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The Impact of Bisphenol A Exposure during Pregnancy on the Heart of Mother and Fetal Rats

(Kesan Pendedahan Bisfenol A kepada Hati Ibu dan Fetus Tikus semasa Bunting)

ZATILFARIHIAH RASDI[1], ROZIANA KAMALUDIN[2], SITI HAMIMAH SHEIKH ABDUL KADIR[3,4,*], MOHD DANIAL MOHD EFENDY GOON[3,4], JESMINE KHAN[3], SHARANIZA AB. RAHIM[3] & MOHD HAFIZ DZARFAN OTHMAN[2]

[1]*Centre of Preclinical Sciences Studies, Faculty of Dentistry, Universiti Teknologi MARA, Cawangan Selangor, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia*
[2]*Advanced Membrane Technology Research Center (AMTEC), School of Chemical and Energy Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor Darul Takzim, Malaysia*
[3]*Department of Biochemistry and Molecular Medicine, Faculty of Medicine, Universiti Teknologi MARA, Cawangan Selangor, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia*
[4]*Institute for Pathology, Laboratory and Forensic Medicine (I-PPerForM), Universiti Teknologi MARA, Cawangan Selangor, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia*

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ABSTRACT

In utero bisphenol A (BPA) exposure has been reported to increase the risk of cardiovascular disease (CVD) in adult life. Thus, this study aimed to investigate the impact of *in utero* BPA exposure on proteins expression related to cardiac function in heart of rat foetuses (*Rattus norvegicus*). In here, pregnant rats were divided into tween-80 (vehicle control), 0.05 mg/mL and 0.2 mg/mL BPA via drinking water for 19 days: from pregnancy day 2 till 21. Caesarean section was conducted on pregnancy day 21 to collect plasma and heart of both mother and foetuses. BPA-exposed pregnant rats showed significant increase in blood pressure (BP) and reduction in glycogen content ($p<0.05$) in comparison to control pregnant rats. Remarkably, reduced expression of cardiac troponin I (cTnI) and redistribution of alpha fetoprotein (AFP) expression were in foetus of BPA-exposed mother in comparison with foetus of control mother. Hypoxia induced factor-1 alpha (HIF-1α) expression was elevated in BPA-exposed foetal heart compared to the control. The findings in here suggest the risk of *in utero* BPA exposure on both foetus and mother, which may increase the risk of CVD in later life by altering the expression of protein crucial for heart development and function.

Keywords: Bisphenol A; cardiovascular disease; foetus; prenatal; protein expression

ABSTRAK

Pendedahan bisfenol A (BPA) *in utero* dilaporkan mampu meningkatkan risiko penyakit kardiovaskular (CVD). Penyelidikan ini dijalankan untuk mengkaji impak *in utero* apabila didedah kepada BPA dari segi ekspresi protein berkait dengan fungsi kardiak dalam jantung fetus tikus (*Rattus norvegicus*). Tikus bunting telah diberi tween-80 (kawalan pembawa), 0.05 mg/mL atau 0.2 mg/mL BPA melalui air minuman selama 19 hari; dari hari kedua bunting hingga hari ke 21. Pembedahan Caesarean dijalankan pada hari bunting ke-21 untuk memperoleh plasma dan jantung daripada fetus dan ibu tikus. Tikus bunting terdedah kepada BPA menunjukkan kenaikan tekanan darah dan penurunan kandungan glikogen yang ketara ($p<0.05$) berbanding kumpulan kawalan tikus bunting. Didapati ekspresi aruhan hipoksia faktor-1 alfa (HIF-1α) meningkat dalam jantung fetus yang terdedah dengan BPA berbanding kumpulan kawalan. Hasil kajian menunjukkan risiko pendedahan BPA dalam rahim ke atas fetus dan ibu tikus boleh meningkatkan risiko penyakit jantung terutamanya dengan peningkatan umur melalui peningkatan ekspresi protein yang penting dalam perkembangan dan fungsi jantung.

Kata kunci: Bisfenol A; ekspresi protein; fetus; penyakit kardiovaskular; pranatal

Introduction

The findings on the prevalence of metabolic diseases development in the offspring have been reported recently. Furthermore, epidemiological and animal studies indicated that the physiology of an individual's body systems is strongly correlated with the influence of intrauterine conditions. Close relationship between maternal diet and changes in the foetus developing system have been reported in the Barker hypothesis. Malnutrition and unfavourable intrauterine environment are hypothesised as the cause of permanent changes in both the structure and function of the organ of the offspring (Barker & Osmond 1986). Foetus may undergo restriction or adaptation to survive during developmental programming, thus lead to alteration in the epigenome through DNA methylation, histone methylation/acetylation and microRNAs (miRNAs) expression. Findings on the restriction of foetal growth based on the weight at the gestational age showed a strong association of *in utero* growth with the increment of CVDs risk (Palinski & Napoli 2008).

Recently, a few studies reported that improper foetal growth (Zanto et al. 2011) increases the risk of developing certain diseases such as type 2 diabetes, obesity, hyperlipidaemia, and cardiovascular problems (Wang et al. 2014). A study reported the differences of ventricles thickness in two different maternal diet groups of control and undernourished (Gray et al. 2014). The left ventricles of an offspring from undernourished mother displayed thicker walls compared to the control, which shows the probability of offspring developing hypertrophy later in life. These observations have led to a term known as 'intrauterine programming'. However, the effect of intrauterine exposure to the insult is determined by the timing, duration and the type of insult (Chen & Zhang 2011).

Endocrine disrupting chemical (EDC) has been detected in almost every environmental media such as in food sources and water. The exposure of EDC such as bisphenol A (BPA) has been shown to impact the infants and early childhood growth as well as reproductive tract development (DiVall 2013). A low level of BPA exposure on the developing foetus has been demonstrated to affect the development of several organs such as heart (Fern et al. 2015), brain (Nakamura et al. 2010), liver (Hijazi et al. 2015) and lungs function (Vanderberg et al. 2012).

Exposure to BPA during prenatal and lactation phase increased the level of serotonin and dopamine in a certain part of brain regions of adult mice, suggesting the impact of BPA towards neurotransmitter system in the brain (Nakamura et al. 2010). In addition, myosin heavy chain 6 (*myh6)*, cardiac myosin light chain 2 (*cmlc2)*, ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2b (*atp2a2b)* and sex determining region Y-box 2 (*sox2)* that are involved in cardiac embryo development were observed to be significantly downregulated in the offspring of paternal BPA-exposed F1 generation (Fern et al. 2015). As the foetal cardiac development is suggested to be altered after being exposed to BPA, there is a high probability of structural and signalling changes would also occur.

The high detection of BPA in the environment proposed to be a risk to public health. BPA is ubiquitous in many consumer products such as baby feeding bottles, plastic food containers and tableware, material in food and beverages lining cans, toys, dental and medical equipment, food packaging and as a material for water pipes (Bondesson et al. 2009). BPA levels have been detected in the umbilical cord, foetal cord serum and amniotic fluid. Results from those studies indicated that BPA could cross the placenta and potentially affect the developing foetus. Therefore, BPA adverse effects should be of greater concern in the developing foetus. Multiple studies in the rodents and primates have demonstrated the detrimental effects of BPA exposure on the development of the foetal organs 'programming'. Moreover, the doses which are currently considered safe for human intake for a tolerable diet intake (TDI) below 50 mg/kg body weight/day (Vanderberg et al. 2012) has been reported to have significant impacts on the foetal organs programming. Several studies reported that long term exposure of BPA to be associated with the onset of CVDs, which is currently at the age between 55 and 65 years old (Allport et al. 2016). Recent studies showed that daily BPA exposure to pregnant primates altered the foetal genes that are important in cardiac pathophysiology and this will impact the cardiovascular fitness during the offspring life including *Myh6* and A Disintegrin and Metalloprotease 12 (*Adam12-l*) (Chapalamadugu et al. 2014). In addition, further experimental studies suggested that even at a low dose of BPA could affect the function of the cardiovascular system promoting abnormal activities of the heart. Although these findings are still limited, it suggests that BPA exposure may contribute to an increased risk of CVDs. Three important miRNAs were reported to be significantly altered in heart of foetuses of mother-exposed to BPA. Those miRNAs are miR-208a-3p, miR-17-5p and miR-210-3p. Following the findings, cTnI expression is suggested to be related with

miR-208a-3p, while AFP expression interrelated with miR-17-5p and HIF1α expression could be observed in relation with miR-210-3p (Białek et al. 2015; Corsten et al. 2010; Vettori, Gay & Distler 2012; Huang et al. 2009). Therefore, this study was conducted to investigate the impact of BPA exposure on the expression of proteins important for cardiac development and function in both mother and foetuses.

Materials and Methods

ANIMAL WORK

All the experiments involving animal handling were conducted according to the Guiding Principle of Care and Use of Laboratory Animals. This study was approved by UiTM Animal Care Unit Committee (ACUC) under protocol approval number; UiTM CARE: 222/7/2017 (8/12/2017). Sample size determination was based on the law of crude method of the diminishing return (Charan & Kantharia 2013). Three groups of investigations were chosen and five rats (*Rattus norvegicus*) in each group to investigate the effect of different dosage of BPA in comparison with control.

The animals used were 5-6 weeks Sprague Dawley rats (*Rattus norvegicus*), with average weight at 200-250 g. They were obtained from Chenur Supplier Enterprise, Selangor, Malaysia. Animals were transported in air-conditioned van to reduce the effect of rat stress responses from Seri Kembangan, Serdang to LACU, UiTM Sg. Buloh (30 minutes to 1 hour). In addition, animals were caged in 1088 cm^2 size cages with food and water. The rats were transferred to 432 cm^2 size cages in the quarantine room of the animal house (LACU) for a week. After mating, the positive mating female rats were housed individually under standard conditions (22 °C, 12-h light-dark cycle), with free access to food and water. Maternal food intake, volume of water consumed, and body weight gain were measured daily. On pregnancy day (PD) 21, blood was drawn from abdominal vein of pregnant rats to measure plasma BPA level and caesarean section was performed with anaesthesia by intraperitoneal injection of pentobarbital. All animals were sacrificed during the caesarean section procedure.

Fifteen Sprague-Dawley rats (*Rattus norvegicus*) were purchased at aged between 150-180 days. Females were mated with male rats in a ratio 2:1 in a cage. Vaginal smears procedure was done as described earlier, and that day was noted as pregnancy day (PD) 1 once sperm was observed. The pregnant rats were randomly divided into 3 groups: Group 1 (vehicle control group; contained 0.4% Tween-80), Group 2 (0.2 mg/mL RO containing BPA water), and Group 3 (0.05 mg/mL RO containing BPA water). They were given vehicle and BPA water according to the respective group. Water bottles and cages made of BPA-free components were used for all these studies to avoid potential contamination from sources other than administered drinking water. Maternal food intake and body weight gain were measured at PD2, PD7, PD14 and PD21. The pregnant rats were weighed using weighing scale (CAMRY, China). They were housed individually under standard conditions (22 °C, 12-h light-dark cycle), with free access to food and water to avoid interruption in normal animal physiology and behavioural patterns as both highly dependent upon light. Pregnant females were administered with RO water containing BPA via drinking water beginning on PD2 until PD21 with a dosage approximately of 5 and 20 mg/kg bodyweight/day. The dosages of BPA given were approximately 5 and 20 mg/kg bodyweight/day. This dosage was chosen as the dosage were said to be lower than the current accepted no observed adverse effect level (NOAEL) and low observe adverse effect level (LOAEL), respectively (Chapin et al. 2008; Doerge et al. 2011). Tween-80 was used as vehicles in *in vivo* study based on preliminary investigation that was done previously. In this study, Tween-80 was used as vehicles in concentration of 0.4% of total volume, which is lesser than previously reported by (Zhang et al. 2003). In cardiac tissue, glycogen is a quick source of glucose for metabolic homeostasis. However, excessive release of glycogen in cardiac tissue may impair cardiac structure and physiology.

BLOOD PRESSURE READINGS

Blood pressure (BP) readings were included to investigate the adverse effect of BPA exposure during pregnancy in increasing risk of hypertension. BP readings were taken twice (PD2 and PD18) throughout the treatment period. Few studies reported BPA exposure increased the BP readings especially systolic BP which surges the possibility of hypertension development. In this study, BP measurement of pregnant rats was conducted using volume pressure recording (VPR) sensor technology. The rats were put in a proper size strainer/holder with the nose protruded through the front of nose cone to allow comfortable breathing while the tail was fully extended and exit through the rear hatch opening of the holder. Prior to procedure, the strainer/holder was put inside the cage to let the rats familiarised with it, thus

avoiding stress factor to the rats which might influence the readings. The strainer then was put on the animal warming plate which has been heated earlier. The warming block may not harm the animals sufficiently in a room below 26 °C and especially when placed the animal in restrainers supplied with spell (CODA) unit. This procedure improved tail blood flow and BP readings. Slide the occlusion cuff as near the base of the tail as possible without force. Forcing should be avoided as it would cause occlusion of vessels and resulted in poor BP readings. The VPR cuff was slide up the tail with the larger end first, until reaching the occlusion cuff. The blood flow and blood volume in the tail were measured by VPR system and BP readings were generated simultaneously. The BP readings (both systolic and diastolic) and heart rate were measured for 20 cycles. The recommended readings were chosen from cycles 11th onward as the 1st until 10th were considered as acclimation cycles especially for unanesthetized rats. Minimum tail volume was tested as 15 by default and the run-time was set at 20 s to obtain best results. The cycles began after clicking 'Finish' at the software.

ANALYSIS OF BPA IN PLASMA SAMPLES USING GC-MS

Three mL of mothers and one mL foetuses blood were collected during caesarean section (n=3). The total bloods were allowed to separate by centrifugation, at 3000 rpm for 3 min. After that, the plasma was transferred to a new 2 mL Eppendorf tube and stored at -20 °C until further use. Plasma of all collected blood was used to measure level of BPA compound. Briefly, sample was added with 5 ng of BPA-d16 before loading on the conditioned cartridge (Supelclean LC-Alumina-B SPE tubes, USA). Two mL methanol was allowed to pass through the cartridge to precondition it and followed by 2 mL phosphate buffer pH 2, 0.04 M. Plasma of all collected blood was loaded at 1 mL/min. The cartridge was then washed with 2 mL of phosphate buffer pH 2, 0.04 M and dried with vacuum pump for 10 min. BPA was then eluted with 3 mL of dichloromethane: ethyl acetate (1:1). Following elution, the collected BPA was dried with vacuum pump for 3 min. The sample collected was then injected to the gas chromatography mass spectrophotometry (GC-MS) for analysis. The standard concentration of BPA was prepared in a range from 0 to 50 ng/mL BPA. GC-MS was set at initial temperature of 50 °C (hold for 2 min). Further increase of 20 °C/min was set until the temperature reached 100 °C. Then, the temperature allowed to increase in every 10 °C/min until it reached 200 °C. Finally, at a rate of 20 °C/min was arranged until the temperature reached 250 °C. The injector port, interface temperature was set at 300 °C and 270 °C, respectively. Column pressure was set at 40 kPa for 5 min and allowed to increase every 2 kPa/min until the pressure reached 70 kPa.

GLYCOGEN ANALYSIS

Glycogen analysis was carried out using glycogen assay kit (Cayman, USA). Additionally, glycogen content in foetal heart was measured to elucidate the effect of prenatal BPA exposure on maturity of the heart (Dawes, Mott & Shelley 1959). The glycogen assay kit was composed of assay buffer, glycogen hydrolysis enzymes, developer, and 96-well plate. The glycogen standard supplied in the kit was prepared according to the table of instruction and the similar volumes (as sample, glycogen hydrolysis enzyme and developer) were added into the well. Frozen heart tissues were weighed and recorded. The tissues were minced into small pieces and homogenised in 2 mL diluted assay buffer. The mixture then centrifuged at 2673 rpm for 10 min in 4 °C to reduce the potential of glycogen degradation. The supernatant was transferred into new 2 mL tube and stored on ice. The samples in new tube were diluted in 1:10 or greater using diluted assay buffer. In 96 well plates, 10 µL samples were pipetted followed by 50 µL glycogen hydrolysis enzymes. Then, the plate was incubated at 37 °C for 30 min. After incubation, 150 µL of developer was added into the wells. The plate was covered and incubated again at 37 °C for 15 min. The plate was read using an excitation wavelength of 535 nm and an emission wavelength of 587 nm. The reading of the absorbance was done using micro plate reader Tecan Model M200 and observed with Magellan software version 6.5. The concentration of glycogen was calculated based on glycogen standard curve and weight of tissues.

IMMUNOHISTOFLUORESCENCE STAINING (IHF)

The heart was extracted during caesarean section on PD21 and fixed with formalin prior to tissue processing and staining. Part of the heart (the left ventricles) was chosen for the histological observation, where the heart was cut longitudinally. The part of heart was put on the tissues embedding cassettes prior to tissue processing. During tissue processing, the tissues were fixed with formalin then washed with xylene and ethyl alcohol (absolute, 90%, 80%, 70% and 50%). This procedure took place

for about 12 h. The next day, the processed heart tissues were embedded in paraffin. It was important to arrange the position of the tissue so that during sectioning, the desired layout was sectioned. The hearts were sectioned; 5-µm sections and transferred to microscope slides (Fisher Scientific, Whitby, ON). Sections were dipped in xylene and ethyl alcohol (absolute, 95%, 80%, 70% and 50%) for breaking down the waxes. Sections of heart tissues were blocked for 30 min with 1% horse serum in PBS then incubated at room temperature for 2 h with primary antibody of troponin (1:400), 4 °C overnight for α-fetoprotein (1:200) or 1 h at room temperature for HIF-1α (1:400). Sections were washed thrice with PBS for 5 min and secondary antibody incubation was carried out using 1:200 FITC or 1:500 goat anti-mouse Alexa Fluor 635. Subsequently they were washed thrice with PBS in 5 min. Tissues were counterstain with DAPI for 1 min, washed as previous. Tissues then were mounted with prolong gold antifade reagent and cover slipped prior to view under fluorescence microscope (Olympus, New York) and confocal microscope (Leica, UK).

STATISTICAL ANALYSIS

A statistical analysis was performed using SPSS ver. 20. The data were presented as mean ± SEM for replicates generate from independent experiments (n≥3). The results were checked for normality and analysed using non-parametric Kruskal Wallis test, one-way analysis of variance (ANOVA), Post Hoc modification for significant ANOVA analysis. The data is considered statistically significant at $p < 0.05$ or $p < 0.001$.

RESULTS

BLOOD PRESSURE

Blood pressure (BP) readings of non-productive mating rats were used as negative control. No significant differences in BP reading were seen between negative control and vehicle control in both BP readings. Moreover, no significant differences in BP were seen from pregnancy day (PD) 2 (115/75 mmHg) to PD18 (125/92 mmHg) in control group. There were significant changes ($p<0.05$) in diastolic blood pressure (DBP) and systolic blood pressure (SBP) (Figure 1) of high dose of BPA-exposed groups in both PD2 and PD18, when compared with control. In DBP, a significant difference was observed in PD18 of 0.2 mg/mL (59%) compared with control group (20%). Moreover, DBP reading were increased from PD2 (71.00 ± 2.91 mmHg) to PD18 (113.00 ± 2.54 mmHg) in 0.2 mg/mL BPA-exposed group ($p=0.009$). No significant differences in DBP were observed between PD2 and PD18 in 0.05 mg/mL BPA-exposed group and the control groups. As for SBP, the results indicate that SBP significantly increased during pregnancy in 0.05 mg/mL and 0.2 mg/mL BPA-exposed rats compared with the control rats ($p<0.05$). The SBP during PD18 were higher in the 0.2 mg/mL BPA-exposed rats compared to SBP reading in control rats (10%) which were 59% differences ($p=0.009$). Furthermore, SBP reading were increased from PD2 (94.00 ± 7.62 mmHg) to PD18 (151.00 ± 4.50 mmHg) in 0.2 mg/mL BPA-exposed group ($p=0.009$). For other groups, no significant differences in SBP were observed between PD2 and PD18.

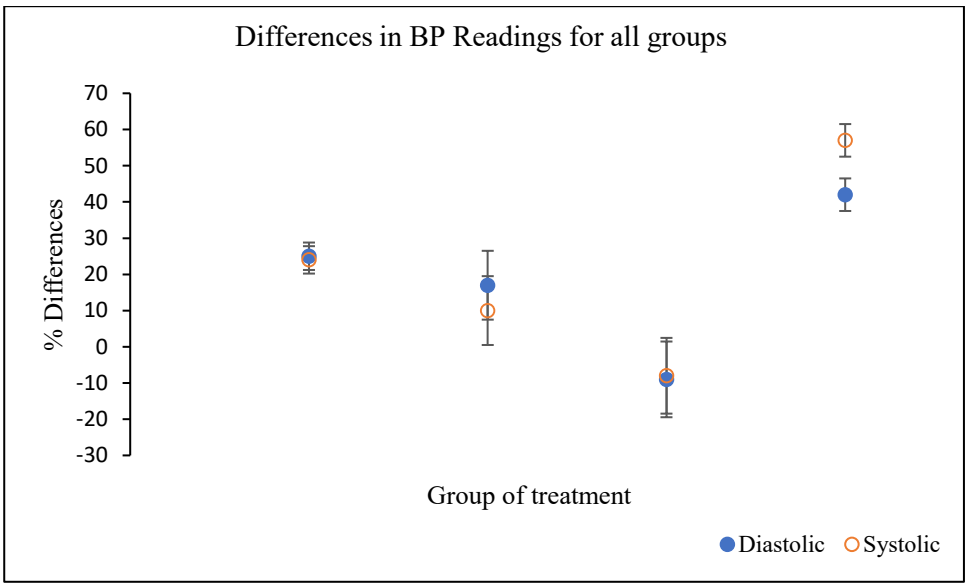

FIGURE 1. Changes of DBP and SBP value in BPA-exposed pregnant rats. Significant increase of DBP and SBP were seen in both BPA-exposed groups compared to BP readings in control group (* denotes $p<0.05$, ** $p<0.01$ on the BPA-exposed versus control pregnant rats' group, n=3-5) (# denotes $p<0.05$ on the changes of BP readings in PD2 to PD18 of 0.2 mg/mL BPA-exposed pregnant rat)

LEVELS OF BPA IN MOTHER AND FOETAL PLASMA

The level of BPA in plasma for both mother and foetuses were measured using GC-MS. Figure 2 shows the range of BPA level detected in both mother and foetuses. BPA was not detected in any of the plasma of control pregnant rats and foetuses. No significant differences were observed in plasma of pregnant rats between control and the two BPA-exposed rats. The plasma BPA level was significantly increased in foetuses exposed to 0.2 mg/mL BPA ($p=0.05$) compared to control, however no BPA was detected in the foetus plasma of 0.05 mg/mL BPA-exposed mother.

GLYCOGEN CONCENTRATION IN BPA-EXPOSED FOETAL HEART

Glycogen content in all heart tissues from control and BPA-exposed groups were quantitated. Figure 3 shows reduction in glycogen for both foetal hearts of BPA-exposed mothers when compared to control hearts. However, only heart of 0.2 mg/mL BPA-exposed mother showed significant reduction ($45 \pm 0.25\%$) in glycogen level ($p=0.047$) compared to control.

EXPRESSION OF CARDIAC TROPONIN I, α FETO-PROTEIN AND HIF1α PROTEIN IN BPA-EXPOSED FOETAL HEART

In here, expression of cardiac troponin I (cTnI), α Feto-protein (AFP) and hypoxia inducible factor 1-alpha (HIF1α) protein in foetal heart of control and BPA-exposed mother were determined by immunohistofluorescence. In the immunohistofluorescence photomicrographs, DAPI (blue stained) was used to stain the nucleus while FITC (in green) stained the protein of interests. Figure 4 shows expression of cTnI protein in all groups. In control group, normal expression and distribution of cTnI were observed. However, the expression of cTnI were reduced and appeared to lose the normal distribution in both foetal hearts from BPA-exposed mother compared to foetal hearts from control mothers.

AFP are highly expressed in developing foetuses liver, thus in here the foetus liver tissues were used as positive control. As expected, high expression of AFP was observed in the foetuses' liver tissue. In heart, foetal heart from control group showed less prominent and well distributed expression of AFP (Figure 5). Meanwhile, foetal heart of BPA-exposed mother 0.05 mg/mL

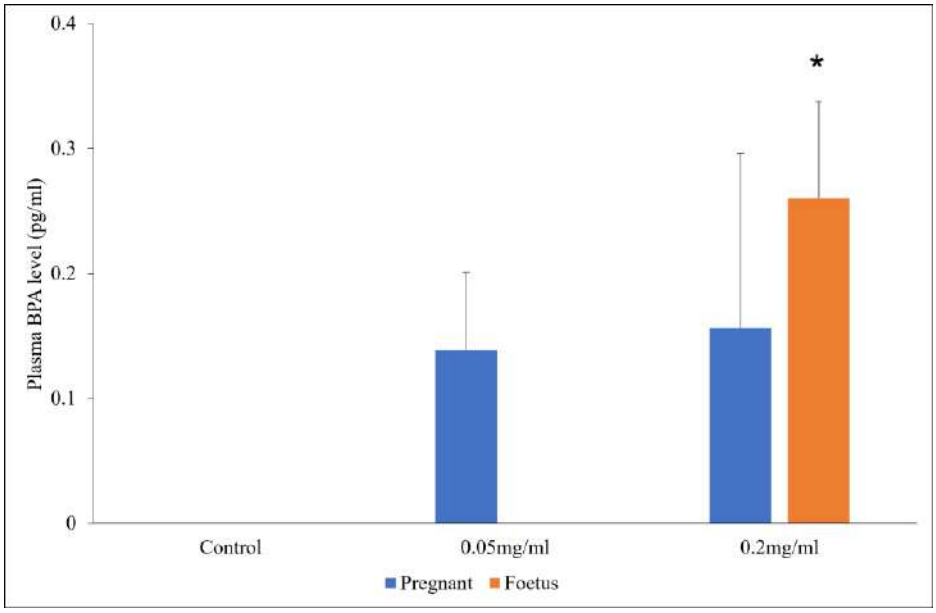

FIGURE 2. Histogram of BPA level detected using GC-MS in BPA-exposed pregnant rats and foetuses. No significant differences in BPA level were detected in the two BPA-exposed pregnant rats with control pregnant rats. Significant increase in BPA level was detected in 0.2 mg/mL BPA-exposed foetuses when compared to control. No BPA was detected in foetuses exposed to 0.05 mg/mL BPA (* denotes $p<0.05$ on BPA-exposed foetuses compared to control, $n=3$)

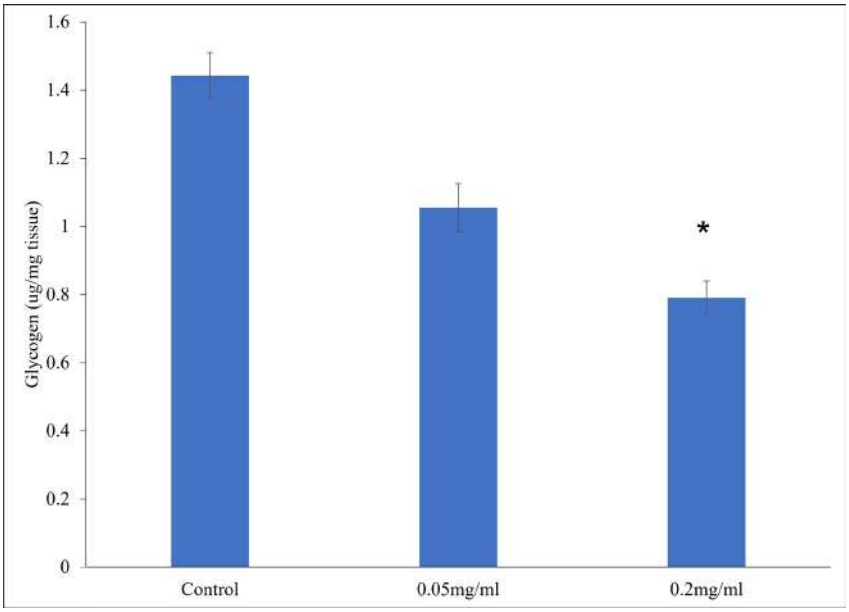

FIGURE 3. Histogram of glycogen level measured in foetal hearts of BPA-exposed mother. Significant reduction in glycogen level of foetal hearts exposed to 0.2 mg/mL BPA were detected compared to control group (* denotes $p<0.05$ compared to control, n=5)

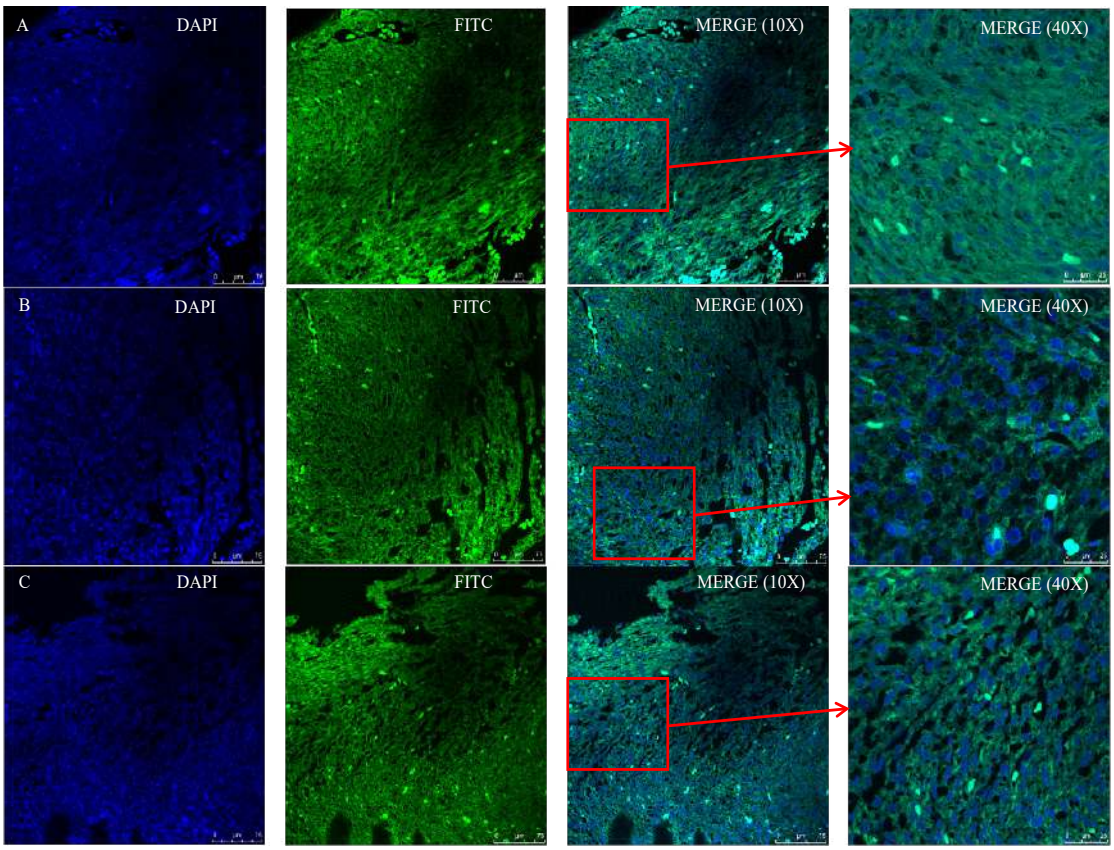

FIGURE 4. Representative of histological slide sections of immunofluorescence staining of foetal heart for cardiac troponin I expression. A - Control foetal heart, B - foetal heart of 0.05 mg/mL BPA-exposed mother, C - foetal heart of 0.2 mg/mL BPA-exposed mother, stained with DAPI and FITC. ZF 2.6 (n=8). Scale bar 25 µm (DAPI, FITC and Merge 10x) and 75 µm. FITC: fluorescein isothiocyanate (stained in green), DAPI: 4′,6-diamidino-2-phenylindole (stained in blue)

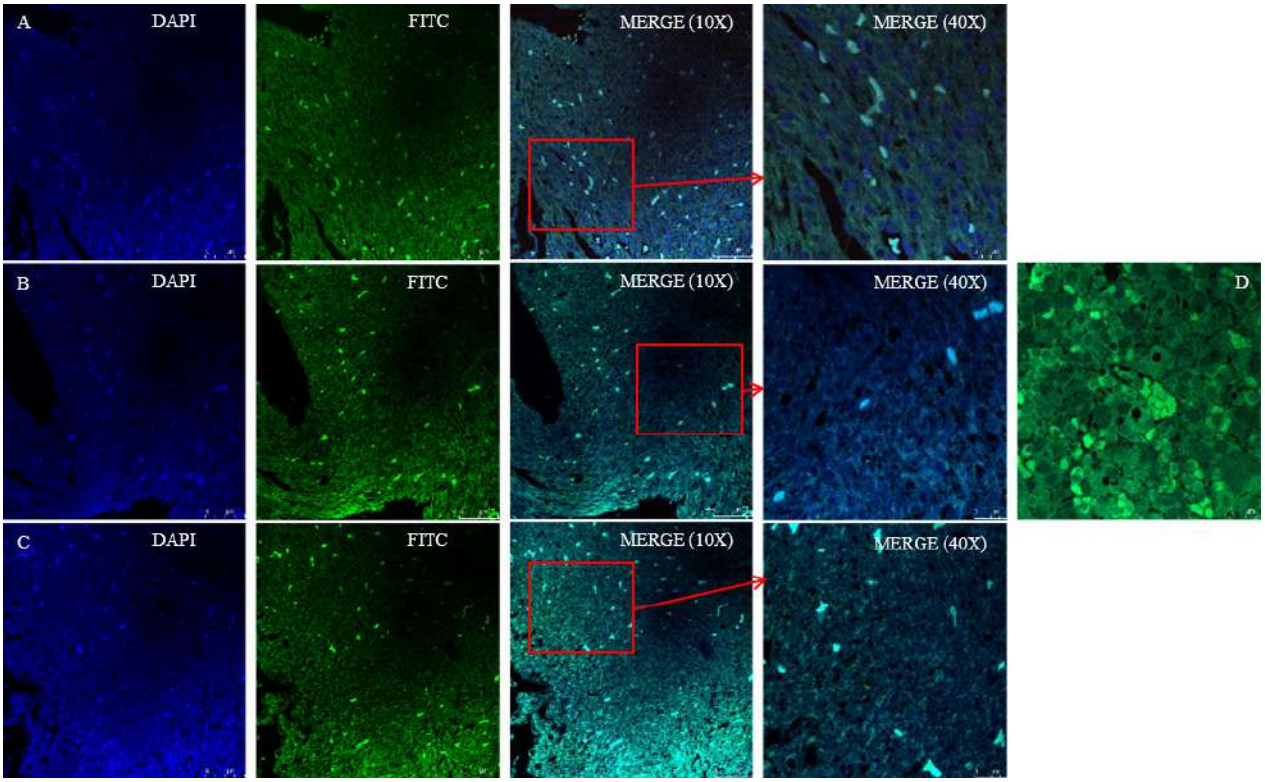

FIGURE 5. Representative of histological slide sections of immunofluorescence staining of foetal heart for AFP expression. A - control foetal heart, B - foetal heart of 0.05 mg/mL BPA-exposed mother, C - foetal heart of 0.2 mg/mL BPA-exposed mother, stained with DAPI and FITC. ZF 2.6 (n=8). D is the positive control for AFP expression in foetal liver, stained with DAPI and FITC. 25 µm (DAPI, FITC and Merge 10x) and 75 µm. FITC: fluorescein isothiocyanate (stained in green), DAPI: 4′,6-diamidino-2-phenylindole (stained in blue)

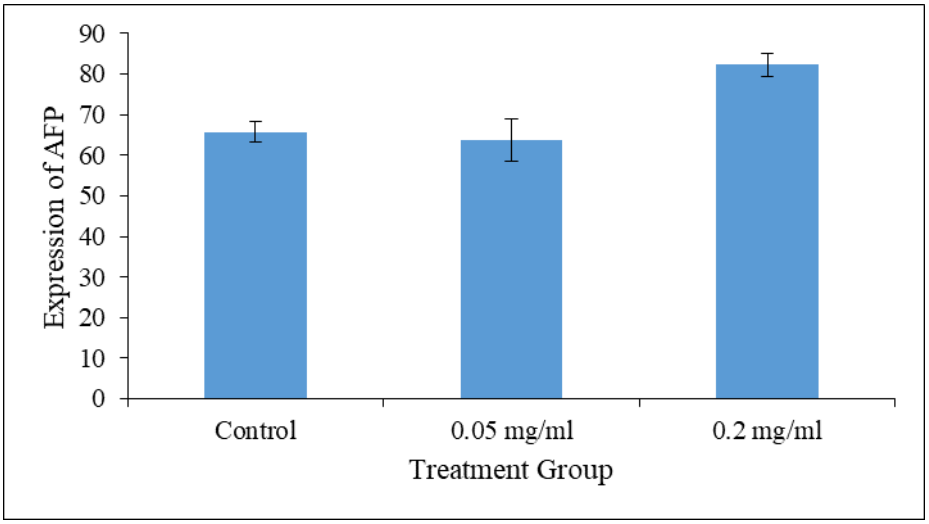

FIGURE 6. Histogram of AFP expression in foetal hearts of BPA-exposed mother. Significant increase in AFP expression of foetal hearts exposed to 0.2 mg/mL BPA were detected in comparison with control group (* denotes $p<0.05$ compared to control)

(64±5.12) and 0.2 mg/mL (82±2.95) showed an increased in AFP expression compared to control (66±2.56) (Figure 6). Nonetheless, the expression was more prominent in foetal hearts of BPA-exposed mother specifically in 0.05 mg/mL BPA and less distributed compared to control foetal hearts.

Expressions of HIF1α were also observed in foetal hearts (Figure 7). Interestingly, the expressions of HIF1α were significantly increased in foetal hearts of BPA-exposed mothers 0.05 mg/mL (65±2.63) and 0.2 mg/mL (81±3.86) in compared to control (36±2.07), the expressions of HIF1α were significantly increased in both foetal hearts of BPA-exposed mothers compared to control (Figure 8). Furthermore, HIF1α protein translocation from the cytoplasm to the nucleus was observed in both foetal hearts of BPA groups. The protein was well distributed in cytoplasm for control foetal heart, meanwhile in BPA-exposed mother, foetal heart showed less distribution of HIF1α expression.

DISCUSSION

The effect of BPA exposure on general health status of pregnant rats was represented by the blood pressure (BP) reading. There were studies reported on the increment of maternal weight during pregnancy in a range of 50 to 75 g, which suggested as healthy pregnancy in small rodents (Hain 1932; Paronis et al. 2015). In addition,

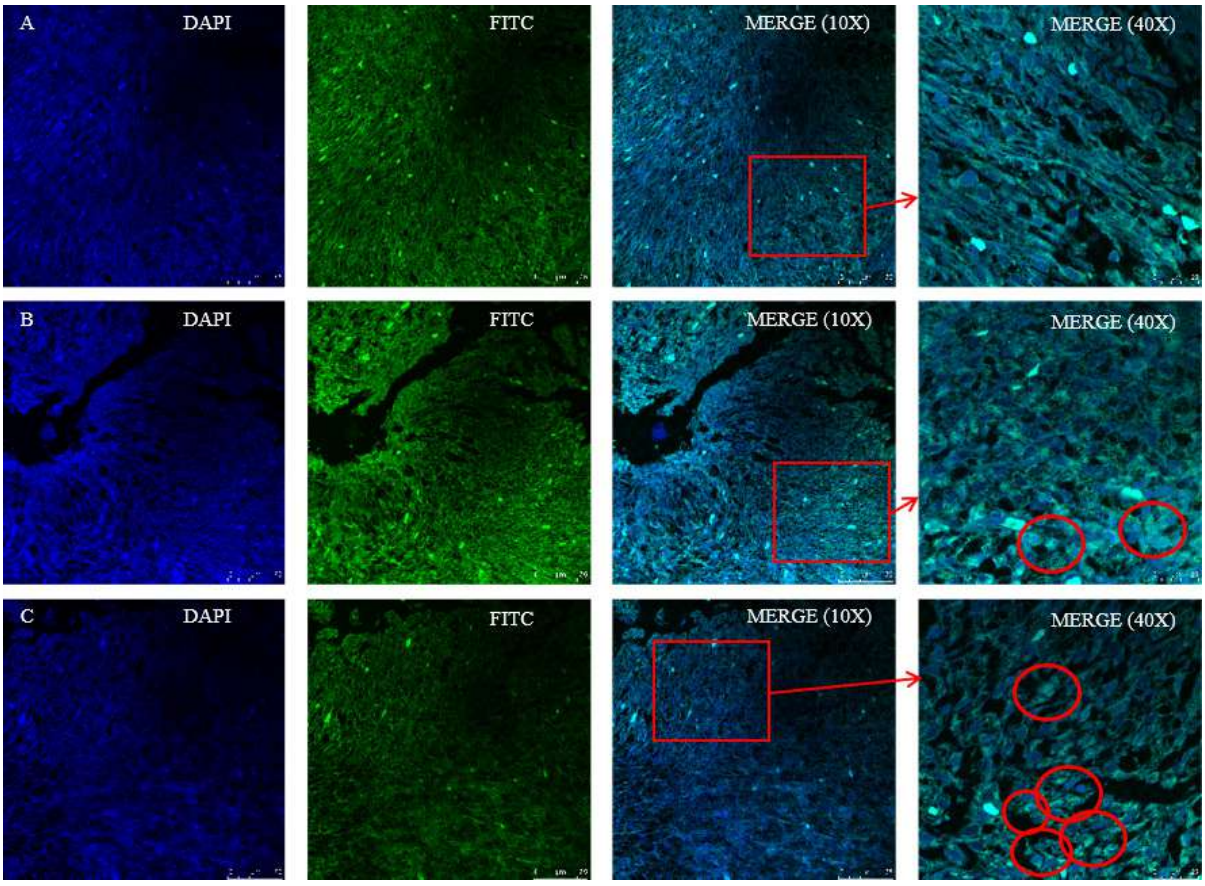

FIGURE 7. Representative histological slides sections of immunofluorescence staining of foetal heart for HIF1α expression. A - control foetal heart, B - foetal heart of 0.05 mg/mL BPA-exposed mother, C - foetal heart of 0.2 mg/mL BPA-exposed mother, stained with DAPI and FITC. ZF 2.6 (n=8). Red circle indicated the expression of HIF1α translocated in nucleus. Scale bar 25 µm (DAPI, FITC and Merge 10x) and 75 µm (Merge 40x). FITC: fluorescein isothiocyanate (stained in green), DAPI: 4′,6-diamidino-2-phenylindole (stained in blue)

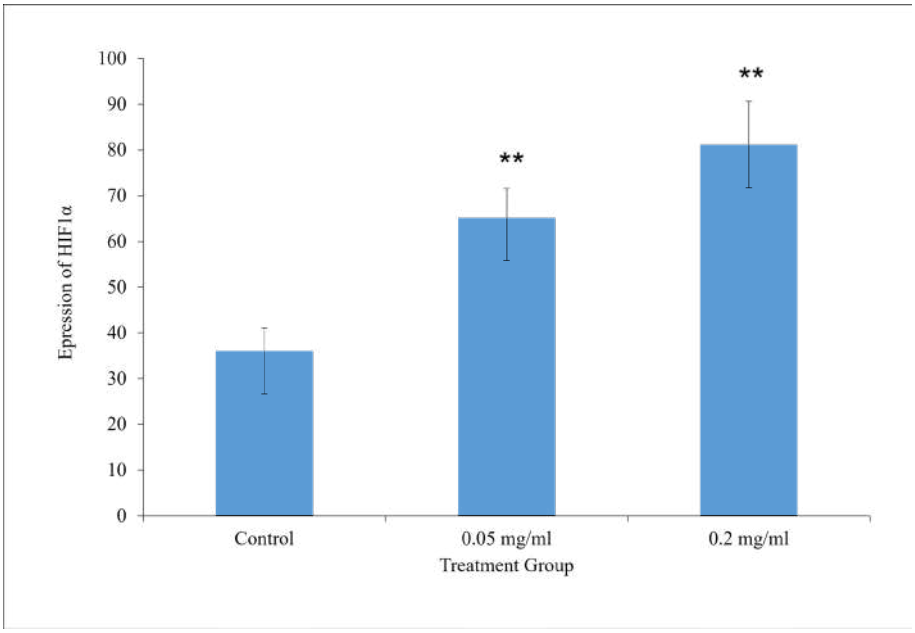

FIGURE 8. Histogram of HIF1α expression in foetal hearts of BPA-exposed mother. Significant increase in the expression of foetal hearts exposed to BPA were detected in comparison with control group (** denotes $p<0.01$ compared to control)

Corvino et al. (2015) documented their findings on physiological and biochemical measurements before, during and after pregnancy of healthy rats. In relation to that, the development of hypertension during pregnancy has been associated with the increased of body weight, a risk factor of CVD development (Nwachukwu et al. 2001). Hypertension could be an independent risk of CVD development. Hypertension or familiarly known as high blood pressure, occurs when there are greater forces of blood flow in blood vessels. Recent study has reported on the association of CVD and hypertension with BPA exposure. These diseases are very common chronic diseases in public health problem (Han & Hong 2016). The study was conducted via randomised clinical trial and clarified that CVD development has a positive relationship with raising level of blood pressure after being exposed to BPA. Surprisingly, the increment of uBPA was also found to be associated with hypertension. This author observed an increment in SBP of 4.5 mmHg after the exposure of BPA which is in parallel with our findings. In our study, a significant rise in BP readings were observed in both BPA-treated pregnant rats compared to control group. However, the association between BPA, hypertension and CVD is still unclear. It is in line with number of studies who reported on the prevalence of hypertension in BPA-exposed subjects among Korean (Bae et al. 2012), and United States adults (Shankar et al. 2012). Those findings suggested that BPA exposure may increase the risk of CVD that is mediated by raised blood pressure readings. Most of the studies reported the increment of blood pressure in SBP. Meanwhile, in this study, significant differences of SBP and DBP in pregnant rats exposed to BPA were observed. The systolic pressure is dependent on the cardiac output based on the volume of blood pumped in aorta when ventricles are contracted (Tin et al. 2002). Meanwhile, DBP is depended on the peripheral resistance, usually occurred when the capillaries constricted. Both changes indicated the disturbance in amount and pressure of blood flow in aorta and capillaries. As the cardiac output increases, the heart rate would eventually increase, thus, contributing to the raising of SBP. Meanwhile, capillaries constriction occurred when there is hypoxic condition or synthesis of endothelin in endothelium which documented to be associated with cardiac hypertrophy, heart failure and other cardiovascular disorders (Kawanabe & Nauli 2011).

In this study, BPA level in plasma of pregnant rats were detected in both BPA-exposed pregnant rats. However, non-significant differences between groups were observed. The level of BPA detected in the rats'

plasma was 1000 times lower than the BPA dosage given to them. These findings suggested that pregnant rats had metabolised BPA efficiently. There were number of studies have documented on detection of BPA in plasma of pregnant women which were highly variable (in a range of 0.3 to 29.4 ng/mL) (Chou et al. 2011; Ikezuki et al. 2002). Those reported values were higher compared with findings observed in this study as those were in humans. BPA metabolism is slower in rats than in humans (less than 6 hours) as BPA conjugation and excretion occur within 6 hours in rats. The differences could be due to the BPA glucuronide molecular weight (MW). In rats, BPA glucuronide MW is above the threshold (350 Da) of biliary elimination, while in humans the MW is below the biliary elimination in rats, thus resulting in delayed elimination of BPA through enterohepatic circulation in rats (Vo et al. 2002). BPA metabolism in foetuses is slower than pregnant mothers even at lower exposure level. Therefore, BPA is potentially more detrimental to foetuses than mothers (Martinez et al. 2017).

On the other hand, several studies reported on the detectable level of BPA in placenta, cord blood and human foetal plasma, thus, demonstrated BPA can cross the placenta and cause adverse effects to the offspring (Schönfelder et al. 2002; Tan et al. 2003). In here, the level of BPA was detected in foetus plasma of pregnant rats exposed to 0.2 mg/mL BPA group only and not detected in 0.05 mg/mL BPA group. There was lack of information reported on level of plasma BPA in foetus of rodent, however, study that was conducted by Yang, Doerge and Fisher (2013) explained the clearance of BPA in rat pups depending on the age of the pups. This was suggested based on the predicted values of maximum biliary excretion and enterohepatic recirculation of the pups itself. In this study, about 55% of BPA passing through from mother to foetus was observed based on the detected BPA plasma level. In foetus, low dose BPA (0.05 mg/mL) was unable to be detected due to below the limit of detection in GC-MS. In accordance, another study based on pharmacokinetic model predicted the factors that caused greater value of area-under-the-curve (AUC) in BPA plasma levels of pups' rat compared to adult (Yang, Doerge & Fisher 2013). Difference in both levels depends on the maximum hepatic reaction velocity and biliary excretion.

In relation to the presence of BPA in plasma, accumulation of BPA may interfere biological process during the development. Cardiac proteins play an important role for the heart to be well-functioned. During contraction, sarcomere is the main component for CMs contraction (Momtahan, Crosby & Zoldan 2019). Maturation of CMs is encouraged via sarcomere development by the expression of sarcomeric proteins such as actin, MHC and cardiac troponin (cTn). Currently, the best validated biomarker for heart failure or disease is cTn (Bialek et al. 2015). Troponin is a crucial regulator in cardiac muscle for contraction. It is a structural protein that can be found in the myocardium, at which the levels of cTn is used as indicators of myocardial injury, heart failure or AMI (Mushtaque et al. 2019; Ravichandran et al. 2019; Yan et al. 2020). cTn comprises of three different subunits; cTnI, C (cTnC) and T (cTnT) where each of the subunits has a specific role in the heart. As for today, the effect of BPA on cTnI has not been well reported. In here, we measured the expression of cTnI in foetal hearts of BPA-exposed mother as compared to control. Surprisingly, cTnI was poorly expressed in foetal heart of BPA-exposed mother in comparison with foetal heart from control mother.

Previous study has documented lung immaturity after being exposed to BPA prenatally (Hijazi et al. 2015). They observed a significant increase of cytoplasmic glycogen content in BPA-exposed foetal lungs (approximately 50% increment compared to control) which is a well-known marker of lung epithelial cell maturation. However, the effect of BPA in heart maturity is not well known. In contrast, our results found significant reduction in glycogen level in high dose compared to low dose of BPA and control group. For instance, study conducted by Chapalamadugu et al. (2014) is in parallel with our finding where they observed the reduced level of glycogen in foetal heart of BPA-exposed mother compared to control. Glycogen is an important fuel source during embryonic development as it provides energy sources during cardiogenesis (Pederson et al. 2004). Aerobic glycolysis and lactate oxidation are the main metabolic pathways in the developing heart (Ritterhoff et al. 2017). Although heart uses fatty acids as a primary substrate of energy production, in certain conditions such as CVD diseases, glucose is still utilised (Szablewski et al. 2017). Nevertheless, newborns adapt to anoxic conditions using stored glycogen as fuel substrate thus indicated higher glycogen level is crucial prior to birth. Moreover, the study conducted by Pederson et al. (2004) suggested lack of glycogen level could affect cardiac development and result in perinatal mortality. In addition, impairment in glycogen synthesis contributed to congenital heart disease. Earlier research found the importance of glycogen or carbohydrates in asphyxiated condition (Dawes, Mott & Shelley 1959).

Apart from glycogen, AFP is also an indicator for maturation of certain tissues such as lung, kidney, and pancreas including heart (Nahon et al. 1988). The study also documented AFP expression in the heart of foetal, newborn and adult. They reported the observation on the AFP expression only in foetal heart, and not in newborn and adult heart. Furthermore, several studies indicated the abnormalities in foetal development based on the levels of AFP in serum of pregnant mother (Adigun et al. 2019; De Mees et al. 2017). Besides that, AFP is also reported as a tumour-associated biomarker for pregnancy and adult clinical samples (Gj 2019). High levels of AFP are associated with neural defects while low levels of the protein expression are reported as an indicator of risk of Down's Syndrome (De Mees et al. 2017). Besides that, the study also pointed out the function of AFP, not only as a marker of developmental abnormalities in foetal and liver tumours but also as a control of female fertility via anti-oestrogenic action.

In here, we observed increased expression of AFP in foetal heart of BPA-exposed mother compared to control. However, they found that the expression of AFP is normally detected in foetal heart and lung, and not in neonatal and adult tissues. The observation clearly explained the presence of AFP in the developmental process of tissue maturation. Interestingly, our findings showed that BPA affects the maturation of heart development, thus supporting our previous results on glycogen level of BPA-exposed foetal heart. It has been reported the presence of AFP as cytoplasmic functional form and reside in liver cancer cells, spinal fluid, immature rodent uterus, and MCF-7 human breast cancer cultured cells (Gj 2019). In addition to that, a similar study stated that in the normal newborn or infant, AFP levels are significantly reduced and decline approaching adulthood (Gj 2019). The study then suggested that increased AFP expression indicated the immaturity of heart development in BPA-exposed offspring.

Furthermore, our previous findings on the altered miR-210-5p (Rasdi et al. 2020) and cTnI expression in BPA-exposed foetal heart are postulated to be associated with prenatal hypoxia. We investigated the expression of HIF-1α protein, which resulted in a significant increase of the protein in foetal heart exposed to BPA. There was study conducted on maternal chronic hypoxia, observed the decrease in cardiomyocytes proliferation and increased of collagen deposition in foetal and neonatal heart of hypoxic subject compared to control (Tong et al. 2011). Their findings are aligned with our H&E and MT staining observation which showed deposited collagen in the foetal heart of BPA-exposed mother (Rasdi et al. 2020).

Conclusions

This study demonstrated the effect of *in utero* BPA exposure to both mother and foetal heart. Our results suggested that BPA may impact foetal cardiac development and increase the risk of CVD development later in life. One of the limitations of this study is that the altered protein expression was observed only in foetus phase. Hence, further study is needed to investigate the targeted proteins and prolong the observation and treatment duration until postnatal day of the offspring to explore the risk of the offspring in developing CVD. Mechanisms underlying CVD development in BPA-exposed subjects remain debatable. Therefore, more extensive studies are needed to fully elucidate the mechanisms underlying the effect of in utero BPA exposure and risks of CVD development in development CVD during adult life.

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*Corresponding author; email: sitih587@uitm.edu.my