systolic (112.3±4.1 versus 143.5±8.4 mmHg; $p<0.05$) and diastolic (90.7±3.3 versus 113.7±6.3 mmHg; $p<0.05$) BP than control on PD7. Additionally, number of male and female offsprings delivered by the BPA group were not substantially different when compared with control and vehicle groups (Table 1).

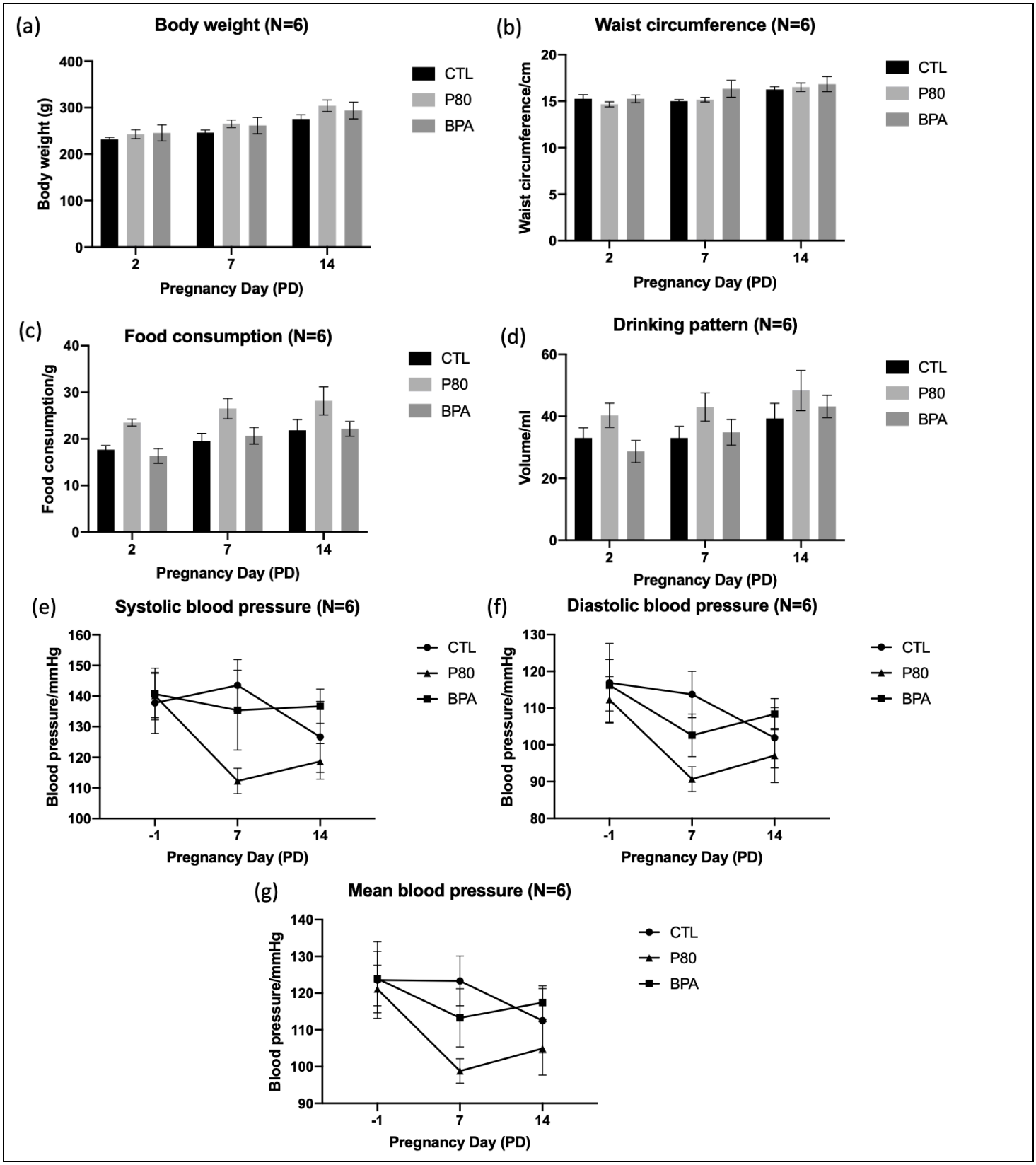

Figure 1. Physiological parameters for obesity in pregnant mothers: (a) body weight, (b) waist circumference, (c) food consumption, (d) drinking pattern, (e) systolic blood pressure, (f) diastolic blood pressure, and (g) mean arterial blood pressure. * CTL versus P80, $p<0.05$; # P80 versus BPA, $p<0.05$. The error bar represents the standard error of the mean for six rats from each group at each timepoint (N=6).

Table 1. Gender distribution of delivered offsprings.

Sex	CTL		P80		BPA	
	Mean	SEM	Mean	SEM	Mean	SEM
Male	4.7	0.3	5.2	0.9	5.3	0.3
Female	6.8	0.5	6.3	0.5	5.3	0.6

Transplacental transfer of BPA from mother to offspring

BPA was detected in plasma of both control and BPA exposed mothers and newborns as shown in Table 3. The median BPA levels in control mother, control newborn, BPA mother and BPA newborn were 0.690 ng/ml, 0.790 ng/ml, 0.738 ng/ml and 1.877 ng/ml, respectively (Table 3). Control newborns showed slightly higher BPA level than their mothers (1.070 ± 0.316 and 0.692 ± 0.050 ng/ml, respectively). Meanwhile, BPA newborns exhibited the highest concentration of BPA (14.031 ± 12.737 ng/ml).

Table 2. BPA levels found in the plasma of control and BPA exposed mothers and newborns.

Sample description	Mean concentration (ng/ml)
Control mothers	**0.692 ± 0.050**
Control newborns	**1.070 ± 0.316**
BPA mothers	**0.738 ± 0.021**
BPA newborns	**14.031 ± 12.737**

Table 3. Descriptive statistics of BPA in dams after spontaneous delivery and their newborns.

Sample	N	GM (GSD)	Percentile				
			Min	**25th**	**50th**	**75th**	**Max**
Control mother (ng/ml)	3	0.6883 (1.135)	0.6060	0.6060	0.6900	0.7800	0.7800
Control newborn (ng/ml)	3	0.9889 (1.602)	0.7200	0.7200	0.7900	1.700	1.700
BPA mother (ng/ml)	3	0.7377 (1.051)	0.7020	0.7020	0.7380	0.7750	0.7750
BPA newborn (ng/ml)	3	3.765 (8.094)	0.7200	0.7200	1.877	39.50	39.50

Maternal BPA exposure does not affect the small intestine morphology of neonates

No difference in the morphology and cellularity (in terms of inflammatory cells such as lymphocytes, plasma cells and eosinophils) had been observed between control, vehicle and BPA exposed neonates (Figure 2).

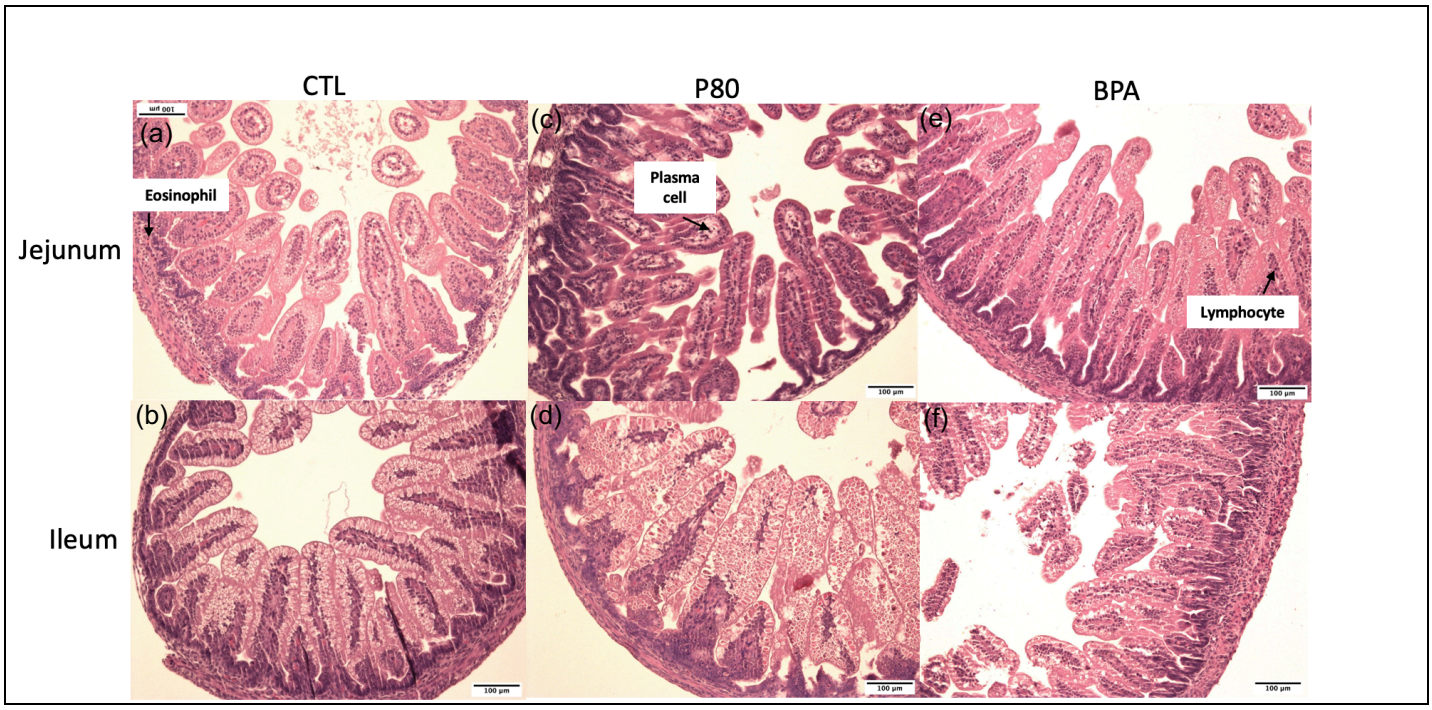

Figure 2. Representative H&E stained jejunum and ileum sections of neonates (PND0–2) from control (a,b), vehicle (c,d) and BPA (e,f) groups exhibit normal morphology and cellularity of the small intestine. (10x magnification, n=6 rats for each group)

Discussion

Many research groups have studied the detrimental impacts of developmental exposure to BPA on the gastrointestinal tract of adult offsprings [16-18, 28]. However, the maternal effects of BPA on the pregnancy and the small intestine of offsprings at birth are somewhat limited. As we know, BPA has been linked to metabolic diseases such as obesity and cardiovascular diseases in both human and animal models [29-34]. Pregnant mothers, in particular, are more susceptible to BPA exposure since Mrp2 and UDP-glucuronosyltransferase, responsible for hepatic BPA glucuronidation, are downregulated during pregnancy [35]. Hence, we assessed the physiological parameters of the pregnant mothers to observe how short-term BPA exposure during pregnancy influences a mother's general health. Interestingly, body weight, waist circumference, water intake, and blood pressures of BPA mothers throughout pregnancy were not statistically significant compared with control and vehicle mothers, consistent with previous findings [36-38]. These results might implicate that BPA exposure has no obesogenic influence on pregnant mothers. Other than that, our results revealed no significant difference in the male to female pups ratio, which is consistent with previous data [39-41]. Nevertheless, some studies have reported perturbed embryonic implantation and pregnancy establishment upon BPA exposure on the uterine tissue, and substantial reduction in the ratio of male to female pups [37, 42, 43].

Slightly higher BPA level in control neonates compared to their mothers may indicate a different metabolism rate of BPA in neonates compared to adults, as demonstrated by previous literature [44-46]. In parallel to Tanaka et al.'s finding on foetal serum BPA concentration after prenatal exposure to the contaminant [47], we also found that BPA neonates have marginally greater BPA level than control neonates. The comparable levels of BPA demonstrated by the control and BPA mothers in our study is consistent with a recent finding [48]. On the other hand, the highest concentration of BPA displayed by BPA neonates when all groups were compared may imply that BPA consumed by the mothers during pregnancy had been passed to their offsprings, as suggested by previous studies [19, 20].

We hypothesized that there might be some anatomical changes to BPA offsprings' gastrointestinal tract,

which could be one of the adverse effects of developmental exposure to BPA (as reflected in their higher plasma BPA level compared to BPA mothers and control pups). For this reason, we performed a histopathological examination of the small intestine (jejunum and ileum) by H&E staining. The small intestine was chosen as our organ of interest because other than its nutrient digestion and absorption function, it also forms a physical and chemical barrier that restricts harmful substances from entering the bloodstream [49, 50]. However, since it is the first site where consumed BPA is absorbed, we believe that the small intestine would be more susceptible to the detrimental impacts of the endocrine-disrupting chemical. Proliferation and differentiation of the intestinal epithelial cells (IECs) could be assessed by villus height and crypt depth measurements [18, 51]. The latter parameters may also indicate the intestinal capacity to digest and absorb nutrients, whereby this capacity would be reduced if IECs proliferation and differentiation were inhibited. Our findings suggested that the inflammatory response and general properties of BPA neonate's small intestine, such as villi length and width, were not substantially different from that of the control and vehicle pups, as reported previously [18]. In contrast, other studies demonstrated focal neutrophilic and eosinophilic infiltration in the colonic lamina propria [52], and a decrease in the jejunum villus height to crypt depth ratio in BPA-exposed offsprings [18]. Meanwhile, adult rats chronically exposed to BPA displayed villous atrophy, hyperplasia of lamina propria, epithelial necrosis, and lesions in the submucosa and mucosa [53]. Nevertheless, we were unfortunate for not being able to measure the general intestinal parameters due to some technical mistakes during organ acquisition of several samples. The mishaps happened because a rat newborn's small intestine is extremely fragile and small, thus requiring extra handling care.

One of the limitations to our study is that we did not observe BPA impacts on the intestine at different stages of offspring's life, such as weaning and adulthood, as conducted in previous studies [16-18, 52]. Perhaps, the effects of foetal exposure to 5mg/kg/day BPA would only manifest at the later part of life. Other than that, a plausible explanation for all of our nonsignificant findings is because of the non-monotonic dose-response relationships exhibited by BPA [54-57]. In one experiment, low concentrations of BPA (≤0.5 mg/kg/day) are more competent at facilitating weight gain and adiposity than high concentrations (>5 mg/kg/day) [40]. In another study, low BPA dosage (50 µg/kg) significantly altered the methylation of *Slc22a12* (linked to metabolic syndrome and obesity in Caucasians when single nucleotide polymorphisms are present in the gene), which was consistent with the strong enrichment of metabolism-related genes [58]. Importantly, the Lowest Observed Adverse Effect Level (LOAEL) of BPA established by the US Environmental Protection Agency was 50 mg/kg/day [54]. Nonetheless, a lot of research has reported adverse impacts of BPA on the gastrointestinal tract at doses lower than LOAEL [16, 52, 59-62], hence why we selected a BPA dose ten times lower than LOAEL (i.e., 5 mg/kg/day). Regardless, an investigation on BPA's effects on pregnancy and the gastrointestinal tract of juvenile offsprings at multiple concentrations and an analysis of its dose-dependence would bring greater benefits to the current understanding.

Conclusions

Our study demonstrated that exposure to BPA during pregnancy may not have obesogenic impacts on pregnant mothers and may not affect the small intestine morphology of neonates. Nevertheless, our future efforts will include how gestational BPA exposure would influence the development and health of offsprings later in life via the intestinal-related mechanisms, and how multiple doses of BPA would affect the different parameters measured.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Funding statement

This work was funded by Fundamental Research Grant Scheme [600-IRMI/FRGS 5/3(387/2019)] and Fundamental Research Grant Scheme for Research Acculturation of Early Career Researchers [600-

IRMI/FRGS-RACER 5/3(008/2019)] from Ministry of Higher Education, Malaysia.

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