Dissertation for the Degree of Doctor of Philosophy

Exposure to bisphenol A during infancy including perinatal period and their association with oxidative stress

주산기를 포함한 영유아기의 BPA 노출 및 산화 손상과의 상관성

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By

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Major in Environmental Health,

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by

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지도교수 김 성 균

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2018년 8월

위 원 장	최 경 호	(인)
부위원장	이 기 영	(인)
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ABSTRACT

Exposure to bisphenol A during infancy including perinatal period and their association with oxidative stress

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Bisphenol A (BPA) is a chemical widely used in numerous consumer products, including baby bottles, reusable water bottles, food container, stretch films, papers, and cardboard. Therefore, BPA has been detected in a variety of environmental samples (i.e., water, air, and dust), foods samples (i.e., plastic container and can linings), and biological samples (i.e., serum, urine, placenta, breast milk, and amniotic fluid). The animal and human studies suggested that BPA could exhibit other modes of endocrine disruption such as androgen, glucocorticoid receptors etc. in addition to binding to estrogen receptors, and results in changes in tissue enzymes and hormone receptors. In addition, BPA can disturb oxidative homeostasis through the direct or indirect pathway, including mitochondrial function, modulation of antioxidant

enzymes in the brain, kidney, and testis.

Fetuses and infants can be biologically more sensitive to the same toxicant exposure on a body weight basis than adults. However, a few epidemiological studies assessed the relationship between exposure to BPA and oxidative stress in women and adults and the information on the BPA exposure levels of fetuses and infants is still limited. Therefore, it is necessary to investigate the BPA exposure and health effects in this susceptible population. In order to address these issues, data on infant BPA exposure and health effect biomarkers in the CHECK study were used. The CHECK study (Children's Health and Environmental Chemicals in Korea) is designed to collect information related to exposure of environmental pollutants (POPs, BPA, Phthalates and heavy metals) during pregnancy and early childhood.

In CHECK study, Pregnant women and their matching infants were recruited from six university hospitals located in four cites of Korea in 2011-2013 (n = 335). For this study, the biological samples (serum, urine, placenta, breast milk, cord serum and neonatal urine) were collected from pregnant women and their babies (n = 318) and then infants' urine (n = 187) and baby food (n = 210) samples were also collected at 9, 12, and 15 months after birth from follow up panel (n = 173). The urine samples (n=271) in the study of association with oxidative stress comprised 190 infants for whom urinary BPA levels, oxidative stress marker levels, and covariate data were available. BPA levels in the biological and food samples were determined with HPLC-MS/MS and GC-MS, respectively. The urinary free cortisol level was analyzed at Samkwang Medical Laboratories and 8OHdG (8-hydroxy-2'-deoxyguanosine) level

was measured using an EIA kit and spectrophotometer.

This study was designed based on the following objectives. The specific objectives of this study were (1) to describe the perinatal BPA exposure using correlations and concentration ratios in mother-neonate paired samples, (2) to assess the BPA exposure of the weaning period through the contamination status in urine and baby food samples, (3) to estimate the health effect on BPA exposure by the correlation between BPA levels and oxidative stress markers in repeatedly collected urine samples.

In chapter II, BPA exposure levels in various body fluids and tissues of pregnant women were determined and fetus and infant exposures to BPA based on associations between mother and their neonate's samples and BPA ratios in mother−neonate paired samples could be described. The median BPA concentration in the samples decreased in the order of neonatal urine (4.75 ng/mL), maternal urine (2.86 ng/mL), cord serum (1.71 ng/mL), maternal serum (1.56 ng/mL), breast milk (0.74 ng/mL), and the placenta (0.53 ng/g). In comparison with other studies, BPA concentration was higher in maternal and neonatal urine but similar in the other samples. There were only significant correlations between maternal urine and cord serum, maternal urine, and placenta. We estimated the ratios of BPA levels in the other sample types to those in maternal serum. The median (95th percentile) cord serum-to-maternal serum ratio was 1.12 (15.2) for mother−fetal pairs (n=160), in which BPA was detected in both matrices. The placenta-, maternal urine-, neonatal urine-, and breast milk-to-maternal serum ratios were 0.28 (5.31), 1.79 (29.9), 1.98 (28.2), and 0.51 (10.5), respectively.

In addition, the 95th percentile values were 14−20-fold greater than the medians. The detection of BPA in cord serum and neonatal urine indicated that fetuses were exposed to BPA via the mother. These variations of BPA ratios suggest that pregnant women are exposed to BPA repeatedly and persistently and that infants could encounter high BPA exposure via placental transfer and breastfeeding.

In chapter III, the BPA levels in baby food and urine of infants were investigated at 9, 12, and 15 months after birth. Median (IQR, interquartile range) levels of BPA were 0.45 ng/g (IQR: N.D (No Detection) -5.16 ng/g wet weight) in homemade baby-food and 0.93 μg/L (IQR: <LOD-2.66 μg/L) in unadjusted urinary BPA [0.94 μg/L (IQR: <LOD-2.80 μg/L) adjusted for specific gravity (SG)]. The BPA concentrations in the baby-food of 15 months old infants (median: 5.09 ng/g) were significantly greater than those detected at 9 or 12 months of age (median: <LOD and 0.47 ng/g, respectively). The urinary BPA concentrations, however, were not affected by the age of the infants. Because the intake rate of foods wrapped in packaging materials and/or containers such as meat, dairy products, and snacks increased, the BPA levels in the baby food at 15 months after birth was higher. However, there was no correlation between BPA levels of urine and baby food samples. Therefore, our results imply that other environmental factors will contribute to urinary BPA of infants, unlike adults whose main source of exposure to BPA is diet, although further study should confirm it.

In chapter IV, BPA, 8-OHdG and free cortisol levels in repeatedly collected urine

samples of infants were examined, and then the correlations between urinary BPA and oxidative stress markers with repeated measures for each individual were estimated. The specific-gravity corrected geometric mean (geometric standard deviation) of all urine samples was 1.9 (4.9) ng/mL (median and 95th percentile: 0.8 and 27.5 ng/mL), and those of 8-OHdG and free cortisol were 63.7 (2.2) ng/mL (median and 95thpercentile: 61.0 and 282 ng/mL), 15.6 (2.6) μg/dL (median and 95thpercentile: 16.7 and 66.8 μg/dL), respectively. The BPA was significantly associated with both 8-OHdG and free cortisol with non-linear relationships ($p<.0001$). The BPA levels were categorized in low, medium, and high group with 25th and 75th percentile, and then the effect of BPA exposure on 8-OHdG and free cortisol using linear mixed effect model after adjustment for infant gender, age, and other chemical levels [phthalate metabolites, lead (Pb) and mercury (Hg)]. BPA was not associated with free cortisol. In the associations of BPA levels with 8-OHdG, the MnBP was also found to interact strongly with BPA exposure. In a high group of MnBP, 8-OHdG levels of a high group of BPA was higher than those of low and medium groups. Therefore, exposure to both compounds affects the urinary 8-OHdG. These results were supported by prior studies that reported the association between BPA exposure and 8-OHdG, although previous associations were found in pregnant women, adults. In addition, the free cortisol levels were significantly correlated with 8-OHdG levels ($\rho=0.42$, $p<.0001$), indicating a high linking of them on the pathway. Our results suggest the possible oxidative stress of BPA during important prenatal and postnatal periods of development.

In conclusion, this study has established that the current levels of exposure to

BPA could be associated with the oxidative stress during infancy. Compared with other studies, the BPA in umbilical cord blood and neonatal urine were high and infant urine was similar. The variations of BPA ratios indicate individual differences in the amounts of BPA delivered from mother to fetus due to differences in life patterns and metabolic function of mothers. BPA in food did not show significant correlation with urinary BPA which suggested there was another source of exposure in environments and behavior of infants. In addition, positive associations of the BPA exposure on 8-OHdG were found, but not free cortisol. These results implied that prenatal and postnatal BPA exposure could induce oxidative stress in fetuses and infants. Therefore, considering the effects of BPA exposure on oxidative stress during gestation and early life stages, further studies are needed to identify the BPA exposure sources of pregnant women and infants through continuous BPA monitoring and to identify the mechanisms of action for BPA on oxidative stress with health implications.

Keywords: Bisphenol A, neonates and infants, BPA ratio, urine, homemade baby-food, oxidative stress, 8-OHdG, free cortisol

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Chapter 1. Background

1.1 Bisphenol A

The substance 2, 2-bis(4-hydroxyphenyl)propane, CAS Number 80-05-7, more commonly known as bisphenol A (BPA), has been widely employed in commercial products since 1957 (EFSA, 2010). With five million tons of BPA produced and one hundred tons of BPA reportedly released into the atmosphere annually. Bisphenol A has a molecular mass of 228.29 g/mol and a molecular formula of $C_{15}H_{16}O_2$ (Chapin et al., 2008). BPA is a colorless solid that is moderately soluble (melting point = 150-155 ℃) and has a greater solubility at alkaline pH values due to its disassociation constants, pKa 9.6 to 10.2, and has a log Kow of 3.32 (Staples et al., 1998).

1.2 Infants as susceptible populations

Over the past decade, the interest in the potential impact of infant health on exposure to environmental chemicals has increased. Infants can be biologically more sensitive to the same toxicant exposure on a body weight basis than adults (Scheuplein et al., 2002). In addition, the National Academy of Sciences (NAS) report concluded that young children might experience quantitatively and qualitatively different

exposure to chemicals than do adults. Although the controversy continues over whether infants are more sensitive than adults, there are many physiologic and pharmacologic reasons why the sensitivity of infants and adults to chemicals exposures may differ.

Although not as dramatic as the growth from conception to birth, human postnatal growth during the first year of life is extraordinary: a typical human infant increases in weight by about 200% and in length by 50% (Sparks, 1998). Therefore, BPA exposure to developing infant is major importance because impact to a physiological system prior to its full development can permanently affect the system (NAS, 1993). The nature and extent of absorption, distribution, metabolism, and elimination are determined by the underlying growth rates and function of the physiological system (Scheuplein et al., 2002).

In absorption, gastrointestinal absorption of xenobiotics overall does not appear to change dramatically with age but several factors that affect absorption do change. Gastric secretion is low in newborns and gastric pH is correspondingly high. These can result in decreased absorption of weak acids and increased absorption of weak bases (Gustaffson, 1962). The skin of the neonate is thinner than that of adults (Evans and Rutter, 1986). Lung alveolar surface area is greater than that of adults on a body weight basis, which, together with children's higher ventilation rates, contributes to children's often greater absorption through inhalation (Burri, 1977). Bile acid metabolism and turnover are not fully developed at birth; primary bile salts exhibit a

transient elevation in the first few weeks and then decline steadily for several years while liver function matures (Heubi et al., 1982). As a result, the absorption of fats and lipid-soluble substances may be affected.

The distribution of a drug is influenced by several factors, including the size of the body water and lipid compartments, regional blood flow, the presence of transport proteins, and the degree to which drugs bind to plasma and tissue proteins (Scheuplein et al., 2002). In the preterm newborn, both albumin and α-acid glycoprotein concentrations and binding affinities are low compared to adults (Nau et al., 1998). Infants have a higher percentage of water in lean body tissues than adults. The additional water is primarily extracellular so that the extracellular water compartment in infants is about twice that of adults (Widdowson and Dickerson, 1964).

In metabolism, although some chemicals are eliminated as unchanged type, most are converted to a wide variety of metabolites before they are excreted in urine, bile, or breath, and most hepatic biotransformation enzymes do not reach adult levels until after birth (Dutton, 1982). Both Phase I and Phase II biotransformation activities generally immature and require additional postnatal maturation. The major Phase I metabolic system comprises various forms of the hepatic microsomal P450 enzyme families, CYP1, CYP2, and CYP3. The P450s interact typically with substrates and molecular oxygen to form hydroxylated metabolites or intermediates. For some substrates, the adult activity of P450 enzymes is surpassed by that of neonates. The most important Phase II metabolic system (glucuronide conjugation) is catalyzed by

uridine diphosphate glucuronosyl- transferase (UDP-GT). The UDP-GTs in the neonatal cluster exhibit low activity at birth, increasing gradually and reaching adult levels by 4 weeks of age. UDP-GTs in the perinatal cluster remain low throughout lactation and increase to adult levels only after weaning (Kopecky and Koren, 1998; Rane et al., 1973).

In excretion, the amount of a substance that is filtered by the kidney depends on renal blood and plasma flow. Renal blood flow is low at birth because the kidney receives a lower proportion of the cardiac output and because of its high intrarenal vascular resistance; renal blood flow increases to adult levels by 5 months of age (Calango and Rubin, 1963).

Infants are disproportionally affected by environmental exposures mainly due to their unique exposure pathways (Leith Sly and Carpenter, 2012). Previous studies reported that BPA can cross the placenta and can transfer to the infants via breastfeeding (Unal et al., 2012; Wan et al., 2010; Kasper et al., 2016). Infants also interact with their environment in a very different manner from adults. They are physically located in a different zone. Although the adult breathing zone is approximately a meter from the floor, the child breathes in a zone much closer to the ground (Tulve et al., 2002).

As BPA has endocrinologic activity, there is concern that BPA may act on hormonally mediated pathways to disrupt normal growth and development. Prenatal and postnatal exposure to BPA may be associated with altered neurodevelopment,

obesity, and precocious puberty (Vom Saal et al., 2007). Gestational exposure is of particular concern given the unique susceptibility of the fetus to environmental toxicant exposures (Mendola et al., 2002).

Infants' organ systems and ability to metabolize dangerous chemicals are not fully developed early in life. They are in a state of growth, and their early exposure may cause disease in adulthood. Therefore, exposure to BPA during the prenatal and postnatal period of infants could have considered.

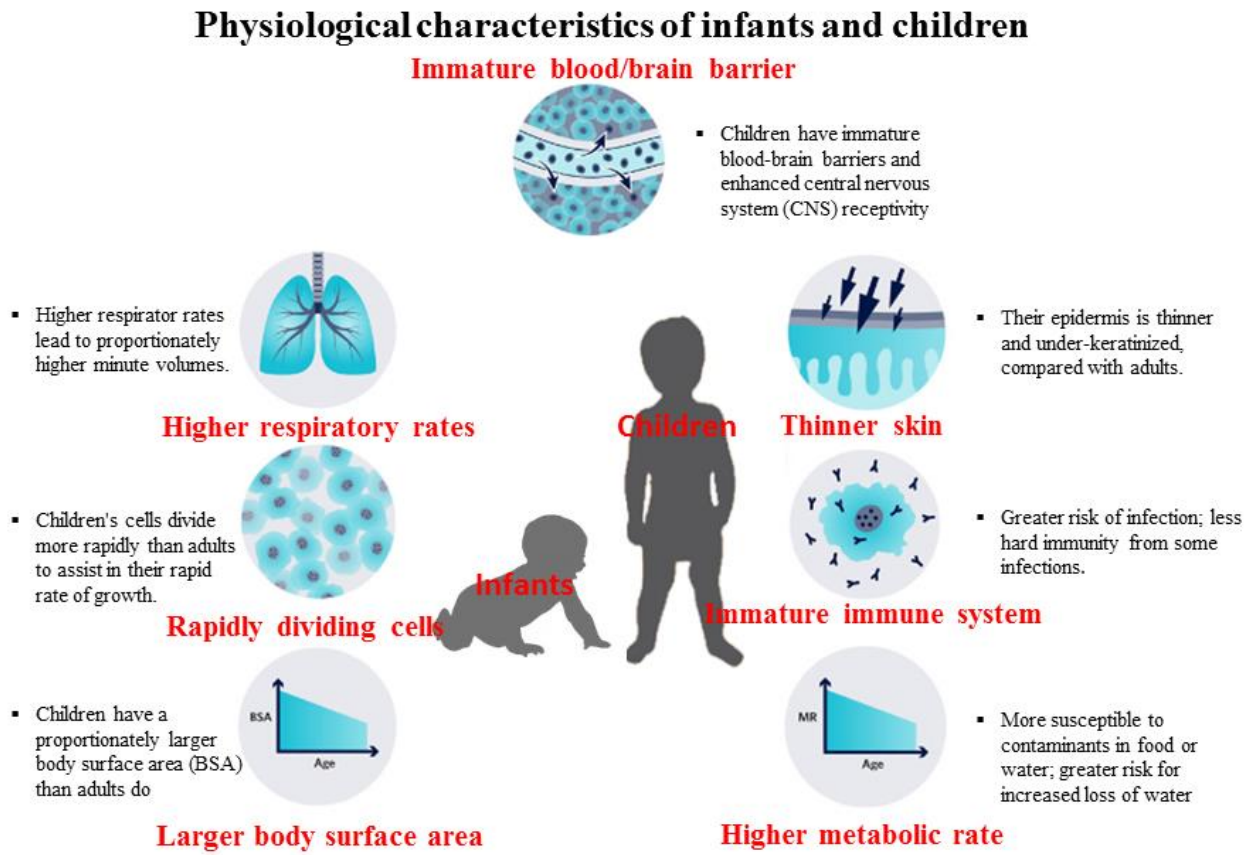

Figure 1-1. Differences of physiological characteristics between infants and adults (Source: COAG, 2009)

1.3 Bisphenol A levels in infants' biological samples

Early-life BPA exposure may be particularly important given the vulnerability of developing fetus and infant. There were a few studies that BPA was detected in umbilical cord blood in the fetus (Ikezuki et al., 2002; Lee et al., 2008; Zhang et al., 2013). A recent study reported that the median BPA level in cord serum was very low at 0.051 ng/mL in Japan (Yamamoto et al., 2016). In the USA, Canada and Germany, median urinary BPA levels were < 0.45 ng/mL, <0.2 ng/mL and 0.49 ng/mL in infant 1-15 months old, one month old and 3-6 days of age, respectively (Volkel et al., 2011; Arbuckle et al., 2015; Nachman et al., 2015). Recently, creatinine-adjusted geometric means were 3.65, 3.39, 3.41, 2.79, 3.36, 4.21 ng /mL at birth and 14, 28, 42 days after birth and 3, 6 months after birth (Wang et al., 2017). The study reported the temporal trend of BPA levels in 337 children with up to six urine samples from 1 to 8 years of age and suggest that exposure to BPA decreased over the period of the study (Stacy et al., 2016). However, another study did not observe a temporal trend in geometric mean of BPA concentrations from 2001 to 2010 in children of an urban minority birth cohort (Hoepner et al., 2013).

In previous studies, young children have higher urinary BPA than adolescents and adults (Becker et al., 2009; Calafat et al., 2008). In Germany, 3-5 years old children had higher geometric mean urinary BPA (3.55 μg/L) than 12-14 years old adolescents (2.42 μg/L) (Becker et al., 2009). This pattern was also observed among

6-11 years old children (3.6 μg/L) and adults (2.6 μg/L) (Calafat et al., 2008). These findings might be related to children's higher food consumption, air inhalation and dust ingestion, and metabolism of BPA (Calafat, 2011). The difference in BPA levels between children and adults in Asia has been reported to be due to high BPA exposure of Asian children through contact with poor quality toys (Huang et al., 2017). However, the study of BPA detection in infant urine is limited. Moreover, concurrent BPA levels in multiple human samples including cord serum and neonatal urine, from a single pregnant woman have not been reported.

1.4 Exposure source and pathway of infants

1.4.1 Placental transfer

In human, variable BPA levels have been reported in the maternal serum, umbilical cord serum, placental tissue, and amniotic fluid (Vandenberg et al., 2010), indicating a transfer of BPA from mother the fetus. Furthermore, perfusion studies using human placenta have demonstrated transfer of BPA across the placenta (Balakrishnan et al., 2010; Mørck et al., 2010).

In several epidemiological studies, few studies have reported low BPA levels in the fetal blood (Schonfelder et al., 2002; Lee et al., 2008; Wan et al., 2010; Zhang et

al., 2013), but they were high in some studies (Ikezuki et al., 2002; Kuroda et al., 2003). These results indicate that there is a larger variation in the BPA levels in the blood samples of mother-fetus pairs. Therefore, it is important to identify BPA levels and variation in other biological samples with maternal and fetal blood. In addition, the relationship between maternal and fetal BPA levels deserves investigation.

1.4.2 Dietary and non-dietary exposure sources

Children are disproportionally affected by children are disproportionally affected by environmental exposures mainly due to their unique exposure pathways and their developing bodies. Children also interact with their environment in a very different manner from adults. They are exposed in the places they spend most of their time, via media like water, air, food, soil, and objects that carry the hazards. They are also exposed to specific age appropriate behaviors (crawling, tasting, and “hand-to-mouth ” behavior in a toddler) (Leith Syl and Carpenter, 2012).

The researcher reviewed the BPA exposure pathways in early childhood as two main categories: dietary and non-dietary (Healy et al., 2015).

Dietary exposure was a major concern in infants and toddlers, who have a significantly higher dietary intake for their body weight than the adults (Landrigan and Garg, 2002). In the first 4–6 months after birth, neonates rely solely on either infant formula or breast milk — or a combination of the two — as their primary

nutritional source. Although BPA has been banned from use in infant feeding bottles, samples of canned and powdered infant formulas in the United States and Canada have been observed to be contaminated with variable concentrations of BPA, ranging up to 113 ng/g (Cao et al., 2009; Kuo et al., 2004; Ackerman et al., 2010). Because of the use of BPA-containing resin coating of several domestic water supply pipes and storage tanks in some region of the world, the practice in the home for reconstitution of powdered infant formulas must also be considered (Fan et al., 2014). In addition, Infant and toddler foods are usually packaged in smaller volumes, leading to a greater surface area to volume ratio between the food and the packaging material (FSANZ report, 2010).

The BPA exposure in young children is not necessarily limited to dietary exposure sources. Sucking and chewing are normal developmental behaviors in early childhood (Mori and Todaka, 2011) and can include mouthing on plastics and other BPA-containing consumer products, which are generally not considered dietary exposure sources, such as pacifiers, teething rings, toys, and books. The frequent hand-to-mouth behaviors of infants and toddlers may indirectly increase ingestion of BPA, following the transfer of BPA to the hands and food, which are then placed in the mouth (Landrigan and Garg, 2002). The study of BPA migration from household plastic products observed that migration concentrations were generally in the range of 150–300 ng/l, which included an infant spoon, a toy, a CD, and a DVD (Vinas et al., 2012). Thermal paper is also extensively recycled and BPA may be present in a range

of paper and cardboard products made from recycled materials, such as magazines, books, and newspapers (Liao et al., 2011). Exposure to BPA has the potential to be significant in young children who ingest BPA containing paper and cardboard products, such as chewing on a magazine or children's book made from recycled thermal paper. As infants and toddlers in most developed countries spend more than 90% of their time indoors, house dust composition may be an under-recognized exposure source of BPA in young children (Loganathan and Kannan, 2011; Liao et al., 2012). The study from Korea report the highest levels of BPA concentrations in household dust, ranging up to 31,900 ng/g (Liao et al., 2012).

Secondhand smoke may be an additional source of BPA exposure since BPA is used as a component of cigarette filters (Braun et al., 2011).

The study reported that BPA has been detected in several media including food, air, and dust samples collected at residences and childcare centers. The result of this study showed that dietary ingestion through the consumption of solid and liquid foods was probably the major route of exposure for 257 preschool children to BPA, and this information suggests that BPA from packing materials and containers of food products affect the internal dose (Wilson et al., 2007). Another study reported the quantitative relationships between the children's intake doses of BPA through the dietary ingestion, non-dietary ingestion, and inhalation routes and their excreted amounts of urinary BPA, and the dietary intake route accounted for > 95 % of the children's excreted amounts of BPA in urine (Morgan et al., 2011). However, the

information for BPA levels in the urine of Korean infants was limited. In addition, we are unaware of any published studies in the Korea that have assessed the quantitative relationships between exposure to BPA and urinary BPA of infants. Thus, it is important to identify the major source of infants is dietary or other sources.

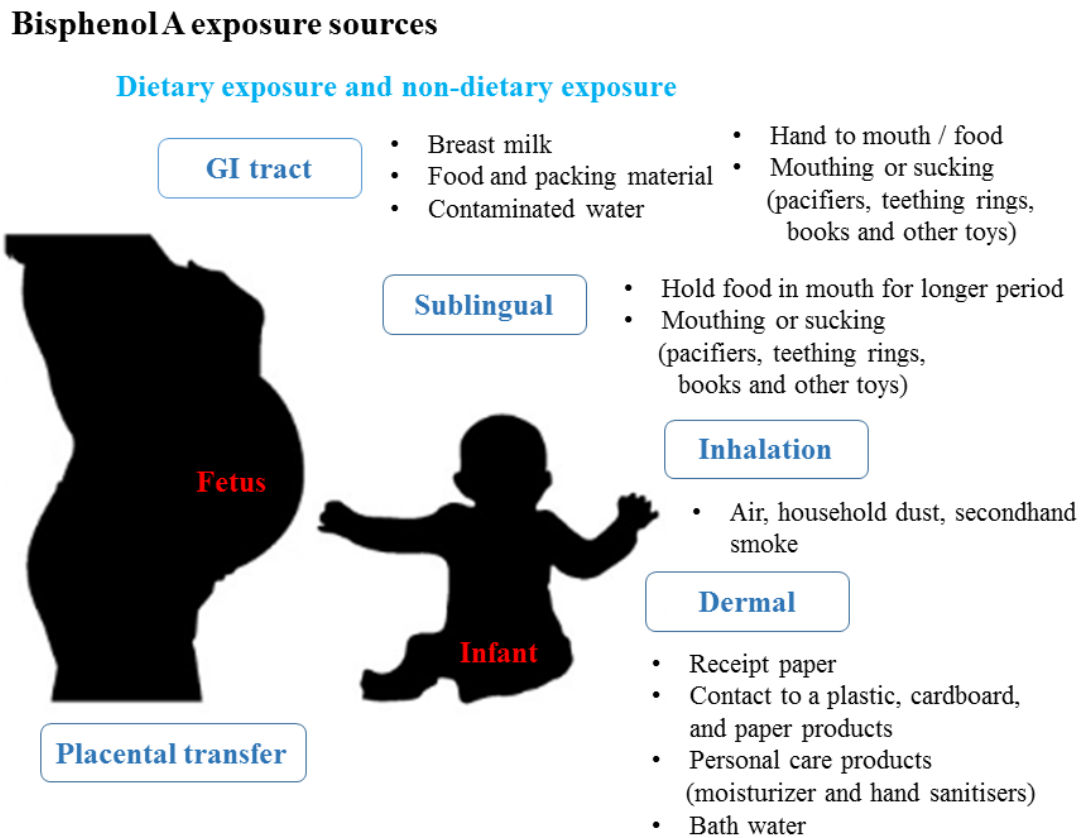

Figure 1-2. Exposure routes and sources for bisphenol A in fetuses and infnats (Healy et al., 2015)

1.5 Importance of Oxidative stress and their association with BPA exposure

A large number of animal and a limited number of human studies suggest that BPA exposure may be associated with adverse health outcomes (Chapin et al., 2008; Vom Saal et al., 2007). The researchers reviewed the health outcomes as categories reproductive system, developmental effects, immune system, Neurobehavioral effect, metabolic effects, genotoxicity, oxidative toxicity and carcinogenicity (Rochester et al., 2013; Srivastava et al., 2015).

Among these health outcomes, oxidative stress is an impaired balance between free radical production and antioxidant capacity resulting in excess oxidative products and has been reported to play an important role in many pathological conditions, including insulin resistance (Grattagliano et al., 2008; Kataria et al, 2017). Recently, BPA exposure causes the generation of oxidative stress, which results in DNA and mitochondrial damage. These damages induced the activation of apoptosis and the p53-p21 pathway, which affect the porcine embryonic development (Guo et al., 2017). In addition, the potential effect of BPA exposure on the development of the hypothalamic-pituitary-adrenal (HPA) neuroendocrine system has been investigated (Panagiotidou et al., 2014; Poimenova et al., 2010), and the association between maternal urinary BPA during pregnancy and infant HPA axis function were reported

(Giesbrecht et al., 2017). 8-OHdG (8-Hydroxy-2˝-deoxyguanosine) is produced by oxidation of the nucleoside, deoxyguanosine, urinary excretion of that has been identified as a sensitive marker for oxidative DNA damage (Erhola et al., 1997). Free cortisol is a hormone that is produced by the adrenal glands, and it is often released in response to stress (Kim et al., 2018). The study suggested that the combined effects of cortisol lead to increased oxidative stress, in which the mitochondrial production of reactive oxygen species (ROS) exceeds the antioxidant potential, thereby causing damage to other molecules such as lipid, proteins and DNA/RNA (Finkel and Holbrook, 2000; Maynard et al., 2009; Joergensen et al., 2011). Several epidemiologic studies reported that 8OHdG were increased by urinary BPA in adults, cashiers and pregnant women (Hong et al., 2009; Lv et al.., 2017; Ferguson et al., 2016). In the association with free cortisol, the study reported that higher maternal BPA was associated with increases in baseline cortisol among infants, because of changes in infant HPA axis function (Giesbrecht et al., 2017).

Considering the effect of oxidative stress by BPA during the developmental stage of fetuses and infants, study for the association between BPA exposure and oxidative stress markers such as 8-OHdG and free cortisol is needed.

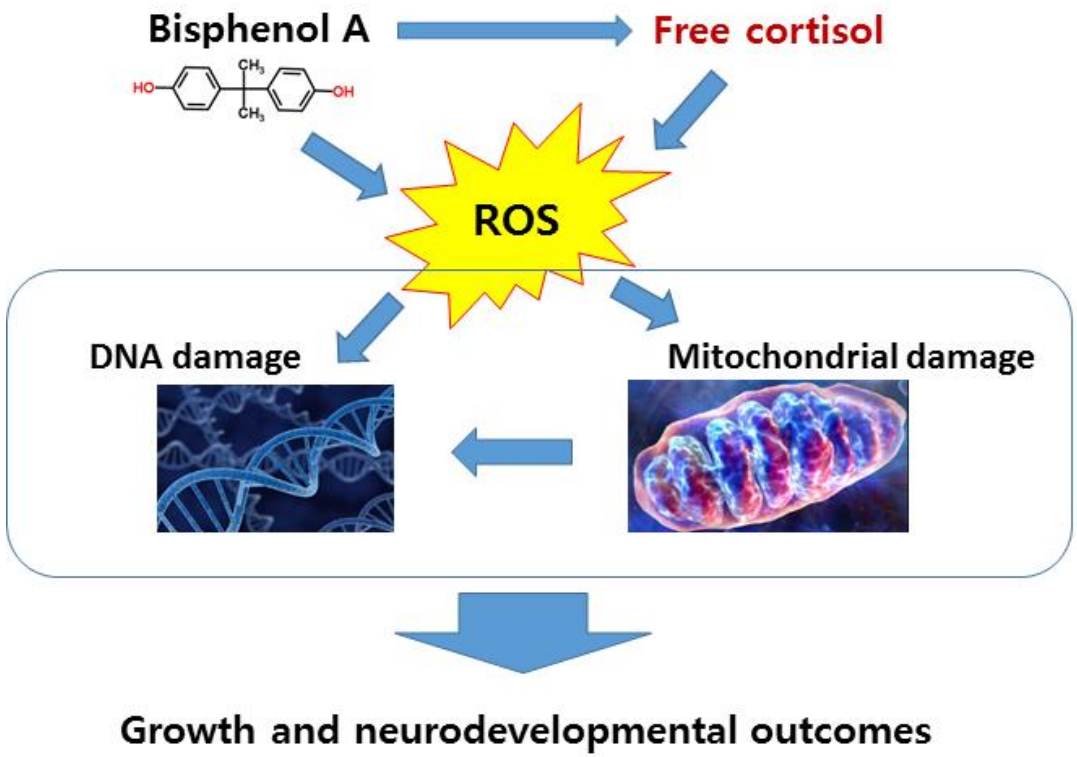

Figure 1-3. Mechanisms of action of BPA in oxidative stress

1.6 CHECK study

In 2011, the CHECK study (Children's Health and Environmental Chemicals in Korea) is designed to collect information related to exposure of environmental pollutants (POPs, BPA, Phthalates and heavy metals) during pregnancy and early childhood.

The objective of the CHECK study is to (1) determine the levels of various environmental pollutants (OCPs, PCBs, PBDEs, BPA, Phthalates, Hg, and Pb) in mothers and their infants (2) to examine how exposure to environmental chemicals affects infants' health (growth, development, and disease).

CHECK cohort is composed of pregnant women-fetus pairs recruited from six university hospitals located in Seoul, Anyang, Ansan, Guro and Jeju of South Korea. By 2013, a total of 335 pairs of mothers and their matching newborn infants have been recruited. This study included pregnant women who had babies with normal gestation age, neonatal weight, and information on birth outcome. We excluded participants with occupational exposure to BPA, a disease, or multiple births, as well as cases with incomplete data necessary for the study.

However, 318 mother-infant pairs were analyzed in chapter 1, excluding 17 for which the urinary sample was not available. The urine (n=224) and serum (n=180) samples of pregnant women were collected the day before delivery. At delivery, the umbilical cord blood (n=230) and placenta (n=257) were collected. The first urine of

neonates (n=152) was collected within 2 days after birth. Breast milk (n=127) samples were collected from breastfeeding mothers at 1 month after delivery. In chapter 2, 173 infants were followed up. Except for participants who did not provide samples for personal reasons and who did not provide enough urine sample, a total of 210 baby-food and 187 urine samples were collected from participating infants. In chapter 3, the urine samples (n=271) in the study of association with oxidative stress comprised 190 infants for whom urinary BPA levels, oxidative stress marker levels, and covariate data were available.

This study (chapter 1~3) found the results related to BPA exposure to fetuses and infants, and association with health effects including 8-OHdG and free cortisol.

On the CHECK cohort study, several studies have been published on exposure, risks, and association with health effects of chemicals including PBDEs, PCBs, OCPs, phthalate, BPA and heavy metals (Kim, 2015 reviewed). In the exposure and risk studies, nineteen PBDEs congeners were determined in 198 maternal and 118 matching umbilical cord blood samples and strong positive correlations were found between them, indicating the importance of placental transfer (Choi et al., 2014). PBDEs, PCBs and OCPs were analyzed in breast milk samples at <7, 15, 30, and 90 days. The estimated daily intakes of ΣPBED, ΣPCB, and ΣOCP were lower than the threshold values proposed by the US EPA and Health Canada (Lee et al., 2013a, 2013b). PBDEs, PCBs, and OCPs were also analyzed in baby food samples. 24 PBDE congeners were determined in 147 homemade baby food samples collected from 97

households for 6-, 9-, 12-, 15-, and from 24 to 27-month-old infant groups, and these results indicates that baby food is an important exposure pathway of PBDEs for over 24-month-old infants (Jeong et al., 2014a; Jeong et al., 2014b). Phthalates were analyzed in breast milk samples. Based on the estimated daily intake, up to 8 % of infants exceeded the reference dose of anti-androgenicity (RfD AA) for DEHP, and 6 % of infants exceeded the tolerable daily intake (TDI) for DnBP (Kim et al., 2015).

In health effect studies, the studies investigated the association between POPs exposure and thyroid hormones or adipokine levels among pregnant women or matching newborns and the current levels of exposure to POPs could be associated with the disruption in thyroid hormones and lipid metabolism among pregnant /lactating women or newborn infants (Kim et al., 2013, 2015a, 2015b). DEHP metabolites were also analyzed in newborns' urine as well as maternal blood, maternal urine, placenta and cord blood samples, and the effects of DEHP exposure on obesity-related markers and body mass change for the first 3 months after birth were evaluated. This study suggests that DEHP exposure may decrease PI and increase TG levels in newborn infants, finally resulting in body mass increase in early life (Kim et al., 2016). The study evaluated the relationships between phthalate metabolites and free cortisol and 8-OHdG in mother-child pairs. MEHHP, MEOHP, MiBP, and MnBP were positively associated with both free cortisol (Kim et al., 2018).

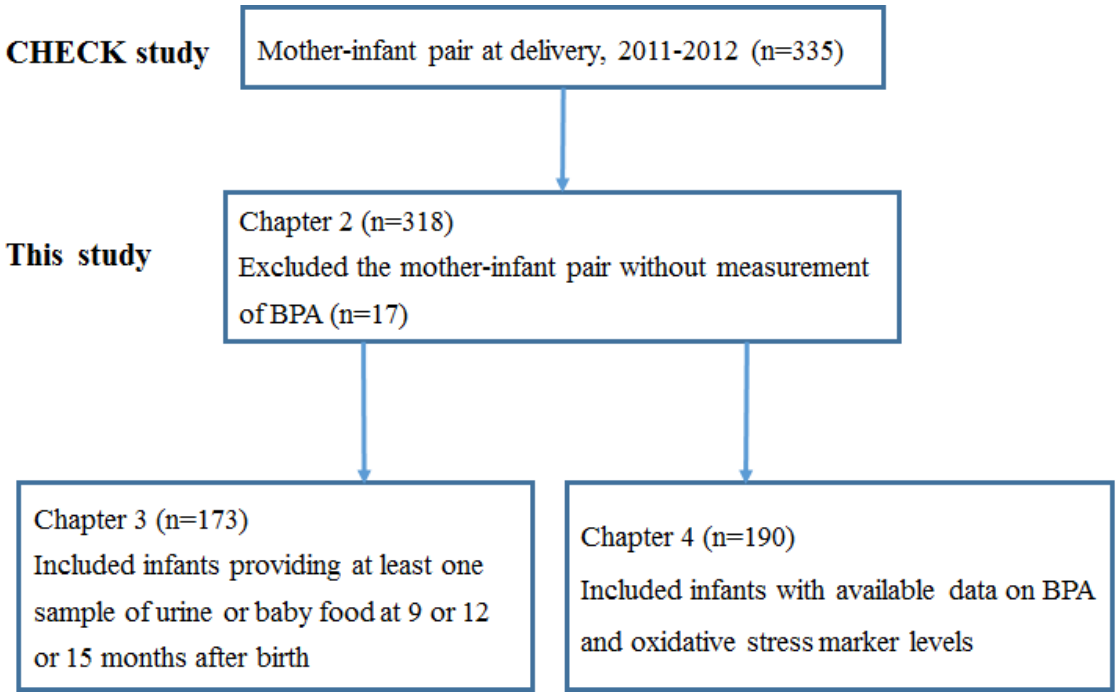

Figure 1-4. Study flow diagram showing the study participants

1.7 Study design and objectives

Fetuses and infants are considered the susceptible population for the exposure to bisphenol A, because of their health effects such as reproductive, development and metabolic outcomes. Therefore, the several researchers reported the BPA levels in maternal serum and cord serum from mother-fetuses pair and baby food samples. However, there are three knowledge gaps for helping to understand BPA exposure in fetuses and infants. First, there is not enough information about how much BPA is delivered from the mother to their fetus because existing studies only show the BPA ratio in serum between pregnant women and their fetuses and inconsistent results of them. Second, the study in Korea that have assessed the BPA exposure through the urine and baby food samples is still limited. Finally, several animal studies reported the health effects on fetal and infant development by oxidative stress, but there is no the information on the relations with oxidative stress during infancy.

This study consists of three studies (Figure 1-5). Following are the specific research contents:

In Chapter 2, to describe the perinatal BPA exposure using correlations and concentration ratios in mother-neonate paired samples.

In Chapter 3, to assess the BPA exposure of the weaning period through the contamination status in urine and baby food samples.

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In Chapter 4, to estimate the health effect on BPA exposure by the correlation between BPA levels and oxidative stress markers in repeatedly collected urine samples.

Taken together, our results from the three chapters can demonstrate the BPA levels and related exposure sources of fetuses and infants, and health effects of BPA on oxidative stress during infancy including the perinatal period.

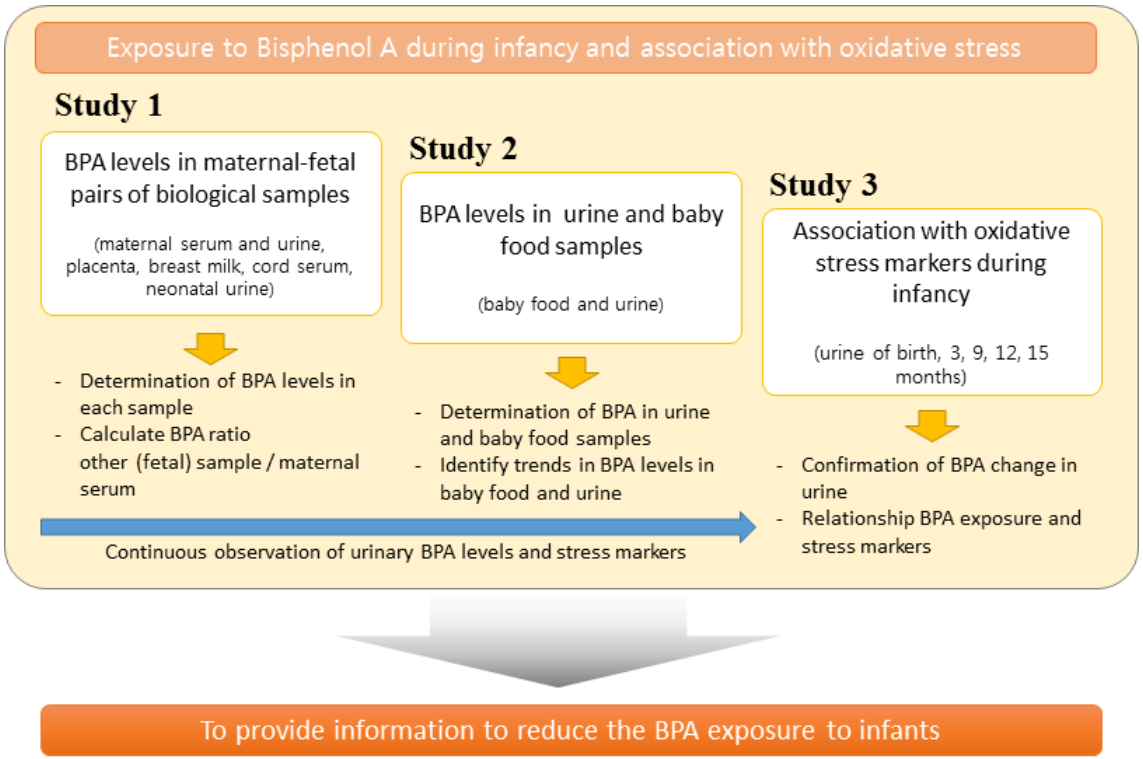

Figure 1-5. Study design of the main studies

Chapter 2. Bisphenol A distribution in serum, urine, placenta, breast milk, and umbilical cord serum in mother-neonate pairs

2.1 Introduction

The general population, including pregnant women, is exposed to bisphenol A (BPA) from consumer products such as polycarbonate water bottles, food storage containers, epoxy-lined food cans, dental sealants, and thermal receipts (Genns et al., 2011). Studies have reported the presence of BPA in the serum, urine, amniotic fluid, follicular fluid, placenta, and breast milk of pregnant women, as well as fetal cord serum (Vandenberg et al., 2010a reviewed). Recently, levels of glucuronide-conjugated BPA in urine up to 11.21 μg/L have been found in neonates within 6 days after birth (Nachman et al., 2015). These biomonitoring data of variable BPA levels indicate that fetuses and neonates are exposed to BPA from their mothers through placental transfer and breastfeeding. Furthermore, perfusion studies using human placenta have demonstrated transfer of BPA across the placenta (Balakrishnan et al., 2010; Mørck et al., 2010). BPA exposure during the perinatal period increases susceptibility to diseases over the life cycle, including childhood, adolescence, and adulthood (Boekelheide et al., 2012). In addition, the early postnatal period is critical for infant growth and development (Pryor et al., 2000). Studies in rodents have found

that prenatal and early postnatal BPA exposure is associated with increased body weight and fat deposition (Miyawaki et al., 2007; Harley et al., 2013). Rodent studies also suggest that early-life BPA exposure may affect physiological functions, including the developmental pituitary-thyroid axis and immune system (Li et al., 2016; Franssen et al., 2016; Fischer et al., 2016).

Considering the potential effects of perinatal exposure to BPA, direct measurements of the amount of BPA transferred to the fetus or neonate via placental transfer and breastfeeding are important. Biomonitoring in humans has been performed for human exposure assessments of environmental chemicals (Calafat et al., 2006). Due to the convenience and accessibility of field studies, many reports have been conducted on BPA levels in urine and blood (Vandenberg et al., 2010a). However, biological sample selection is more restricted in mother-infant (or neonate) pairs, as in birth cohort studies. Physiologically based pharmacokinetic (PBPK) modeling coupled with Monte Carlo simulation could provide greater insight into the distribution of chemicals of concern; however, precise estimations depend on actual measurements from the population of interest (EPA, 2006). There is little information on the distribution of the BPA ratio in mother-infant pairs during the perinatal period. Moreover, many studies have not reported concurrent BPA levels in multiple human samples, such as serum, urine, breast milk, placenta, and amniotic fluid, from a single pregnant woman. A few studies have analyzed the BPA levels in maternal and fetal samples on a pairwise basis, offering limited assessments of the inter-individual

variability of the BPA distribution (Schonfelder et al., 2002; Lee et al., 2008; Zhang et al., 2013; Braun et al., 2011).

To address these issues, the distribution of BPA in pregnant women and their fetuses were identified by BPA levels in maternal urine and serum, placenta, breast milk, cord serum, and neonatal urine. In addition, fetal and neonatal BPA exposure were described using correlations of BPA levels and BPA ratios in mother–neonate paired samples. BPA concentration ratios were calculated with their individual monitoring data.

2.2 Materials and Methods

2.2.1. Study population and sample collection

Pregnant women and their babies were recruited from six university hospitals located in four cities in Korea (Seoul, Anyang, Ansan, and Jeju) between February 2011 and December 2012 (Choi et al., 2014; Kim et al., 2015a). At the time of recruitment, participating women completed a detailed questionnaire that included general demographic parameters and food intake frequency, while information on birth weight, birth length, and gestational age at delivery, delivery mode, parity, and infant sex were collected from medical records (Table 2-1). This study included pregnant women who had babies with normal gestation age, neonatal weight, and

information on birth outcome. Participants with occupational exposure to BPA, a disease, or multiple births, as well as cases with incomplete data necessary for the study were excluded. Among the 355 pregnant women recruited, 318 mother-infant pairs were included in the final analysis. The urine and serum samples of pregnant women were collected the day before delivery. At delivery, the umbilical cord blood and placenta were collected. The first urine of neonates was collected by nurses using polyethylene urine collection bags (Urine Collector; ROOTICS Corp., Seoul, Korea) within 2 days after birth. Maternal and cord serum were collected in serum separation tubes (BD Vacutainer SST II Advance, ref #367953; Becton-Dickinson, Plymouth, UK), using a Vacutainer (Kim et al., 2016). Approximately 15 g placenta were collected from the central section, which included the maternal and fetal sides, and was fragmented and placed in a 50-mL falcon tube. Breast milk samples were collected from breastfeeding mothers at 1 month after delivery. Because the maternal and cord serum samples were used for the detection of persistent organic pollutants (e.g., organochlorine pesticides, polychlorinated biphenyls, and polybrominated diphenyl ethers), some samples had insufficient volume for testing. In addition, there were fewer samples of breast milk and newborn urine, because not all mothers did breastfeed, and it was difficult to collect urine sample from infants within 2–3 days after birth. Therefore, a total of 180 maternal serum, 224 urine, 257 placenta, 127 breast milk, 230 cord serum, and 152 neonatal urine samples were included in the BPA analyses; 20 participants provided at least one sample, and only 40 participants

provided all samples. Before the analysis, all samples were stored at −70 °C. All samples and data were processed in a blinded manner. The present study was approved by the institutional review board at the School of Public Health, Seoul National University, Korea (IRB no. 8-2012-04-20), and informed consent were obtained from all participating women.

2.2.2 Analysis of BPA

Urine

The experimental procedures of BPA in urine were optimized with some modifications from previous studies (Koch et al., 2003a; Kho et al., 2008). In brief, urine samples (500 μL) were thawed at room temperature, and vortex mixed. Next, 10 μL of 13C-labeled internal standard (Cambridge isotope laboratory, USA) 1 μg/mL were spiked, and 100 μL of 1M ammonium acetate (Sigma-Aldrich, USA) and 10 μL of β-glucuronidase enzyme (Roche, Biomedical, Mannheim, Germany) were added. The sample was incubated at 37 ◦C for 2 hr to allow for the deglucuronidation of the BPA. After hydrolysis, 300 μL of urine samples were mixed with 50 μL 0.1 % acetic acid in acetonitrile and centrifuged at 3000 rpm for 10 min.

Total BPA was quantified by liquid chromatography-mass spectrometry with an AB Sciex 4000 tandem mass spectrometer (Framingham, MA, USA), coupled to a

Shimazu HPLC system (Kyoto, Japan). Separation was achieved using a Shiseido ACR C18 column (3um, 2.0 X 150mm). However, urine samples were analyzed by online enrichment with C18 pre-column (Waters, Milford, MA, USA) and column-switching techniques. The mobile phase composition used in the chromatographic separation was optimized by binary mixtures of 0.1 % acetic acid in water (solvent A) and 0.1 % acetic acid in acetonitrile (solvent B). The flow rate of the mobile phase was 0.2 mL/min. A volume of 10 µL of each sample was injected into the HPLC system.

Serum and breast milk

The experimental procedures of BPA in serum were optimized with some modifications from previous studies (Calafat et al., 2004). In brief, serum (500 µL) or breast milk (1mL) samples were thawed at room temperature, treated with 1 M H3PO4 and vortex mixed. Next, 10 µL of the 13C-labeled internal standard 1 µg/mL were spiked, and 500 µL of 1M ammonium acetate and 10 µL of β-glucuronidase enzyme were added. The sample was incubated at 37 ◦C for 120 min to allow for the deglucuronidation of the BPA. The samples were extracted by solid phase extraction (SPE) using an Oasis-HLB cartridge (60mg/3cc; Waters, Milford, MA, USA). The cartridge was conditioned with 2 mL HPLC-grade methanol and 2 mL water. The incubated samples were diluted with 2 mL of 0.1M formic acid and loaded onto the

SPE cartridge at a rate of 1 mL/min. The cartridge was washed with 1 mL of water and 1 mL of 40 % methanol in water. The BPA was eluted with 3mL of acetonitrile. The eluents were concentrated and dissolved in 100 μL of 70 % methanol in water for HPLC-MS/MS.

BPA was quantified by liquid chromatography-mass spectrometry with an AB Sciex 4000 tandem mass spectrometer (Framingham, MA, USA), coupled to a Shimazu HPLC system (Kyoto, Japan). Separation was achieved using a Shiseido ACR C18 column (3um, 2.0 X 150mm). The mobile phase composition used in the chromatographic separation was optimized by binary mixtures of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B). Gradient conditions were 0.0-2.5 min, 10 % B; 2.5-3.0 min, 10-30 % B; 3.0-5.0 min, 30-40 % B; 5.0-7.0 min, 40 % B; 7.0-11 min, 40-45 % B; 11-16 min, 45-100 % B; 16-20min, 100 % B and return to 10 % in 20.1-23 min. The flow rate of the mobile phase was 0.2 mL/min. A volume of 10 μL of each sample was injected into the HPLC system.

Placenta

The experimental procedures of BPA in placenta were optimized with some modifications from previous studies (Jimenez-Diaz et al., 2010; Vela-Soria et al., 2011). In brief, placenta samples (1g) were placed into a 15mL falcon tube and homogenized with 1mL of water. Next, 10 μL of 13C-labeled internal standard 2

μg/mL were spiked, and 100 μL of 1M ammonium acetate and 10 μL of β-glucuronidase enzyme were added. The sample was incubated at 37 ℃ for 2 hr to allow for the deglucuronidation of the BPA. The samples were extracted by adding 5 mL ethyl acetate and shaking again for 10min and then the mixture was centrifuged for 10 min at 3000 rpm. The organic layer was transferred to a clean falcon tube and repeated this extraction procedure. The transferred samples were evaporated to dryness at room temperature under the nitrogen stream. The residue was dissolved in 100 μL of 90 % methanol in water and then centrifuged for 10 min at 3000 rpm, prior to its injection into the LC-MS/MS. The analysis method for LC-MS/MS was mentioned in serum and breast milk

2.2.3 Quality Assurance and Quality Control

For each batch of 20 samples analyzed, a method blank, and a pair of matrix-spiked (QC check) samples were analyzed. Calibration curves for measurement of BPA in biological sample media were constructed with method blanks - Milli-Q water for urine, bovine serum (Sigma-Aldrich, USA) for serum, pooled extra-samples for placenta and breast milk. We made three calibration curves and applied them to determine the BPA at the beginning, middle and the end of the analysis, respectively. BPA was not detected or ignorable (less than LODs) in the methods blanks (Figure S1 of Appendix). In order to avoid any possibility of contamination (Calafat et al.,

2013; Geens et al., 2009; Liao et al., 2012), Lab supplies such as containers and tubes were pre-washed with Milli-water and acetonitrile and were monitored to confirm them prior to each assay (Table S1 of Appendix). To evaluate the accuracy and precision of the method, the intra-day variations were assessed with five consecutive injections of the spiked samples and inter-day variations were determined by measuring the spiked samples with five injections on three consecutive days. They both were tested at four different levels of spiked samples, i.e., LOQ level, low, medium and high (Table S2, S3 of Appendix). The accuracy of the validated method ranged from 83.9 to 128 % in serum, 95.8 to 108 % for urine, 80.3 to 103 % for placenta, and 85.8 to 106 % in breast milk. The precision (% coefficient of variation) ranged from 4.7 to 24.8 (% CV) in serum, 4.5 to 13.5 (% CV) for urine, 3.3 to 17.1 (% CV) for placenta and 3.6 to 19.6 (% CV) for breast milk. The regression coefficient of calibration standards, injected at concentrations ranging from 1.0 to 250 ng/mL (9 levels included) was greater than 0.99 in urine (from 1.0 to 100 ng/mL in serum, placenta and breast milk). As a check for instrumental drift in response factors, a midpoint calibration standard was injected before sample injection. A pure solvent (methanol) was injected as a check for carry-over of all chemicals from sample to sample the limit of detection (LOD) was calculated with a standard deviation of the response and slope of the calibration curve. The limit of detection (LODs) was determined at 0.4 ng/mL for BPA serum, urine and 0.3 ng/g or ng/mL for BPA in placenta, breast milk.

2.2.4 Creatinine and specific gravity

Creatinine (Cr) and specific gravity (SG) levels in urine were analyzed at Sam-Kwang Medical Laboratories (Seoul, Korea). Because creatinine concentrations may be confounded by muscularity, physical activity, urine flow, time of day, diet, and disease states, creatinine adjustment may not be appropriate (Boeniger et al., 1993). So, we used specific gravity rather than creatinine and urinary dilution was normalized with following equation (Mahalingaiah et al., 2008; Meeker et al., 2013);

$$Pc = P \times [(\text{median of SG}-1)/(SG-1)]$$

Where Pc is the SG-adjusted BPA concentration (μg/L), P is the observed BPA concentration (μg/L), and SG is the specific gravity of the urine sample. The median of SG is 1.015 for the pregnant women's, and 1.009 for the neonates' urine. But Creatinine adjustment was also performed for comparability with other studies that previously reported. Creatinine-adjusted concentrations were calculated as the quotient of the measured the BPA and the creatinine levels in urine samples.

2.2.5 Statistical analysis

For chemicals that were detected no chromatogram, N.D. (no chromatogram detected) used to replace the zero, and for chemicals that were detected chromatogram below the Limit of detection (LOD), a proxy value, i,e, LOD divided by square root 2 (Hornung and Reed, 1990), was used. Kruskal–Wallis test was used for testing significant differences between categorical groups. Mann–Whitney U test was used for testing significant difference between pregnant women and their neonates. Non-parametric correlations between biological samples, i.e., maternal urine, maternal serum, and placenta were assessed using Spearman correlation. All statistical analyses were conducted using SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

2.3 Results

2.3.1. Participant characteristics

In the BPA analyses, 318 pregnant women provided serum, urine, breast milk, and placenta samples, as well as cord serum and urine samples from their infant. Table 2-1 provides a description of the characteristics of the participants. The mothers were primarily young (66.1 % < 40 years old), and a small percentage of the mothers were overweight (12.9 %, BMI > 25.0). Approximately 39 % of the participants were expecting their first child, while the other 61.3 % had at least one child already. Approximately 69.5 % of the mothers had their baby by vaginal birth, and 28.5 %

gave birth within 37 weeks. Approximately 51 % of the neonates were male, and 73.3 % had birth weights < 3.5 kg. BPA levels were significantly higher in the serum of 40-year-old pregnant women and in the urine of women who delivered by cesarean section (Table 2-1).

Table 2-1. Bisphenol A levels in pregnant women by Characteristics groups

Variable	N (%)	Median (Interquartile range, μg/L)	
		Serum	Urine (adjusted specific gravity)
All	318	1.56 (1.20, 2.45)	3.31 (1.52, 9.36)
Pregnant women			
age (years)			
22-29	47 (14.8)	1.59 (1.16, 2.22)	5.23 (1.45, 15.7)
30-39	248 (78.0)	1.54 (1.21, 2.58)	3.31 (1.73, 8.85)
40-46	23 (7.2)	2.42 (2.14, 6.20)*	1.20 (0.47, 2.12)
Body mass index (kg/m^2)			
<18.5	113 (35.5)	1.58 (1.08, 2.35)	3.88 (1.87, 7.04)
18.5-24.9	164 (51.6)	1.55 (1.29, 2.45)	2.95 (1.38, 9.12)
25.0+	41 (12.9)	1.58 (1.16, 2.63)	3.11 (1.27, 33.9)
Parity			
0	123 (38.7)	1.56 (1.23, 2.81)	3.15 (1.56, 7.17)
≥ 1	195 (61.3)	1.57 (1.20, 2.35)	3.33 (1.50, 9.36)
Cesarean section			
NSVD[a]	221 (69.5)	1.69 (1.29, 2.66)	2.94 (1.44, 6.15)
Cesarean section	97 (30.5)	1.42 (1.09, 2.05)	5.46 (1.56, 34.6) *
Gestational period (days)			
≤ 259 (37 weeks)	2 (0.6)	1.19 (0.84, 1.54)	38.9 (-)
260-294 (37-42 weeks)	316 (99.4)	1.58 (1.20, 2.47)	3.23 (1.52, 8.85)
Income (US$/month)			
<2900	77 (28.2)	1.46 (1.20, 2.35)	3.67 (1.73, 6.45)
2900-5800	121 (44.6)	1.67 (1.29, 3.03)	2.93 (1.63, 8.54)
>5800	72 (27.3)	1.71 (1.37, 2.47)	2.58 (1.31, 6.90)
Neonates			
gender			
Female	155 (48.7)	1.54 (1.16, 2.39)	3.33 (1.52, 8.39)
Male	163 (51.3)	1.61 (1.29, 2.60)	3.15 (1.63, 13.4)
Birth weight (Kg)			
<3.0[b]	59 (18.6)	1.54 (1.06, 2.35)	2.61 (1.45, 6.35)
3.0-3.5	174 (54.7)	1.61 (1.22, 2.52)	4.08 (1.94, 12.6)
>3.5	85 (26.7)	1.54 (1.20, 2.39)	2.29 (1.09, 10.2)

Birth length (cm)			
<50[b]	130 (40.9)	1.54 (1.20, 2.35)	4.11 (1.73, 8.85)
≥ 50	188 (59.3)	1.64 (1.23, 2.69)	2.73 (1.45, 9.36)
Head circumference (cm)			
<34 [b]	121 (38.1)	1.63 (1.23, 2.60)	3.82 (1.63, 7.17)
≥34	197 (61.9)	1.54 (1.20, 2.35)	2.98 (1.51, 12.6)

[a] NSVD: Normal spontaneous virginal delivery, [b] Median or quartile value of the present study population

2.3.2. BPA levels and relationships in maternal and neonatal samples

Table 2-2 shows the geometric means and selected percentiles of BPA levels in the participants' biological samples. BPA was detected in 100% of the maternal and neonatal serum samples and in 90.2%, 82.2%, 82.1%, and 79.5% of the maternal urine, neonatal urine, placenta, and breast milk samples, respectively. The median BPA levels in maternal serum and urine were 1.56 μg/L and 2.86 μg/L [specific gravity (SG)-adjusted levels: 3.31 μg/L; creatinine-adjusted levels: 4.55 μg/g creatinine], respectively. The median BPA levels in cord serum and neonatal urine were 1.71 μg/L and 4.75 μg/L (SG-adjusted levels: 3.66 μg/L; creatinine-adjusted levels: 10.1 μg/g creatinine), respectively. BPA levels in maternal serum were lower than in cord serum ($p < 0.05$). Although there were no significant differences in either unadjusted or SG-adjusted urinary BPA levels between mothers and infants, creatinine-adjusted urinary BPA levels differed significantly between mothers and infants ($p < 0.05$) (Table 2-2). In addition, there appeared to be significant associations between maternal serum and

cord serum, maternal urine (either SG or creatinine-adjusted) and cord serum BPA levels ($p < 0.05$) (Table 2-3). Lower median BPA levels were found in placenta (0.53 μg/kg) and breast milk (0.73 μg/L) than in the other samples. There was a significant association between maternal urine and placenta BPA levels ($p < 0.05$).

Table 2-2. Descriptive statistics of BPA in pregnant women and their neonates

Sample		N	n>LOD	GM (GSD)	Percentile					
					Min	25th	50th	75th	95th	Max
Pregnant woman										
Serum (μg/L)		180	180	1.99 (2.39)	0.44	1.20	1.56	2.45	17.9	47.1
Urine	Uncorrected (μg/L)	224	202	4.37 (4.46)	<LOD	1.29	2.86	9.88	71.8	206
	SG corrected (μg/L/L)	195	174	4.58 (4.01)	<LOD	1.52	3.31	9.36	51.5	238
	Cr corrected (μg/g creatinine)	213	192	6.10 (3.72)	<LOD	2.18	4.55	12.0	74.6	207
Placenta (μg/kg)		257	211	0.62 (2.83)	<LOD	0.35	0.53	0.83	10.2	53.1
Breast milk (μg/L)		127	101	0.85 (3.19)	<LOD	0.34	0.74	1.79	7.74	43.2
Neonate										
Serum (μg/L)		230	229	2.08 (2.50)	<LOD	1.22	1.71	2.52	20.0	51.5
Urine	Uncorrected (μg/L)	152	125	5.27 (4.18)	<LOD	0.93	4.75	14.5	48.2	118
	SG corrected (μg/L)	108	87	4.62 (4.26)	<LOD	0.68	3.66	12.1	34.9	146
	Cr corrected (μg/g creatinine)	125	102	13.0 (4.06)	<LOD	2.30	10.1	28.3	116	493

LOD: Limit of detection; GM: geometric mean; GSD: geometric standard deviation; SG: specific gravity; Cr: Creatinine.

There was statistical significant difference in serum BPA and creatinine-corrected urinary BPA between pregnant women and neonate (non-parametric Mann–Whitney U test, $p < 0.05$).

Table 2-3. Correlations of BPA levels among biological samples

Covariate	Spearman correlation coefficient N									
	1	2	3	4	5	6	7	8	9	10
1. Maternal serum (μg/L)	1	0.016 156	0.019 144	-0.024 151	0.080 149	0.154 75	0.196* 160	-0.098 99	-0.038 72	-0.102 81
2. Maternal urine (μg/L)		1	0.943** 195	0.872** 213	0.232** 181	-0.112 97	0.142 170	0.250** 133	0.161 97	0.164 112
3. SG corrected maternal urine (μg/L)			1	0.947** 195	0.197* 159	-0.135 83	0.196* 150	0.147 121	0.057 92	0.085 105
4. Cr corrected maternal urine (μg/g creatinine)				1	0.211** 173	-0.036 92	0.217** 161	0.182* 129	0.090 96	0.113 110
5. Placenta (μg/kg)					1	-0.011 111	0.021 186	0.137 125	0.026 87	0.044 102
6. Breast milk (μg/L)						1	-0.049 88	-0.168 64	-0.176 42	-0.123 49
7. Umbilical cord serum (μg/L)							1	0.045 110	0.008 77	0.042 91
8. Neonatal urine (μg/L)								1	0.957** 108	0.906** 125
9. SG corrected neonatal urine (μg/L)									1	0.951** 108
10. Cr corrected neonatal urine (μg/g creatinine)										1

*p<0.05, **p<0.01

2.3.3 BPA ratios among the biological samples

Table 2-4 lists the BPA ratios for the samples from the pregnant women and their infants. The BPA ratios were calculated as the BPA level in the other biological sample types to that in maternal serum. The median (interquartile range) cord-to-maternal serum ratio was 1.12 (0.73–1.58) for the 160 mother-infant pairs in whom BPA was detected in both samples. The placenta-, maternal urine-, neonatal urine-, and breast milk-to-maternal serum ratios were 0.28 (0.16–0.46), 1.79 (0.69–5.86), 1.98 (0.13–8.04), and 0.51 (0.19–0.89), respectively. Furthermore, the median (interquartile range) cord serum-to-placenta ratio was 4.03 (2.12–6.95) and neonatal urine-to-cord serum ratio 1.95 (0.23–6.20).

Table 2-4. BPA concentration ratio for maternal and neonatal tissues

Tissue	N	Mean±SD	GM (GSD)	Percentile			
				25th	50th	75th	95th
Maternal tissue distribution							
Maternal urine / maternal serum	144	8.21±21.7	2.23 (5.31)	0.69	1.79	5.86	29.9
Placenta[a] / maternal serum	149	1.03±3.68	0.28 (3.70)	0.16	0.28	0.46	5.31
Breast milk / maternal serum	75	1.42±2.83	0.51 (4.06)	0.19	0.51	0.89	10.5
Cord serum / maternal serum	160	2.83±5.94	1.17 (3.42)	0.73	1.12	1.58	15.2
Neonatal urine / maternal serum	72	5.90±9.73	2.78 (5.11)	0.13	1.98	8.04	28.2
Neonatal tissue distribution							
Cord serum / placenta[a]	186	9.20±17.1	3.80 (3.88)	2.12	4.03	6.95	45.8
Neonatal urine / cord serum	77	5.01±8.01	2.35 (4.78)	0.23	1.95	6.20	25.6

[a] The unit of placenta was ng/g, ng/mL for serum and breast milk

2.4 Discussion

BPA biomonitoring in a mother and her fetus is generally performed using maternal serum, urine, or cord serum samples; however, there is limited information on the distribution of BPA in other biological sample types collected from the mother–infant pair. The total (free and conjugated) BPA levels were determined in various tissues and bodily fluids obtained from mother–infant pairs, including maternal serum and urine, breast milk, and the placenta, as well as cord serum and the first urine of neonates. Spot urine samples are usually used to monitor exposure to chemicals; however, the urinary volume influences the extent of urinary dilution and thus the concentration of excreted substances (Carrieri et al., 2001). Therefore, urinary BPA levels in mother and infant urine must be adjusted to account for this dilution. Studies have confirmed an influence of age on creatinine concentrations in spot urine samples, and creatinine in the infant at birth is probably maternal in origin (Carrieri et al., 2001; Finney et al., 2000). Consequently, urinary BPA levels were adjusted by specific gravity instead of by creatinine in the correlation analysis and BPA ratio calculation. Significant correlations were found between some biological sample types (i.e., maternal and cord serum, placenta and maternal urine, and maternal and neonatal urine) (Table 2-3). Meanwhile, the BPA concentrations in the sample types of interest relative to maternal or cord serum were diverse (Table 2-4).

As for the BPA level correlations among the various samples, maternal serum and cord serum showed a significant association, in agreement with a previous study (Lee et al., 2008; Aris, 2014). Moreover, there was only a significant correlation between maternal urine and cord serum, maternal urine, and placenta. Such correlations can be influenced by various factors (e.g., medium characteristics, sampling time, and detection level). Maternal serum and urine were collected on the same day before delivery. Because of BPA metabolism, which is excreted in urine within 24 h, serum levels, which reflect current exposure, might not be correlated with urine levels. Although we did not find in this study, a study in China reported a positive association between BPA levels of serum and urine in the general population. These paired samples were the first morning voids after an over 8 h fast (Zhang et al., 2013). Since BPA exposure was no longer within the fasting time, BPA in maternal serum might contribute directly to urine level. Placenta, umbilical cord serum, and maternal urine reflect BPA exposure before delivery. Therefore, there were significant correlations between maternal urine and umbilical cord serum, maternal urine, and placenta. In addition, maternal serum and urine levels were not correlated with levels in breast milk, which was collected after 1 month. Moreover, unlike urine and serum, breast milk has different characteristics, such as lipid content and route of excretion from the body, which could affect BPA concentrations. Considering the associations between BPA level of maternal urine and other samples, it is important to evaluate

maternal and fetal BPA exposure using urinary BPA. Nonetheless, future studies should consider using replicate samples and total urine samples collected after 24 h.

In addition, it is necessary to determine the amount of BPA transferred via the placenta and breast milk. The ratios of BPA in various sample types were calculated using the same method as that used for the other paired samples, expressed relative to maternal serum BPA levels. Few studies have reported BPA concentrations in paired urine and blood samples from adults or in the cord and maternal blood. The median fetal serum-to-maternal serum BPA ratio was 0.035 (Zhang et al., 2013). , indicative of low concentrations of BPA present in fetal blood, as shown in previous studies (Schonfelder et al., 2002; Lee et al., 2008). However, in this study, BPA concentrations were higher in fetal serum than in maternal serum (median cord serum-to-maternal serum ratio: 1.12). These findings are in agreement with reports from Japan (Ikezuki et al., 2002; Kuroda et al., 2003). The median placenta-to-maternal serum BPA ratio and cord serum-to-placenta BPA ratio were 0.28 and 4.03, respectively. These results indicate that the fetus may be exposed to BPA through trans-placental absorption via diffusion and active transport, and the placenta is not an effective barrier against fetal exposure to BPA. In agreement with this, several studies have demonstrated the transfer of BPA across the placenta in perfusion experiments using human tissues (Balakrishnan et al., 2010; Mørck et al., 2010). In addition, the 95th percentile of the fetal serum-to-maternal serum ratio was 15.2, which was 13.5-fold higher than the median. Therefore, it is of primary concern to

determine the rate at which BPA crosses the placenta and whether serum BPA levels are higher in the fetus than in pregnant women. The range of BPA urine-to-serum ratios for adults was 3.0–250 (Teeguarden et al., 2011), which was similar to our results (median: 1.79, range: 0–205). Meanwhile, the median neonatal urine-to-cord serum ratio was 1.95 (range: 0–46.1). The urine-to-serum BPA ratio was indicative of BPA elimination from the body; however, there was no significant difference between the mother and her fetus.

The median breast milk-to-maternal serum ratio was 0.51 (range: 0.01–14.9), which was lower than the cord serum-, maternal urine-, and neonatal urine-to-maternal serum ratios. In general, chemicals with a log octanol–water partition coefficient (Kow) value > 5.0 tend to demonstrate high accumulation, and BPA has a log Kow of 3.32 (Nakao et al., 2015). Therefore, the accumulation rate of BPA in breast milk appears to be relatively low. Even though BPA has a low accumulation rate in the body, it has been detected in maternal serum–breast milk dyad samples, with the 95th percentile of 10.5, which was 20-fold higher than the median. These findings suggest that pregnant women are exposed repeatedly and persistently to BPA and that infants could encounter high BPA exposure via breastfeeding. In our results, when analyzing the BPA ratio for mother–infant pairs, we had to consider potential inappropriate associations, such as those caused by sampling time. The various sample types were collected before delivery, at delivery, and 1 month after delivery. However, a previous study reported that a single spot sampling approach might adequately

reflect the average exposure of a population to BPA, because the diet is the main source of exposure to BPA, and we are exposed continually to BPA (Ye et al., 2011). In addition, some samples were missing in this study. Therefore, we presented the BPA ratios for the 40 participants who provided a complete sample set. The 95th percentile of cord serum in complete data set of the 40 participants was higher than those of full data set of 318 participants. However, the other media samples did not differ between the two groups.

In the present study, the correlations between maternal and fetal BPA levels were found in various biological samples, and the BPA ratio and the variation in BPA levels provided information on the amount of BPA transferred from the mother to the neonate and infant via cord serum and breast milk. However, information on the partition coefficient calculated from human studies is limited, and maternal and fetal distribution coefficients have been determined from animal experimental data using conventional gestation PBPK models (Shin et al., 2011). Thus, the ratios of BPA levels in other samples types to those in maternal serum determined in this study in the mothers and their neonates are an important parameter for determining the partition coefficient in PBPK models and could be used to build gestation and lactation PBPK models for BPA. Monte Carlo simulations based on the distributions of input parameters are frequently used for such studies. This method consists of repeated computations using inputs selected at random from statistical distributions of each parameter to generate a statistical distribution for the output (EPA, 2006).

Using the Monte Carlo approach, the partition coefficient corresponding to the median and 95th percentile were determined. These parameters could be used to estimate BPA concentrations indirectly in biological samples other than maternal serum and urine.

In comparison with other studies, BPA concentrations were higher in maternal and neonatal urine but similar in the other samples (Table 2-5). These differences may be explained by lifestyle factors, such as dietary habits and use of plastic materials, and other sociodemographic considerations (Hoepner et al., 2013; Braun et al., 2011; Casas et al., 2013). Regarding the age of pregnant women, this study has shown a positive association with serum BPA levels, whereas study from China reported that older people had lower BPA levels (He et al., 2009). These higher values were found in pregnant women who had a cesarean section and highlight a potential contamination from medical devices, because of higher levels of free BPA and 95th percentile in their group (Vandentorren et al., 2011).

This study documented the BPA concentrations in various biological samples, including maternal serum, urine, placenta, breast milk and, cord serum and neonatal urine. In addition, we reported BPA levels in neonatal urine collected within 3 days of birth. Nevertheless, there are several limitations to this study. First, the BPA monitoring results reported in pregnant women did not include amniotic fluid or colostrum samples. So, BPA transfer from maternal blood to amniotic fluid and fetal exposure by amniotic fluid could not be identified. Second, these results demonstrate that different sample collection times, such as those used for spot urine and serum,

breast milk, and placenta, have the potential to confound the BPA exposure levels. Therefore, we assumed that BPA concentrations were constant in the bodies of the pregnant women. Thus, further studies with additional monitoring data for amniotic fluid and colostrum are needed. Moreover, we should standardize or adjust the timing of sample collection or measure BPA at multiple times to account for the variability in BPA levels.

Table 2-5. Comparison of median BPA levels reported other studies

Sample type	Country	Year of collection	N (age) [a]	BPA (μg/L or μg/kg)		References
				Median	Range	
Maternal serum	Korea	2011-2012	180	1.6	<0.4-47.1	Present study
	Japan	1989-1998	248	2.2	0.6-14.4	Yamada et al., 2002
	Germany	2000-2001	37	3.1	0.3-18.9	Schonfelder et al., 2002
	USA	2003-2004	86	2.7	-	Woodruff et al., 2011
	Korea	-	300	2.7	<0.6-66.5	Lee et al., 2008
	USA	-	27	14.1	0-154	Unal et al., 2012
	China	2010	30	0.8	<0.1-29.0	Zhang et al., 2013
Cord serum	Korea	2011-2012	230	1.7	<0.4-51.5	Present study
	Germany	2000-2001	37	2.3	0.2-9.2	Schonfelder et al., 2002
	France	2002-2005	106	0.9	0.1-4.8	Fenichel et al., 2011
	Korea	-	300	<0.6	<0.6 -8.9	Lee et al., 2008
	China	2010	30	<0.1	<0.1-0.8	Zhang et al., 2013
	USA	2010-2012	85	0.8	0.1-62.8	Gerona et al., 2013
	USA	-	27	1.3	0-25.6	Unal et al., 2012

Table 2-5. (Continued)

Sample type	Country	Year of collection	N (age) [a]	BPA (μg/L or μg/kg)		References
				Median	Range	
Maternal urine	Korea	2011-2012	224	2.9	<0.4-206	Present study
	USA	1998-2006	375	1.8	-	Donohue et al., 2013
	Netherland	2002-2006	100	1.2	0.3-46.0	Ye et al., 2008
	USA	2003-2006	344	1.3	-	Braun et al., 2011
	Spain	2004-2006	120	2.2	<0.1-123	Casas et al., 2013
	USA	2005-2008	71	1.2	-	Philippat et al., 2013
	Denmark	2010-2012	200	1.4	<0.1-25.2	Tefre de Renzy-Martin et al., 2014
Neonatal urine	Korea	2011-2012	152 (3day)	4.8	<0.4-118	Present study
	USA	2003	54	28.6	1.6-946	Calafat et al., 2009
	Germany	-	47 (1-5 months)	<0.45	<0.45-17.4	Volkel et al., 2011
	Canada	2009-2010	1 month	<0.2	<0.2-12.3	Arbuckle et al., 2015+
	USA	2012-2013	39 (3-6 days)	0.49	<0.1-11.2	Nachman et al., 2015

[a], Information on age was provided only in child urine, The subjects of Calafat's study were low-birth-weight infants from level Ⅲ NICUs (Neonatal intensive care unit)

Table 2-5. (Continued)

Sample type	Country	Year of collection	N (age) [a]	BPA (μg/L or μg/kg)		References
				Median	Range	
Placenta	Korea	2011-2012	257	0.5	<0.3-53.1	Present study
	Canada	1998-2006	21	3.0	0.6-64.0	Zhang et al., 2011
	Germany	2000-2001	37	12.7	1.0-105	Schonfelder et al., 2002
	Spain	2010	47	<0.2	<0.2-34.9	Jimenez-diaz et al., 2011
	Spain	2012	50	3.1	1.2-15.4	Vela-soria et al., 2011
Breast milk	Korea	2011-2012	127	0.7	<0.3-43.1	Present study
	USA	2003-2006	21	0.7	<0.2-10.8	Zimmers et al., 2014
	USA	2006-2008	27	0.8	<0.3-23.6	Mendonca et al., 2012
	USA	-	20	1.1	<0.3-7.3	Ye et al., 2006
	Japan	-	23	0.6	0.3-1.0	Sun et al., 2004
	Korea	-	100	10.4	0.7-29.9	Yi et al., 2010

Chapter 3. Occurrence of bisphenol A in urine and baby-food samples for 9~15-month infants

3.1 Introduction

Infants are regarded more vulnerable to chemical exposure than adults due to immature organ systems such as nervous, immune and respiratory system, rapid physical development, higher rates of respiration and metabolism, etc. (Healy et al., 2015; Heffernan et al., 2014; Vandenberg et al., 2011), and chemical exposure in infants can lead to adverse health outcomes later in life. (Wilson et al., 2007). Previous studies in rodents have found that early-life BPA exposure may affect the physiological functions including the developmental pituitary-thyroid axis and immune system (Li et al., 2016; Franssen et al., 2016; Fischer et al., 2016).

BPA was predominantly used in numerous consumer products, including reusable water bottles and food containers, thermal papers, and metal food can linings (Vanderberg et al., 2007; CDC, 2010). Because of its extensive use and widespread occurrence in the environment, exposure to BPA is ubiquitous among general populations in the world (Vandenberg et al., 2010a).

Infants are exposed through exposure routes such as ingestion, sublingual, skin, and inhalation from the dietary and non-dietary sources (Healy et al., 2015), but

dietary intake is of primary concern (Wilson et al., 2007; Zalko et al., 2011). BPA levels in foods including infant formula have been reported (Kuo and Ding, 2004; Schecter et al., 2010; Vandenberg et al., 2007; Wilson et al., 2007). Most BPA in food is excreted in the urine as metabolites, bisphenol-A glucuronide, and sulfate within about 6 hours, and BPA is eliminated within 24 hours (Volkel et al., 2002, 2005). Several studies have reported urinary BPA of infants (neonates), preschool children (2~5 years old) and school-aged children (>5 years old) in the U.S., Germany and China (Mendonca et al., 2014; Morgan et al., 2011; Becker et al., 2009; Calafat et al., 2008; Wang et al., 2014). However, BPA monitoring data is very limited for toddlers under two years old.

According to a US study, diet contributes 95 % of exposure to BPA were observed in preschool children aged 23–64 months, which suggested that solid food was a significant contributor (Morgan et al., 2011). In Korea, mothers usually feed their children homemade baby-foods such as soup of boiled rice soup, minced vegetable or fruits, etc. as weaning foods (Lee et al., 2006), and children have gradually consumed more and more solid food as they grow. To our knowledge, there were no published studies that have assessed simultaneously the BPA levels in urine and baby food samples, especially under two years old. In order to do so, the concurrent samples of urine and food–duplicate from the table were collected, and then BPA levels were measured in them.

3.2 Materials and Methods

3.2.1. Study population and Sample collection

The baby-food and urine samples were collected from Korean infants in the Children's Health and Environmental Chemicals in Korea Panel, or CHECK panel. CHECK panel is comprised of pregnant women-fetus pairs recruited from Seoul, Pyeongchon, Ansan, and Jeju during the period from 2012 to 2013 (Jeong et al., 2014b; Kim et al., 2017; Lee et al., 2018). Among the 318-paired mother and their infants recruited, 173 infants were followed up. Except for participants who did not provide samples for personal reasons and who did not provide enough urine sample, a total of 210 baby-food and 187 urine samples were collected from participating infants. The sampling times were 9-month-old (n=77), 12-month-old (n=74), 15-month-old (n=59) for baby-food; 9-month-old (n=83), 12-month-old (n=69), 15-month-old (n=35) for urine. Among them, 141 and 132 infants provided at least one sample for baby-food and urine, respectively. Demographic information is summarized in Table 3-1.

In sample collection, baby food samples were collected using the plastic bag (Zip-loc®) and 50mL falcon tube. For this purpose, tubes were pre-washed with methanol in the laboratory. Mothers collected one more set of the same kind and quantity of baby foods that the infants had consumed during the day and stored them in the plastic bag provided, and the actual amount of ingredients was recorded. Liquid

food samples such as breast milk, commercial milk, and beverage were also collected, but these samples were excluded in this study. For analysis, a baby food sample of each individual was pooled and homogenized using a blender. After one sample was homogenized, the blender was washed with soapy water and the organic solvents such as acetonitrile, methanol, and acetone.

The urine samples were collected using cloth diapers. Detailed information for sample collection can be found elsewhere (Kim et al., 2017). Briefly, cloth diapers were pre-washed four times with tap water in the laboratory. And diapers were dewatered using a commercial dehydrator and air-dried. Baby wipes and diaper cream were prohibited before and during the urine collection. Their urine-absorbed diapers were centrifuged at 1300 rpm for 10 min to extract urine.

Mother of the participating infants was instructed with a detailed guide with pictures describing sampling, storing, and delivery methods. The collected baby-food and urine samples were stored at -20 ° C in the refrigerator. The frozen samples were subsequently delivered to the laboratory in a cooler with an ice pack. Questionnaires to collect demographic information and data on self-reported consumption amounts of baby food and its ingredients in the 24 h preceding urine sample collection were administered.

All samples and data were processed blind. All participants provided written informed consent. The present study was approved by the institutional review board at School of Public Health, Seoul National University, Korea (IRB no. 8-2012-04-20).

3.2.2 Analysis of BPA

Urine

The experimental procedures of BPA in urine were optimized with some modifications from previous studies (Koch et al., 2003; Kho et al., 2008). In brief, urine samples (500 μL) were thawed at room temperature, and vortex mixed. Next, 10 μL of 13C-labeled internal standard (Cambridge isotope laboratory, USA) 1 μg/mL were spiked, and 100 μL of 1 M ammonium acetate (Sigma-Aldrich, USA) and 10 μL of β-glucuronidase enzyme (Roche, Biomedical, Mannheim, Germany) were added. The sample was incubated at 37 ℃ for 2 hr to allow for the deglucuronidation of the BPA. After hydrolysis, 300 μL of urine samples was mixed with 50 μL 0.1 % acetic acid in acetonitrile and centrifuged at 3000 rpm for 10min.

Total BPA was quantified by liquid chromatography-mass spectrometry with an AB Sciex 4000 tandem mass spectrometer (Framingham, MA, USA), coupled to a Shimazu HPLC system (Kyoto, Japan). Separation was achieved using a Shiseido ACR C18 column (3μm, 2.0 X 150 mm). However, urine samples were analyzed by online enrichment with C18 pre-column (Waters, Milford, MA, USA) and column-switching techniques. The mobile phase composition used in the chromatographic separation was optimized by binary mixtures of 0.1 % acetic acid in water (solvent A) and 0.1 % acetic acid in acetonitrile (solvent B). The flow rate of the mobile phase

was 0.2 mL/min. A volume of 10 μL of each sample was injected into the HPLC system.

Homemade baby-food

The experimental procedures of BPA in baby-food were optimized with some modifications from previous studies (Sanchez-Brunete et al., 2009). In brief, homogenized baby-food samples (1 g) were placed into a 15 mL falcon. Next, 10 μL of 13C-labeled internal standard (Cambridge isotope laboratory, USA) 2 μg/mL were spiked, and 20 μL of HCl (36 %) (Sigma-Aldrich, USA) was added. The samples were extracted by adding 5mL ethyl acetate and shaking again for 10 min and then the mixture was centrifuged for 10 min at 3000 rpm. The organic layer was transferred to a clean falcon tube and repeated this extraction procedure. The transferred samples were evaporated to dryness at room temperature under the nitrogen stream. The residue was dissolved in 100 μL of ethyl acetate followed by the addition of 50μL of BSTFA containing 1 % TMCS (Sigma-Aldrich, USA). The vials were closed and the mixture left react for 10 min at 60 ◦C. After the derivatization process, an aliquot (2 μL) of these solutions was injected in GC–MS.

GC–MS analysis was performed with an Agilent 7890A (Santa Clara, CA 95051, USA) gas chromatograph equipped with a PAL combi automatic injector, Model 300W Xe Light Source Set, and a mass spectrometric detector (MSD), Model 5975C,

equipped with an inert ion source. A fused silica capillary column DB-5MS (30 m×0.25 mm i.d. and 0.25μm film thickness), from Agilent (Santa Clara, CA 95051, USA), was used. The injector port of the GC was set at 250 ◦C. The samples were automatically injected using the splitless-injection mode. The transfer line of the GC to the MS was set at 270 ◦C, and the electron impact (EI) ion source of the MS was set at 250 ◦C. The ionization energy was 70 eV. The GC oven temperature program applied was as follows: the initial oven temperature was set at 60 ◦C, ramped at 20 ◦C/min to 220 ◦C, then ramped at 5 ◦C/min to 300 ◦C and held for 3.0 min. The carrier gas was high-purity helium (99.9 %) with a constant flow of 1mL/min. A solvent delay time of 5 min was used to protect the ion multiplier of the MS instrument from saturation.

3.2.3 Quality Assurance and Quality Control

For each batch of 20 samples analyzed, a method blank, and a pair of matrix-spiked (QC check) samples were analyzed. Calibration curves for measurement of BPA in urine and baby food media were constructed with method blanks - Milli-Q water for urine, cooked rice extracted with hexane for baby food. BPA was not detected or ignorable (less than LODs) in the methods blanks. To determine the level of contamination during sample collection, BPA levels were measured in blank sample (n =5) following the same procedure as urine sample collection using Milli-Q

water, but not detected. To prevent possible contamination, we washed all glassware materials used for pretreatment with detergent, rinsed with Milli-Q water, methanol and acetone, and subsequently dried in a furnace at 400 °C for 8 hours. In addition, the containers and tubes that were used for sampling, storing, and analysis of BPA were confirmed to be free from contamination with the target BPA.

To evaluate the accuracy and precision of the method, the intra-day variations were assessed with five consecutive injections of the spiked samples and inter-day variations were determined by measuring the spiked samples with five injections on three consecutive days. The accuracy and precision were tested at four different levels of spiked samples, i.e., LOD level, low, medium and high, but low and high in baby food (Table S2, S3 of Appendix). The accuracy of the validated method ranged from 95.8 to 108 % and, 82.2 to 116 % for urine and baby food, respectively. The precision ranged from 4.5 to 13.5 (% CV) and, 2.2 to 14.3 (% CV) for urine and baby food, respectively. The linearity of the method for urine was measured over the established working concentration range of 1.0-250 μg/L, with duplicated working standards for each concentration levels. (1 to 400 ng/mL for baby food). The regression coefficient of calibration standards was greater than 0.99 in urine and baby food. A pure solvent (methanol) was injected as a check for carry-over of all chemicals from sample to sample. The limit of detection (LOD) was calculated with the standard deviation of the response and slope of the calibration curve. The limit of detection (LODs) was determined at 0.4 μg/L for urine and 0.2 μg/kg for baby food.

3.2.4 Measurement of specific gravity and adjustment

Specific gravity levels in urine were analyzed at Sam-Kwang Medical Laboratories (Seoul, Korea). The urinary BPA levels were adjusted using specific gravity to avoid the influence of urine volume fluctuation and were normalized with following equation (Mahalingaiah et al., 2008; Meeker et al., 2013);

$$Pc = P \times [(\text{median of SG} - 1) / (SG - 1)]$$

Where Pc is the SG-adjusted BPA concentration (μg/L), P is the observed BPA concentration (μg/L), and SG is the specific gravity of the urine sample. The median of SG was the median SG value for this group of urine samples.

3.2.5 The composition of baby-food ingredients

The individual baby-food samples such as water, beverages, rice, vegetables, fruits, meat, fish and other baby-food were collected from mothers of the participating infants, and consumption amounts of the food for each subject were collected from questionnaires. Mothers reported on the amounts (weight) of the components of baby food or the actual food type, such as half of the apple, a piece of sliced cheese. If they reported actual food type, we converted into weight according to the food exchange table of the public health center. The ratio of components of each baby-food was

calculated as the quotient of the components and total consumption amounts of baby-food, and we averaged the ratio by infant's age.

3.2.6 Statistical analysis

For chemicals that were detected no chromatogram, N.D. (no chromatogram detected) used to replace the zero, and for chemicals that were detected chromatogram below the Limit of detection (LOD), a proxy value, i.e., LOD divided by square root 2 (Hornung and Reed, 1990), was used. Kruskal–Wallis test was used for a testing significant difference between categorical groups. The association of BPA between the baby-food and urine (9, 12 and 15 months) was tested with Spearman correlation coefficients (PROC CORR of SAS). The BPA levels in repeatedly collected urine and baby-food showed lognormal distribution, they were natural log-transformed before construction of statistical models for multiple comparisons among gender and collection months (PROC MIXED of SAS using random intercept) with Tukey's adjustment. All statistical analyses were conducted using SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

3.3. Results

3.3.1 Information of participants

Of the 173 subjects with demographic information, about a half of subjects who did not provide samples of food-duplicates with concurrent urine at 9, 12 and 15 months were excluded in the analysis. The characteristics of the participants were described in Table 3-1. The mothers were primarily young (66.5 % <35 years of age) and of normal weight (86.2 %, <25.0 of BMI). About forty percent of them were expecting their first child, while the other had at least one child. About 26.6 % of women made household income less than the equivalent of US $ 2,900 (Table 3-1).

Table 3-1. Characteristics of participants

Variables	N (%)
All[a]	173 (100)
Maternal age (years)	
25-29	18 (10.4)
30-34	97 (56.1)
35+	58 (33.5)
Body mass index (kg/m^2)	
<18.5	52 (30.1)
18.5-24.9	97 (56.1)
25.0+	24 (13.8)
Number of children	
1	68 (39.3)
≥ 2	108 (60.7)
Income (US$/month)	
<2900	39 (26.5)
2900-5800	75 (51.0)
>5800	33 (22.5)
Neonatal gender	
Female	85 (49.1)
Male	88 (50.9)

3.3.2 BPA levels in baby-food and urine

The median levels (>LOD detection frequencies, %) of BPA in baby-food were ND (not detected, 32.5 %), 0.47 (54.1 %) and 5.09 (76.3 %) ng/g wet weight for the 9th, 12th and 15th month of infants, respectively (Figure 3-1). There was a statistically significant increasing trend between baby-food BPA and the collection time points ($\rho = 0.426$, $p < 0.0001$); however, there was no statistical difference of food BPA between for boys' and girls' before the 15th month. The median levels (>LOD detection frequencies, %) of BPA in urine were 0.91 (85.5 %), 0.96 (85.5 %), 0.93 (85.7 %) μg/L, respectively [specific gravity (SG)-adjusted levels – 0.74, 1.11, 1.47 μg/L; creatinine-adjusted levels – 5.29, 3.94, 5.44 μg/g creatinine, for 9, 12, 15 months, respectively] (Figure 3-1). There were no significant differences among urinary BPA by neither collection time nor gender regardless of urine adjustment. Based on a linear mixed-effect model where the logged level of urinary BPA (either unadjusted or SG-/creatinine-adjusted) was a dependent variable, the corresponding body weight, gender, and food consumption amount were not significant. There were no significant correlations of BPA levels between baby food and urine samples.

The percentage composition of various food groups to total baby-food was shown in Figure 3-3. In the baby-food of 9-month-old infants, rice pap made the largest composition, accounting for 79.9 %. At 15^{th} month, cooked rice was the major composition, accounting for 51.0 % and rice pap was decreased by 19.4%. In other

words, the percentage of more solid foods such as cooked rice, meat, fish, dairy, egg, and other food increased from 20.1% to 80.6% between the baby-food of 9- and 15-month-old infant (Figure 3-3).

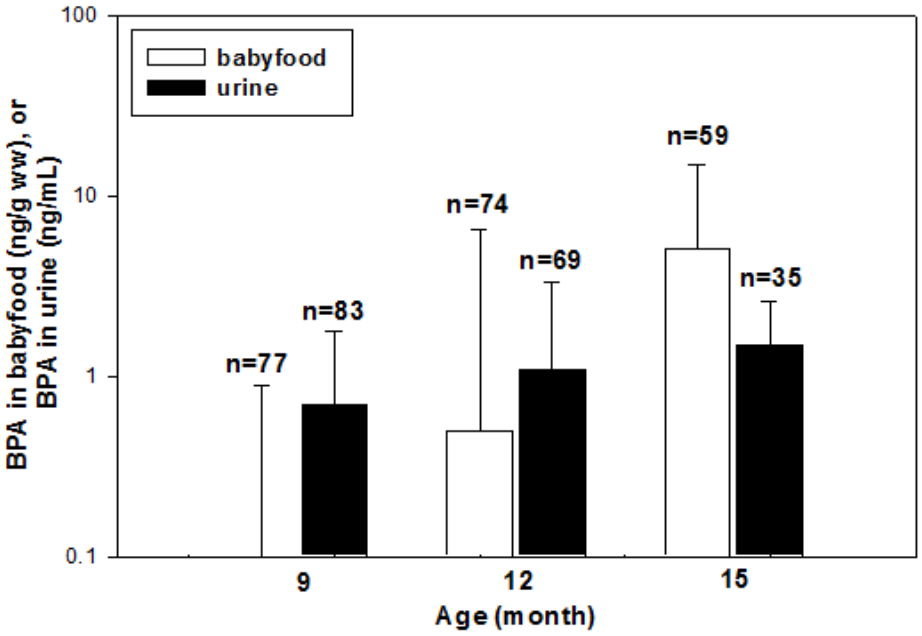

Figure 3-1. BPA levels in of baby-foods and urine by infants' age. Value is the median and Inter-quartile range.

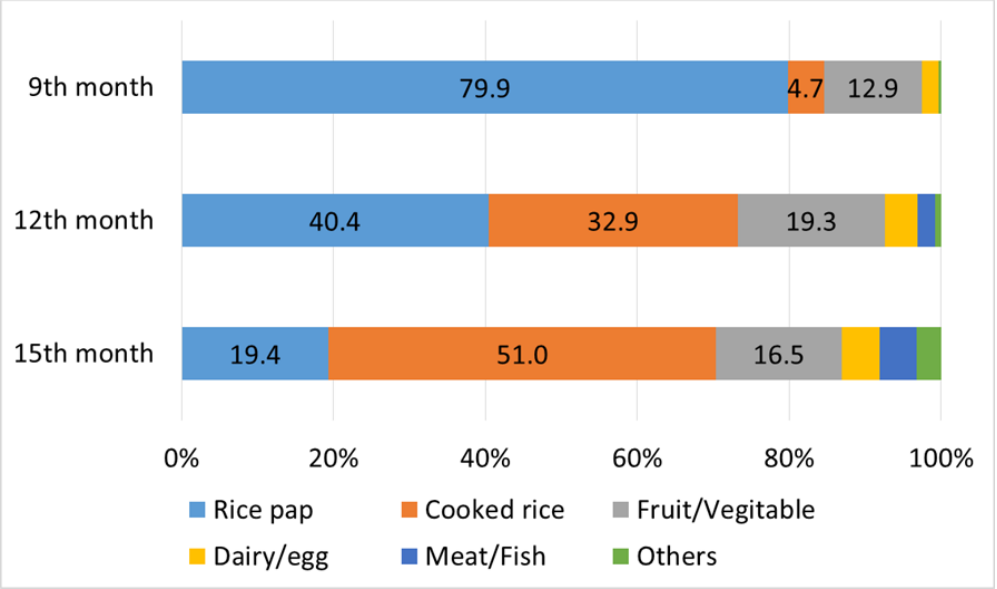

Figure 3-2. The daily average of the baby-food composition by infant's age. Total 210 table duplicates were used to make the baby-food composition from 60 subject families.

3.4 Discussion

One of the main exposure sources of BPA for the children are known as diet and environment (Geens et al., 2011; Morgan et al., 2011); however, data on BPA levels in infant urine and baby food samples collected simultaneously are very limited. Moreover, Korean infants have been fed with homemade baby-food traditionally at weaning, but few studies have been made with them. We made measurements of BPA in the weaning foods as table duplicates in infants of 9, 12 and 15 months in a Korean birth panel.

Among 210 weaning food samples, BPA was detected more frequently and more amounts as collection month up ($p < 0.0001$), which contrasted with constant urinary BPA (no difference, $p = 0.933$) at the same period. Although we just focused on infants between 9 and 15 months old, when they were 3-month old, they showed about three-fold lower urinary BPA [median (IQR), ND (ND ~ 0.283 ng/g wet), n=81] and they consumed breastmilk or formulas mostly.

In addition, this study showed only solid food BPA levels because the previous study reported that solid food was 7 times higher and significantly contributing to the BPA intake dose of children compared to liquid foods (Morgan et al., 2011). In addition, there are no significant correlations between BPA levels in infants' urine and breastmilk (Mendonca et al., 2012). In our CHECK study panel, the median BPA levels of breast milk at first month were 0.74 ng/g (IQR, 0.34-1.79), similar to BPA

levels in baby food at 12th month (Lee et al., 2018). According to the questionnaire, which was obtained from each subject family in the present study, breastfeeding was made at the 1st and 3rd month for each but it was reduced about half as much at 9th month and about one of ten at 15th month (Supplemental Table S4). The water contents of breast milk (about 87.5 %) and early weaning foods (about 90 %) are high (Vaughan et al., 1979, Ku et al., 2013). Figure 3-3 shows types of weaning food preferred by some of the participants illustrated with an average selection of menu and ingredients. Although they came from a convenient sampling of repeated questionnaires (n = 99) from sixty volunteers (30 boys and 30 girls), it was enough to show a cross-section of diet-changes in infants at weaning up to 15th month. Most popular food was rice pap (gruel, usually 90% water-contents) at 9th month and it was replaced by cooked rice (as in adult food, 60% water-contents) gradually while fruit and vegetable were selected at 13~19% during the period (Ku et al., 2013; Lee S and Lee G, 2011). Moreover, the intake rate of foods wrapped in packaging materials and/or containers such as meat, dairy products, and snacks increased at the 15th months. This is similar to the previous study on the diet of infants from 4 to 24 months, breast milk and commercial milk were predominantly consumed until 7-8 months; Fruits and juices were consumed after 9 months; Meat, dairy products, and snacks etc. were consumed after 12 months (Skinner et al., 2004). The BPA concentration levels of these foods have been previously reported. (Sakhi et al, 2014; EFSA 2006). Considering time track of diet change from solid food with high water contents to

solid food as infants grow, there are no differences in urinary BPA among age of infants. These results suggest solid food with high water contents did not drive the internal dose, or there were another source of exposure in the baby diet (other than weaning-food) or other environments although further study should confirm it.

Although there were no significant correlations of BPA levels between baby food and urine samples in all collection time, the significant association at 15^{th} month were shown in small sample size (Table S5; $\rho = 0.6$, $p = 0.028$).

The present observation that consumption of solid food explained body burden of BPA at the 15^{th} month was supported in the context of diet as the main exposure source with other studies. According to Children's Total Exposure to Persistent Pesticides and Other Persistent Organic Pollutants (CTEPP) study, dietary ingestion was probably the major route of exposure for 257 preschool children (130 North Carolina and 127 Ohio children) to BPA at their residences and daycare centers in 2000-2001 (Wilson et al., 2007). The study also showed that the 81 CTEPP Ohio children's consumption of both solid and liquid foods contributed to their exposures to BPA through the dietary ingestion route. In addition, their statistical modeling pointed that solid food were significant predictors of urinary BPA excretion (Morgan et al. 2011). Considering their research covered children' age for preschool (2~5 years old), the present study bridged exposure assessment of BPA for young children between newborn and children.

Compared with other studies, BPA levels of baby-food and infant urine in this

study are comparable or lower (Table 3-2 and 3-3). One thing to point out is the baby-food (weaning food) in the present study reflected Korean traditional culture. Although the current lifestyle of Korea became westernized and young parents follows a new trend from the US, and EU, feeding their babies with first meal or weaning foods seems to follow tradition: more homemade baby-food with rice pap or cooked rice with high water contents (Figure 3-2), which could explain relatively low BPA in the concurrent urine in part.

The present findings can fill the knowledge gaps in measurements of BPA in baby foods and corresponding urine samples for infants aged 9~15 months in Korea. However, there are some perceivable limitations to interpreting our data in the present study. The data only applies to infants aged 9-15 months in Korea since the difference in the type of baby-foods among the countries. In addition, the infant's food and urine samples were collected on the same day, but some measurement errors were likely to occur as the urine collection period could partly reflect BPA intake from the prior day. The present study also needs to consider the survey's estimation error with the actual intake of the infants and estimated intake provided by the parent. Furthermore, this study did not analyze the BPA level content in liquid food such as breast milk, powder milk, and other beverages. We took account of a report that the median levels of BPA were at least seven times higher in the solid food samples (3.5_3.6 ng/g) than in the liquid food samples (0.4_0.5 μg/L), and solid food significantly contributing to the children's dietary doses of BPA (Morgan et al., 2011). Thus, this content level of BPA

in liquid food was not considered in the present study. However, further studies about the assessment of all BPA exposure sources using quantitative relationship should be needed for infants.

Table 3-2. Comparison of bisphenol A in baby-food with other studies

Country	Year of sample collection	N	Infants' age	Baby-food type	Container type	BPA concentration (ng/g ww)		References
						AM	Median	
Korea	2012-2013	210	9-15 months	Solid food	Homemade	5.7	0.5	This study
USA	2010	9	-	Milk-based	Canned	0.4	N.D	Lorber et al., 2015
USA	2010	8	-	Milk-based	Canned	1.1	-	Schecter et al., 2010
USA	2008-2009	293	-	Milk-based	Canned	5.4	4.4	Ackerman et al., 2010
Canada	2008	99	-	Solid food	Jar	-	0.7	Cao et al., 2009
USA	2001	81	2-5 years	Solid food	Homemade	5.7	3.6	Morgan et al., 2011
USA	1997	14	-	Milk-based	Canned	5.0	4.2	Biles et al., 1997

Table 3-3. Comparison of urinary Bisphenol A of infants with other studies

Country	Year of sample collection	N	Infants' age	Urinary BPA (μg/L)		References
				GM	Median	
Korea	2012-2013	187	9-15 months	1.3	0.9	This study
China	2014	100	3-6 years	1.1	1.4	Lv et al., 2016
USA	2012	12	7-44 days	-	0.7	Nechman et al., 2012
China	2012	666	9-12 years	1.1	1.0	Wang et al., 2014
Canada	2009-2010	45-55	1-3 month	<0.2	0.2	Arbuckle et al., 2015
Canada	2007-2009	1031	6-11years	1.3	-	Bushnik et al., 2010
Germany	2008	47	1-5 months	-	<0.15	Volkel et al., 2011
USA	2006-2008	29	3-18 months	2.3	1.8	Mendonca et al., 2014
USA	2003-2004	314	6-11 years	3.6	3.7	Calafat et al., 2008
Germany	2003-2006	599	3-5 years	2.7	3.5	Becker et al., 2009
USA	2003-2006	213	1 year	-	3.9	Braun et al., 2011
USA	2001-2010	568	3 years	3.7	3.8	Hoepner et al., 2013
USA	2001	81	2-5 years	4.8	5.2	Morgan et al., 2011

Chapter 4. Associations between urinary BPA and biomarkers of oxidative stress in infants of Korea

4.1 Introduction

Although the use of bisphenol A (BPA) in infant feeding bottles has been banned, BPA is still used in polycarbonate plastic items designed for use by infants, such as toys, pacifiers, teething rings, and food storage containers (Becker et al., 2010; Sajiki et al., 2010; Geen et al., 2011). Because of the physiology and unique susceptibility of infants and observations of higher urinary BPA in young children, there has been increasing concern about the potential health effects of exposure during critical windows of early childhood development (Healy et al., 2015; Heffernan et al., 2014; Vandenberg et al., 2011).

In epidemiological studies, prenatal and postnatal BPA exposure has been associated with the adverse effects, including metabolic, pubertal development, infant growth and neurodevelopment outcomes (Braun et al., 2009, 2014; Wolff et al., 2010; Valvi et al., 2013; Casas et al., 2015). The study has highlighted endocrine disruption as a primary pathway of BPA action due to structural similarities between BPA and estradiol (Takayanagi et al., 2006). However, a few animal studies have also reported a capacity for BPA to induce oxidative stress (Bindhumol et al., 2003; Chitra et al.,

2003). In addition, the potential effect of BPA on hypothalamic-pituitary-adrenal (HPA) axis has been investigated in rodents' models (Panagiotidou et al., 2014; Poimenova et al., 2010). The primary end product of the HPA system in human is cortisol, a steroid hormone. The cortisol and corticotropin-releasing hormone (CRH) play key roles in mobilizing energy sores, modulating immune system activity, and orchestrating behavioral and physiological responses to potentially threatening events (Munck, Guyre, and Holbrook, 1984). The combined effects of cortisol lead to increased oxidative stress, in which the mitochondrial production of reactive oxygen species (ROS) exceeds the antioxidant potential, thereby causing damage to DNA/RNA (Joergensen et al., 2011). 8-OHdG (8-Hydroxy-2-deoxyguanosine) is produced by oxidation of the nucleoside, deoxyguanosine, and is well-documented usefulness as a systemic biomarker of oxidative stress (Il'yasova et al., 2012).

Several epidemiological studies have also reported a capacity for BPA to induce oxidative stress including 8-OHdG and free cortisol although previous associations were found in other populations or different study design (Asimakopoulos et al., 2016; Ferguson et al., 2016; Hong et al., 2009; Lv et al., 2017; Yang et al., 2009; Giesbrecht et al., 2017).

The study showed the associations between urinary BPA and 8-OHdG levels in four repeated urine samples of pregnant women (Ferguson et al., 2016). However, information was limited for associations between urinary BPA and oxidative stress such as 8-OHdG and free cortisol, particularly in infancy.

Therefore, the goal of this study was to explain the health effects of BPA by the relation with 8-OHdG and free cortisol during infancy, and evaluate the association between free cortisol and 8-OHdG. For this study, the BPA and oxidative stress biomarkers including 8-OHdG and free cortisol in repeatedly collected urine samples of the infant were analyzed, and then the association between urinary BPA and oxidative stress biomarker and the association between free cortisol and 8-OHdG were estimated.

4.2 Materials and Methods

4.2.1. Study population and sample collection

The monitoring data used in this study were provided by the Children's Health and Environmental Chemicals in Korea (CHECK) study that was launched in January 2011 and continued to November 2013. The CHECK cohort was composed of pregnant women and their babies recruited from Seoul, Pyeongchon, Ansan, and Jeju, and was expected to represent the general population of Korea (Kim et al., 2017; Lee et al., 2018). From a total of 318 mother-infant pairs, 190 infants for whom urinary BPA levels, oxidative stress marker levels, and covariate data were considered in the current analysis. In total, 271 urine samples were collected: 103, 53, 58, 42, and 15 samples at birth, 3 months, 9 months, 12 months, and 15 months after birth,

respectively. The demographic characteristics of the participating infants are shown in Table 4-1. The first urine sample from infants was collected within two days postpartum using a polyethylene urine collection bag (Urine Collector, ROOTICS corp., Korea). At 3, 9, 12, and 15 months after birth, urine samples were collected via extraction from a cloth diaper using a centrifuge. Detailed methods of urine collection can be found in a previous study (Kim et al., 2017). All samples were stored at - 80 ℃ until analysis. All mothers provided written informed consent, and all procedures were approved by the Institutional Review Board at the School of Public Health, Seoul National University, Korea (IRB no. 8-2012-04-20).

4.2.2 Chemical analysis

The experimental procedures for determining BPA in urine were optimized by modifying those used in previous studies (Kho et al., 2008; Koch et al., 2003a). In brief, urine samples (500 μL) were thawed at room temperature and vortex mixed. Next, 10 μL of 13C-labeled internal standard (Cambridge Isotope Laboratory, USA; 1 μg/mL) was spiked, and 100 μL of 1 M ammonium acetate (Sigma-Aldrich, USA) and 10 μL of β-glucuronidase enzyme (Roche, Biomedical, Mannheim, Germany) were added. The sample was incubated at 37 ℃ for 2 h to allow for the deglucuronidation of the BPA. After hydrolysis, 300 μL of the urine sample was

mixed with 50 µL of 0.1 % acetic acid in acetonitrile and centrifuged at 3000 rpm for 10 min.

Total BPA was quantified by liquid chromatography mass spectrometry with an AB Sciex 4000 tandem mass spectrometer (Framingham, MA, USA) coupled to a Shimazu HPLC system (Kyoto, Japan). Separation was achieved using a Shiseido ACR C18 column (3 µm, 2.0 × 150 mm). Urine samples were analyzed by online enrichment with C18 pre-column (Waters, Milford, MA, USA) and column-switching techniques. The mobile phase composition used in the chromatographic separation was optimized by binary mixtures of 0.1 % acetic acid in water (solvent A) and 0.1 % acetic acid in acetonitrile (solvent B). The flow rate of the mobile phase was 0.2 mL/min. A volume of 10 µL of each sample was injected into the HPLC system.

4.2.3 Quality Assurance and Quality Control

To validate the method, we followed the guidance of the US FDA (1998). For each batch of 20 samples analyzed, a procedural blank, a spiked blank, and a pair of matrix-spiked (QC check) samples were analyzed. Calibration curves for measurement of BPA in biological sample media were constructed with method blanks–Milli-Q water for urine. Calibration curves for measurement of BPA in biological sample media were constructed with method blanks–Milli-Q water for urine. To determine the level of contamination during sample collection, BPA levels

were measured in the blank sample (n =5) following the same procedure as urine sample collection using Milli-Q water, but not detected. To evaluate the accuracy and precision of the method, the intra-day variations were assessed with five consecutive injections of the spiked samples, and inter-day variations were determined by measuring the spiked samples with five injections on three consecutive days. Both of these were tested at four different levels of spiked samples, i.e., LOQ level, low, medium, and high (Table S2, S3 of Appendix). The accuracy of the validated method ranged from 95.8 to 108 %. The precision ranged from 4.5 to 13.5 % CV. The regression coefficient of the calibration standards injected at concentrations ranging from 1.0 to 250 µg/L was greater than 0.99. A pure solvent (methanol) was injected as a check for carry-over of all chemicals from sample to sample. The LOD was determined as 0.4 µg/L (calculated as 3.3 × slope / standard from the regression curve, ranging from 0 to 12.8 µg/L).

4.2.4 Measurement of urinary 8-OHdG and free cortisol

The level of 8-OHdG was measured using enzyme immunoassay by Cayman chemical company (Ann Arbor, MI, USA) following manufacturer's instructions. A spectrophotometric plate reader (TECAN infinite® M200, TECAN Group LTD., Mannedorf, Switzerland) was used for this purpose. Each experiment was carried out in duplicates and average values were used. The level of free cortisol was analyzed at

Samkwang Medical Laboratories (Seoul, Korea). This measurement was conducted using a Unicel DxI 800 Immunoassay System (Beckman Coulter, Brea, CA, USA).

4.2.5 Measurement of specific gravity and adjustment

Specific gravity levels in the urine were analyzed at Sam-Kwang Medical Laboratories (Seoul, Korea). Because creatinine concentrations may be confounded by muscularity, physical activity, urine flow, time of day, diet, and disease states, creatinine adjustment may not be appropriate (Boeniger et al., 1993). Therefore, we used specific gravity rather than creatinine, and urinary dilution was normalized with the following equation (Mahalingaiah et al., 2008; Meeker et al., 2013):

$$Pc = P \times [(\text{median of } SG-1) / (SG-1)]$$

where Pc is the SG-corrected BPA concentration (μg/L), P is the observed BPA concentration (μg/L), and SG is the specific gravity of the urine sample. The median SG for the urine of pregnant women was 1.015 and that for neonate urine was 1.009.

4.2.6 Statistical analysis

The distribution of urinary BPA, 8-OHdG and free cortisol for the entire sample were described using the geometric mean (geometric standard deviation) and median percentile with specific gravity corrected urinary BPA. For the analyses, urinary BPA

levels below the LOD were imputed by assigning a value equal to the LOD divided by the square root of 2 (Hornung and Reed, 1990). The correlations between urinary BPA and oxidative stress markers or other chemicals were found using Spearman correlation coefficients (Table 4-3).

The distribution was not normal, so log10-transformed urinary BPA levels were used in statistical analyses. The differences of urinary BPA among sample collection time were analyzed by regression analysis and analysis of variance (ANOVA) with Tukey's test for multiple comparisons.

To explore non-linear associations of urinary BPA with free cortisol and 8-OHdG, penalized regression splines of BPA exposure on free cortisol and 8-OHdG were evaluated using generalized additive models (GAM) after adjustment for infants' gender, age. All infants' gender, age were treated as categorical variables.

The random-intercept linear mixed models were used with adjustment for infants' gender, age and urinary levels of phthalate metabolites (MEP, MiBP, MnBP, MEHHP, MEOHP) and heavy metals (lead and mercury) to estimate the effect of BPA exposure on oxidative stress biomarkers.

Variable selection in the model was performed with reference to the method of previous studies (Cordell and Clayton, 2002). A strategy of this analysis was based on backward selection, in which we start with a model that includes all variables such as infants' gender, age, and chemicals with co-exposure and co-mechanism. Each variable was then deleted in turn from this full model, and the one that gives the least-

significant deterioration in fit is removed from the current model. At the next stage, each variable still in the model is again deleted in turn, and the one that gives the least-significant deterioration is again removed from the current model. In practice, at each stage, a variable that has previously been removed may be added again if they offer a significant improvement to the current model. The adjusted variables were selected among the significant variables in case the $p < 0.05$, and interaction terms were also investigated. Competing models were compared using Akaike information criteria (AIC), Bayesian information criteria (BIC), and the $R^{2.}$

Several sensitivity analyses were performed to verify the accuracy of the model. First, to further explore the interaction effects of urinary BPA and phthalate metabolites on oxidative stress biomarkers, we stratified the levels by the quartile of the urinary BPA into low ($\leq 25^{th}$ percentile), medium ($> 25^{th}$ percentile but $\leq 75^{th}$ percentile), and high subgroups. Second, to examine whether the association between BPA exposure and oxidative stress differed based on time point during infancy, we examined associations stratified by age of infants at sample collection in an attempt to identify sensitive time point. In addition, the associations between BPA exposure and oxidative stress at each sample collection point (infants' age) were further estimated based on linear mixed effect models adjusted for multiple urinary phthalate metabolites and heavy metals. These chemicals were included in the multivariate model based on their correlations with one another as well as their individual associations with the oxidative stress biomarkers.

All other data handling and analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and R.version 3.3.2 (The Comprehensive R Archive Network: http://cran.r-project.org).

4.3 Results

4.3.1 Characteristics of the study participants

The basic characteristics of the participants were shown in Table 4-1. A total of 318 mother-infant pairs were recruited in this study, and 190 infants [107 boys (56.3 %) and 83 girls (43.7 %)] were followed to 15 months after birth. The mothers were primarily young (66.3 % < 40 years old), and a small percentage of the mothers were overweight (10.0 %, BMI > 25.0). Approximately 36.8 % of the participants were expecting their first child, while the other 63.2 % had at least one child already. Approximately 51 % of the neonates were male, and 73.3 % had birth weights < 3.5 kg.

Table 4-1. Characteristics of participants

Variables	N (%)
All	190 (100)
Infants' gender	
Female	83 (43.7)
Male	107 (56.3)
Mothers' age (years)	
22-29	28 (14.7)
30-39	98 (51.6)
40-46	64 (33.7)
Mothers' BMI (kg/m^2)	
<18.5	61 (32.1)
18.5-24.9	110 (57.9)
25.0+	19 (10.0)
Parity	
1	70 (36.8)
≥2	120 (63.2)
Family monthly income (USD)	
<2900	43 (26.4)
2900-5800	73 (44.8)
>5800	47 (28.8)

4.3.2 Concentration of BPA, 8-OHdG, and free cortisol

BPA was detected in 66.3–82.2 % of samples and the specific gravity (SG) corrected geometric mean (GSD) of all urine samples was 1.9 (4.9) μg/L, and those in the 8-OHdG and free cortisol were 63.7 (2.2) μg/L, 15.6 (2.6) μg/dL, respectively. There was no significant difference in urinary BPA between girls and boys (Table 4-2). The SG corrected median (interquartile range, IQR) values of those at birth and at 3, 9, 12, and 15 months after birth were as follows: 3.9 (0.6-12.1) ng/mL, <LOD (<LOD~<LOD), 0.7 (<LOD~1.8) ng/mL, 1.1 (0.3~3.3) ng/mL and 1.6 (0.4~2.9) ng/mL for BPA, 83.3 (57.6~132) ng/mL, 56.2 (36.3~69.8) ng/mL, 33.7 (25.5~61.6) ng/mL, 55.7 (38.6~82.2) ng/mL, and 52.7 (31.4~99.8) ng/mL for 8-OHdG, 38.2 (27.5~52.5) μg/dL, 9.9 (6.4~14.1) μg/dL, 8.0 (4.2~13.1) μg/dL, 13.5 (8.1~24.1) μg/dL, and 15.7 (8.0~23.1) μg/dL for free cortisol. Urinary BPA was found to be lowest at 3 months after birth and highest at birth, with no difference of those from 9 to 15 months after birth. Urinary 8-OHdG and free cortisol levels were highest at birth and lowest at 9 months after birth (Figure 4-1).

Table 4-2. Descriptive statistics of specific gravity adjusted urinary BPA, 8-OHdG and free cortisol in infants (N=190)

Sample	N	GM (GSD)	Percentile					
			Min	25th	50th	75th	95th	Max
BPA (ng/mL)	271	1.88 (4.85)	<LOD	<LOD	0.77	3.69	27.5	146
8-OHdG (ng/mL)	271	63.7 (2.20)	5.70	37.2	61.0	95.7	282	1180
Free cortisol (ug/dL)	271	15.6 (2.57)	0.70	7.98	16.7	34.5	66.8	84.0

LODs for BPA, 8-OHdG and free cortisol were 0.4 ng/mL, 0.4 μg/dL, and 33 pg/mL, respectively.

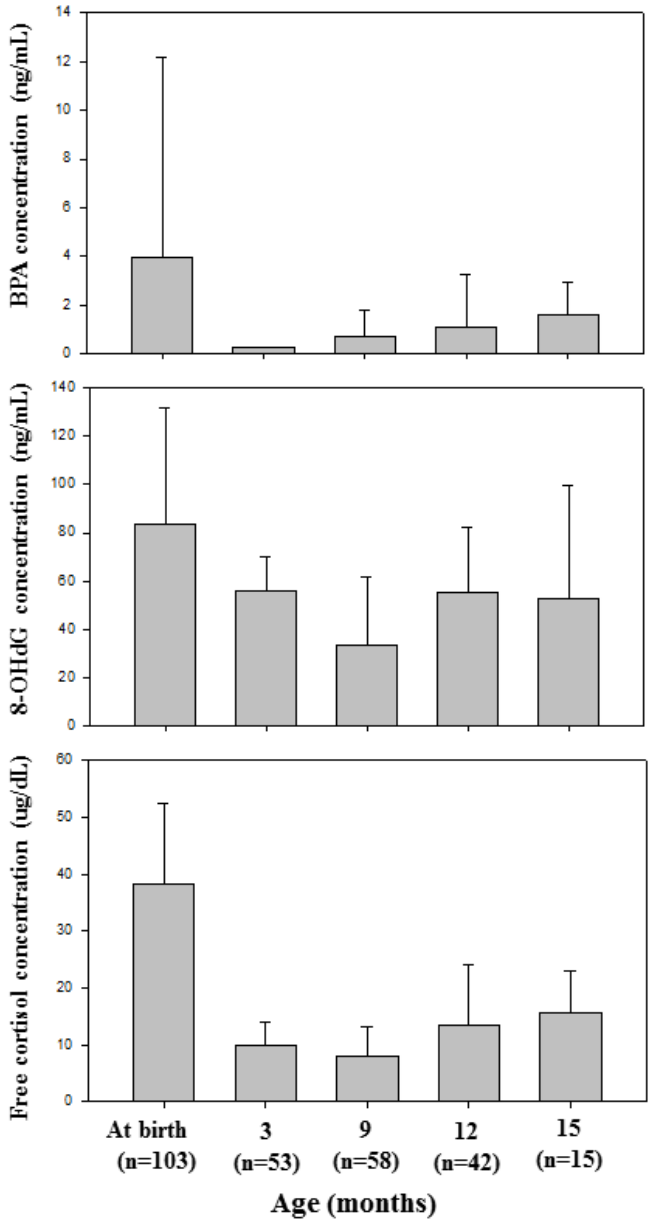

Figure 4-1. Urinary concentrations of BPA, 8-OHdG and free cortisol by infant's age (each bar and error bar represent p50 and p75, respectively).

4.3.3 Relationship among the urinary levels of BPA, 8-OHdG, and free cortisol

To investigate the possible non-linear associations between the urinary BPA levels and oxidative stress markers, penalized regression splines of urinary BPA on 8-OHdG and free cortisol levels were evaluated (Figure 4-2). In this study, the BPA levels were significantly associated with both 8-OHdG and free cortisol levels with non-linear relationships ($p < .0001$). The non-linear relationships with upward curvature were observed between the urinary BPA and oxidative stress marker levels.

The associations between BPA levels and each oxidative stress biomarker are presented in Table 4-3 and 4-4. Urinary BPA levels showed weak to moderate but significant correlations with phthalate metabolites including MEP, MiBP, MnBP, MEHHP, MEOHP, and lead. The strongest correlation coefficients were between BPA and MnBP. BPA was significantly correlated with 8-OHdG and free cortisol (Table 4-3). In the linear mixed effect model, adjusted for phthalate metabolites and lead, BPA was significantly associated with increases in 8-OHdG. MnBP was remained statistically significant, but coefficients for other chemicals lost statistical significance when models were additionally adjusted for covariates that were associated with BPA (Table 4-4). In addition, the BPA was also found to interact strongly with MnBP exposure ($p<0.05$) in the levels by the quartile of the urinary BPA. In a low group of BPA, 8-OHdG levels in a high group of MnBP was higher than

those of low and medium groups of MnBP (Figure 4-3). In the free cortisol, there was a significant association between urinary BPA and free cortisol levels in correlation (Table 4-3; $\rho = 0.3$, $p < .0001$), but no association in single exposure models after adjustment for infants' gender and age.

In sensitivity analyses, BPA levels showed statistically significant correlations with urinary phthalate metabolites, including: MnBP, MiBP at birth and MnBP at 3 months after birth, and MEP, MnBP at 9 months after birth. 8-OHdG was significantly correlated with compounds, including: BPA, MiBP, MnBP, and Pb at birth and MEHHP at 3 months after birth and MEP at 9 months after birth (Table S6). Therefore, we considered the effect of including multiple phthalate metabolites and Pb in the model due to co-exposure to these compounds. In a model of 8OHdG, effect estimates of BPA were diminished compared with the single-exposure model, (β: 0.17, 95% CI: 0.05, 0.28), but BPA remained the significant coefficient after adjustment for other compounds (β: 0.11, 95% CI: 0.03, 0.20) (Table S7).

However, to identify potentially sensitive time points for the relationship between BPA exposure and oxidative stress, we examined the associations at collection time. BPA was significantly associated with oxidative stress biomarkers, and coefficients for Pb were remained the statistical significance at birth. The significant associations between 8-OHdG and BPA were observed at 9 months after birth (β: 0.26, 95% CI: 0.08, 0.44) and other compounds lost statistical significance. However, there were no associations between BPA exposure and oxidative stress

biomarkers in other time points. The free cortisol levels were not associated with higher BPA levels at all collection points (data not shown). In stratified analyses, the association between BPA and oxidative stress were variable and imprecise. However, in a total of data at 9, 12, 15 months after birth, the association between BPA and 8-OHdG were observed, and not with other compounds (Table S7).

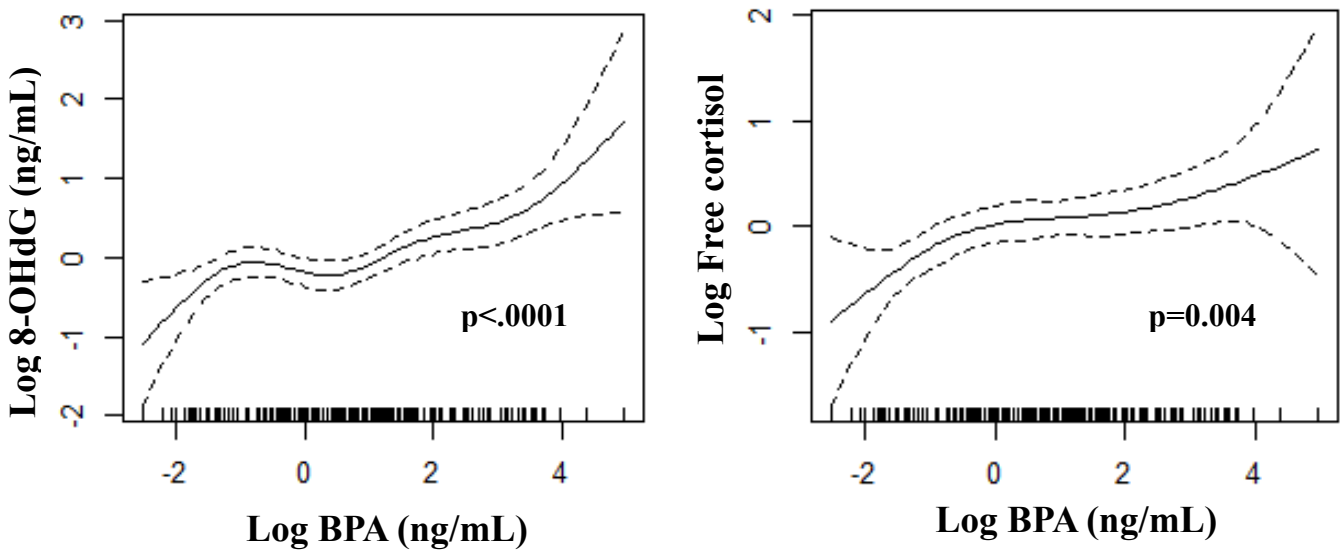

Figure 4-2. Effects of urinary BPA on 8-OHdG and free cortisol. Each graph and p-values is a generalized additive model (GAM) output after adjusted for infant's gender and age. The solid lines depict the regression lines form the GAM analyses, the area between the broken lines represent the 95% CI of these regression.

Table 4-3. Spearman correlations among specific gravity-adjusted chemicals

	Spearman correlation coefficient N								
Covariate	1	2	3	4	5	6	7	8	9
1. BPA (ng/mL)	1	0.249*** 271	0.212** 271	0.438*** 271	0.243*** 271	0.157** 271	0.249*** 243	0.242*** 271	0.303*** 271
2. MEP (ng/mL)		1	0.494*** 271	0.596*** 271	0.428*** 271	0.419*** 271	0.042 243	0.174** 271	0.197** 271
3. MiBP (ng/mL)			1	0.720*** 271	0.608*** 271	0.650*** 271	-0.162* 243	0.084 271	-0.123* 271
4. MnBP (ng/mL)				1	0.667*** 271	0.632*** 271	0.035 243	0.251*** 271	0.196** 271
5. MEHHP (ng/mL)					1	0.968*** 271	0.034 243	0.104 271	0.063 271
6. MEOHP (ng/mL)						1	-0.072 243	0.018 271	-0.071 271
7. Pb (ng/mL)							1	0.286*** 243	0.473*** 243
8. 8-OHdG (ng/mL)								1	0.422*** 271
9. Free cortisol (μg/dL)									1

*P<0.05, **p<0.01, ***p<.001

Table 4-4. Parameter estimates for 8-OHdG and free cortisol in association with urinary BPA (n=271).

Outcomes	Covariate	Estimate	95% confidence limit	*p* value
Ln 8-OHdG	Intercept	3.73	(3.37, 4.10)	<.0001
	Ln BPA	0.12	(0.03, 0.20)	0.01
	Infant gender (female)	reference	-	-
	(male)	-0.06	(-0.28, 0.15)	0.550
	Infant age (at birth)	reference	-	-
	(3 months)	0.09	(-0.36, 0.54)	0.682
	(9 months)	-0.54	(-0.83, -0.23)	0.001
	(12 months)	-0.32	(-0.64, 0.01)	0.057
	(15 months)	-0.31	(-0.75, 0.13)	0.163
	Ln MnBP	0.22	(0.10, 0.34)	0.001
Ln free cortisol	Intercept	3.54	(3.33, 3.75)	<.0001
	Ln BPA	0.02	(-0.05, 0.09)	0.522
	Infant gender (female)	reference	-	-
	(male)	-0.04	(-0.24, 0.16)	0.693
	Infant age (at birth)	reference	-	-
	(3 months)	-1.46	(-1.84, -1.08)	<.0001
	(9 months)	-1.57	(-1.83, -1.31)	<.0001
	(12 months)	-0.89	(-1.16, -0.63)	<.0001
	(15 months)	-1.00	(-1.39, -0.62)	<.0001

The levels of BPA, 8OGdG, and free cortisol were adjusted by specific gravity. Results from linear mixed effect models with subject-specific random intercepts.

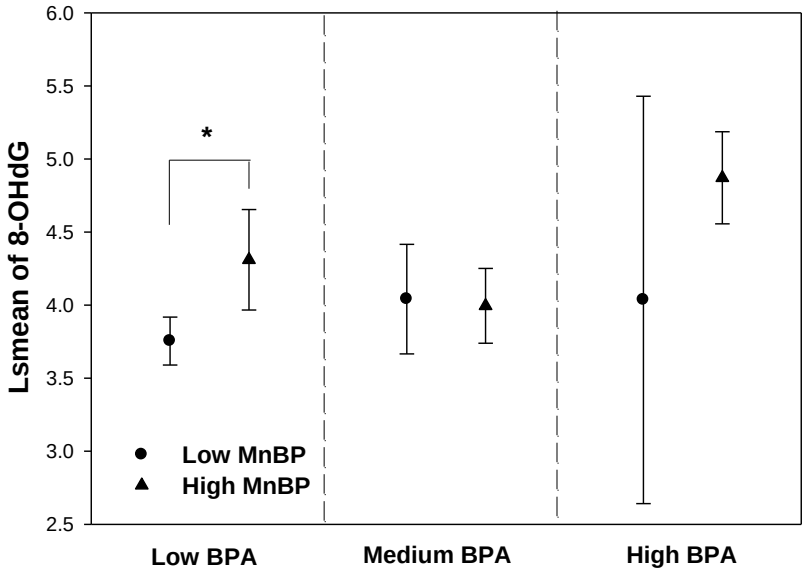

Figure 4-3. Interaction effects of urinary BPA and MnBP on 8-OHdG among infants. Values showed were the least squares means and 95% confidence interval. *p<0.05

4.4 Discussion

Infants may be exposed repeatedly to BPA from mother before birth and from breast milk, baby foods, and other sources after birth (Healy et al., 2015). BPA is an endocrine-disrupting chemical that may affect the development of infants (Harley et al., 2013; Wetherill et al., 2007). Recently, the oxidative stress effects of BPA on porcine embryonic development was reported (Guo et al., 2017). However, there is little information for oxidative stress of BPA exposure during early life stage.

BPA also generate ROS by decreasing the activities of antioxidant enzymes (Bindhumol et al., 2003; Ooe et al., 2005), and affect the function of mitochondria (Guo et al., 2017; Moon et al., 2012). Free cortisol is a hormone that is produced by the adrenal glands, and it is often released in response to stress (Kim et al., 2018). The studies suggested that possible mechanism is that the combined effects of cortisol lead to increased oxidative stress, in which the mitochondrial production of reactive oxygen species (ROS) exceeds the antioxidant potential, thereby causing damage to other molecules such as lipid, proteins and DNA/RNA (Finkel and Holbrook, 2000; Maynard et al., 2009; Joergensen et al., 2011). 8-OHdG is formed by hydroxyl radical attack on the C-8 position of deoxyguanosine in DNA and, among the many types of oxidative DNA damage, is the most common in cellular DNA (Kasai, 1997)., and is well-documented usefulness as a systemic biomarker of oxidative stress for establishing an association with adverse health outcomes (Il'yasova et al., 2012).

Therefore, in the present study, we examined the association between repeated measures of urinary BPA and 8-OHdG and free cortisol as biomarkers of oxidative stress during infancy including the perinatal period, and between free cortisol and 8-OHdG. However, we observed that urinary BPA levels were associated with significant increases in 8-OHdG, but not in free cortisol.

Because we did not find any association between of parental characteristics and oxidative biomarkers, only infants' gender and age were considered in our models to determine the association between urinary BPA and oxidative biomarker, including 8OHdG and free cortisol. Urinary BPA was significantly correlated with phthalate metabolites and lead, but only MEP, MnBP and Pb were significantly correlated with 8-OHdG and free cortisol (Table 4-3). Because of these result indicated that infants were simultaneously exposure to BPA, phthalate, and lead and then these chemicals affected the 8-OHdG and free cortisol levels, and were considered in these models. In addition, the studies on the association between phthalate metabolites or lead and 8--OHdG have already been reported (Kim et al., 2018; Ni et al., 2014). Of the phthalate metabolites examined in association with 8-OHdG, the greatest effect estimate for 8-OHdG was for MnBP. Thus, we built an additional model including BPA and MnBP as well as covariates. Resultantly, BPA with MnBP was associated with an increase in 8-OHdG (Figure 4-3).

In several epidemiologic studies, 8-OHdG were increased by urinary BPA in adults, cashiers and pregnant women (Ferguson et al., 2016; Hong et al., 2009; Lv et

al.., 2017; Watkins et al., 2015). These relationships were specific to post-menopause women because higher estrogen levels of pre-menopausal women might have inhibited BPA from binding to ERs, yet this hypothesis needs to be further investigated. (Yang et al., 2009). Although these epidemiologic studies targeted pregnant women, adults, the associations were supported by prior results indicating associations between BPA exposure and 8-OHdG.

In the association between urinary BPA and free cortisol, although there was a significant correlation, no association in a linear mixed effect model. The period between 2 and 4 months after birth is one of tremendous biobehavioral reorganization in infants. This period sees major changes in the circadian or day-night organization of sleep, in EEG patterning, attention and irritability (Larson et al., 1998; Barr, 1990; Emde, Gaensbause, and Harmon, 1976). In addition, the study reported that co-sleeping and breastfeeding contributed positively to cortisol regulation (Beijer et al., 2013). Therefore, the association between BPA and free cortisol might not be found by these confounding factors.

Meanwhile, the urinary free cortisol levels were correlated with 8-OHdG level, indicating a high relationship between them in the mechanism of health effects (Joergensen et al., 2011; Kim et al., 2018). The study suggested that the combined effects of cortisol lead to increased oxidative stress, in which the mitochondrial production of reactive oxygen species (ROS) exceeds the antioxidant potential. Therefore, cortisol may be another pathway causing damage to DNA/RNA. When

estimating the correlation between BPA and 8-OHdG, free cortisol needs to be considered with other chemicals.

Although source and mechanism by which 8-OHdG enter fetal urine and amniotic fluid are unclear (Rejc et al., 2017), infants before and after birth were continuously exposed to BPA from maternal and environmental conditions. In addition, the changes in BPA and 8-OHdG concentrations are similar in this study. Therefore, the associations between BPA exposure and oxidative stress were examined adjustment for infants' gender and age in whole data, and there was a positive correlation between BPA and 8-OHdG levels. However, sensitivity analysis was performed for the accuracy of the results. In results of stratified analysis by collection time (infants' age), the significant levels from each collection time indicated that for urinary BPA at birth the strongest associations with overall 8-OHdG. The study reported that 8-OHdG levels of neonates were found to be at a higher level than infants or children, and this was implied to be more vulnerable to oxidative stress due to relatively rapid growth. In other words, DNA can be more vulnerable to oxidative stress by chemical exposures as nuclear membranes and histones are removed, because of the high rate of cell division until 4 to 6 weeks after birth (Drury et al., 1998). Therefore, at birth, urinary BPA levels were higher through placental transfer and they would have contributed significantly to DNA damage. However, the relationship between exposure to BPA and oxidative damage variable and imprecise after birth, because there are individual differences in exposure to BPA of infants by

various environmental factors, and their DNA repair capacity (Kataria et al., 2017). In particular, at 3 months after birth, urinary BPA concentration was low, while 8-OHdG was high, which is might be due to consumption of breastmilk or formula milk. Previous studies reported that 8-OHdG was detected in breast milk (4.0 to 9.1 ng/mL) and formula milk (18.1 to 43.3 ng/mL) (Shoji et al., 2003). It is, therefore, necessary to consider these factors when using 8-OHdG as oxidative stress biomarker during the lactation period.

The strength of this study was its examination of the effects of BPA on oxidative stress biomarker including 8-OHdG and free cortisol with repeated measurements (birth, 3, 9, 12, and 15 months) during infancy. Associations were positive between BPA and 8-OHdG in that analysis. However, the study had some limitations. First, only phthalate metabolites and heavy metal among other environmental exposure were adjusted in this models. Second, other biomarkers and different biological pathways on oxidative stress were not considered. Finally, a linear mixed effect model was used to account for the individual BPA and oxidative stress variation in the repeatedly collected samples, but the sample size was small. Further studies should investigate the effect of BPA on oxidative damage by considering potential confounders, and co-exposed compounds in a large number of repeated urine samples.

Chapter 5. Conclusions

The identification of BPA exposure and health effects for the prenatal and postnatal period of the mother and matching infants was determined in a series of three studies. For this purpose, biological samples (serum, urine, placenta, breast milk, and cord serum) were collected from pregnant women and their babies and then urine and baby food samples were also collected from them at 9, 12, and 15 months after birth in CHECK panel. We analyzed the urinary BPA and oxidative stress biomarkers such as 8OHdG and free cortisol and estimated the correlations between urinary BPA and oxidative stress biomarkers.

In chapter 2, we present evidence that pregnant women in Korea are widely exposed to BPA, based on the detection of BPA in various biological samples (e.g., maternal serum, urine, placenta, and breast milk). The detection of BPA in cord serum and neonatal urine indicated that fetuses were exposed to BPA via the mother. Significant associations in BPA concentrations were found between maternal serum and cord serum, maternal urine and cord serum, and maternal urine and placenta based on differences in BPA exposure and time of sampling. The BPA ratios and variations showed the distribution of BPA in each tissue or bodily fluid and the differences in levels among individuals. These results can be used as a parameter in BPA gestation

PBPK models. However, further studies are required to determine the factors that influence inter-individual variability in BPA levels and BPA ratios.

In chapter 3, compared to the levels of BPA in baby-food and infants' urine, the ones in the present study were comparable or lower. The present observation that consumption of solid food explained the body burden of BPA at the 15th month was supported in the context of diet as the main exposure source for other studies. However, BPA in food did not show significant correlation with urinary BPA in all collection time, which suggests the baby food with high water content did not drive the internal dose, or there was another source of exposure in the baby diet (other than weaning-food) or other environmental sources although further study should confirm it.

In chapter 4, we measured the BPA and oxidative stress biomarkers levels including 8-OHdG and free cortisol in urine samples that were repeatedly collected from infants and estimated the relationships between urinary BPA and oxidative stress. We found positive associations of the BPA exposure on 8-OHdG including indications of increasing ROS and oxidative DNA damage.

Overall, these findings can fill the knowledge gaps on BPA exposure and the association with oxidative stress in Korean infants from CHECK panel. However,

there are some limitations. First, we could not collect all samples from participants and a relatively small sample size at 12, 15 months after birth in the follow up study. Second, an error in the interpretation of data by sample collection time and estimation of intake dose through a questionnaire (study 3). Third, Liquid food such as breast milk and powdered milk were not considered. Fourth, we considered only phthalate and heavy metal in the model about the association between BPA exposure and oxidative stress. In addition, other biomarkers and different biological pathways on oxidative stress were not considered

Although not all samples could be collected from 318 participants, it was enough to show the variation of BPA levels and ratio. The second limitation can be overcome by the assumption that BPA concentrations were constant in the bodies of the pregnant women and their fetuses.

This content level of BPA in liquid food does not significantly affect the interpretation of our results because the study reported that solid food was 7 times higher and significantly contributing to the BPA intake dose of children compared to liquid foods (Morgan et al., 2011). To overcome the fourth limitation identified above, the combined effect of multiple exposure factors inducing oxidative stress should be further studied.

In the BPA exposure during the prenatal and postnatal period, placental transfer and baby food are a critical factor. However, other exposure sources (life pattern and metabolic function of the mother) may contribute to the BPA levels of fetus and infant,

because BPA ratio (maternal serum to other samples) variation was large. Especially, changes in concentration and variability of BPA during infancy up to 15th month indicate that other exposure sources such as behavior as well as diet contribute to urinary BPA. In addition, BPA exposure during infancy including perinatal period increased the ROS, thereby may affect the growth and development of infants by DNA and mitochondrial damage. However, our findings should be confirmed further in future studies.

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Appendix

Table S1. Analytical results of BPA in hexane-cleaned Milli-Q water stored in the tubes that were used in the present study*.

Tube type	Use	BPA (n=5)
8.5 mL-SST tube	Blood sampling	<LOD
1.0 mL-Screw cap tube	Blood storage	<LOD
15mL-falcon tube	Blood analysis	<LOD
Urine collection bag	Urine sampling	<LOD
1.5 mL-Cryo Tube	Urine storage	<LOD
1.5mL-Glass vial	Urine analysis	<LOD
50 mL-Falcon Tube	Placenta and breast milk sampling	<LOD
15 mL-Falcon Tube	Placenta and breast milk storage or analysis	<LOD

*Hexane-cleaned Milli-Q water was prepared by 1 hr mechanical shaking of 400 mL Milli-Q water with 100 mL hexane. For sample preparation, the hexane-cleaned Milli-Q water was added to each type of tube and was stored in a freezer for 1 day. Frozen water samples were thawed and pretreated by the preparation method of each media.

Table S2. Accuracy (recoveries) of BPA in biological samples

Media	**Spiking Level** (μg/L or ng/g)	**Intra-day recovery (n=5)** (%)	**Inter-day recovery (3 days)** (%)
Serum	LOQ (1.0)	84.1	83.9
	2	128	119
	20	105	108
	80	98.2	97.6
Urine	LOQ (1.5)	108	108
	5	106	106
	50	100	99.9
	200	95.8	98.2
Placenta	LOQ (1.0)	81.1	101
	2	80.3	96.8
	20	102	99.8
	80	103	96.4
Breast milk	LOQ (1.0)	102	88.4
	2	85.8	88.4
	20	106	105
	80	99.9	99.0
Baby food	10	90.7	96.6
	100	108	106

Accuracy was determined as the percent difference between the mean of observed concentrations and the theoretical concentration, and was required to be within ± 20 % for the LOQ and within ±15 % for the other concentrations. Accuracy (%) = analyte found / analyte spike * 100

Intra-day variation was assessed by five consecutive injections of LOQ, low, medium, high concentration standard solution, and inter-day variation was determined by measuring the same standard solution on three different days.

Table S3. Precisions of BPA in biological samples

Media	**Spiking Level** (μg/L or ng/g)	**Intra-day recovery (n=5)** (%)	**Inter-day recovery (3 days)** (%)
Serum	LOQ (1.0)	13.0	24.8
	2	14.4	19.1
	20	6.6	12.6
	80	5.0	4.7
Urine	LOQ (1.5)	13.5	8.9
	5	7.4	11.6
	50	10.0	10.1
	200	5.6	4.5
Placenta	LOQ (1.0)	10.8	11.4
	2	10.0	17.1
	20	3.4	8.9
	80	3.3	10.3
Breast milk	LOQ (1.0)	17.9	19.6
	2	9.4	13.0
	20	3.6	7.2
	80	5.8	6.2
Baby food	10	13.5	13.2
	100	8.3	6.8

CV: Coefficient of variation.

The coefficient of variation (CV) was the standard deviation of replicate measurements expressed as a percentage of the mean value, and should not exceed 20 % for the LOQ and 15 % for other concentrations. CV (%) = standard deviation of observed concentrations / Mean of observed concentrations * 100.

Table S4. Daily amounts of breastfeeding and breastfeeding rates in the subject families

Age	No. subjects	p25	p50	p75	p95	Feeding rate (%)
1	67	480	672	960	1344	100 %
3	48	540	840	1075	1320	100 %
9	52	0	210	1050	1470	52 %
12	26	0	0	960	1440	26 %
15	10	0	0	0	960	10 %

*Age – collection month, p25 – 25th percentile, p50 – 50th percentile (median), p75 – 75th percentile, p95 – 95th percentile . unit – mL.

Table S5. Correlations of BPA levels between urine and baby food

Age	Covariate	Spearman correlation coefficient (*p*-Value) N		
		1	2	3
9th month	1. BPA in urine (corrected SG,ng/mL)	1	-0.204 0.190 43	-0.055 0.759 34
	2. BPA in baby food (ng/g)		1	0.976 <0.001 55
	3. Daily BPA intake amounts (ng/day)			1
12th month	1. BPA in urine (corrected SG,ng/mL)	1	0.391 0.059 24	0.456 0.0657 17
	2. BPA in baby food (ng/g)		1	0.969 <0.001 46
	3. Daily BPA intake amounts (ng/day)			1
15th month	1. BPA in urine (corrected SG,ng/mL)	1	0.608 0.0276 13	1 <0.001 3
	2. BPA in baby food (ng/g)		1	0.952 <0.001 22
	3. Daily BPA intake amounts (ng/day)			1

Table S6. Spearman correlations among specific gravity-adjusted chemicals by age of infants

Covariate	At birth (n=103)	3months (n=53)	9months (n=58)	12months (n=42)	15months (n=15)
MEP (ng/mL)	0.147	0.194	0.316*	-0.146	0.477
MiBP (ng/mL)	0.380**	0.223	0.129	-0.005	0.442
MnBP (ng/mL)	0.301***	0.333	0.261*	-0.049	0.293
MEHHP (ng/mL)	0.046	0.164	0.147	0.041	0.302
MEOHP (ng/mL)	0.040	0.217	0.181	-0.021	0.225
Pb (ng/mL)	0.046	-0.069	0.063	0.320	0.273
8-OHdG (ng/mL)	0.244*	-0.120	0.083	0.064	0.172
Free cortisol (μg/dL)	-0.064	-0.212	-0.085	-0.105	0.429

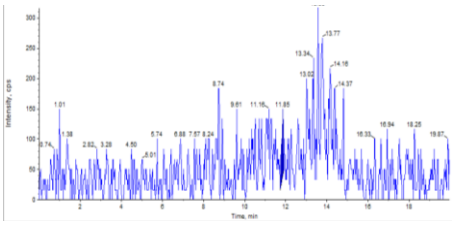
*P<0.05, **p<0.01, ***p<.001

Table S7. Relationships between 8-OHdGand BPA levels in urine samples by age of infants

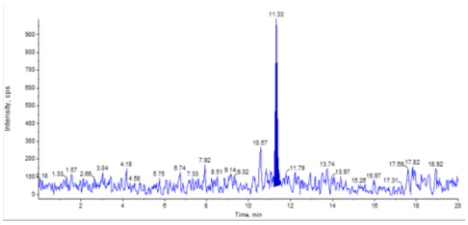
	Univariate				Mutivariate[a]			
	β	95% CI		*P* value	β	95% CI		*P* value
		Lower	Upper			Lower	Upper	
At brith	0.17	0.05	0.28	0.004	0.18	0.06	0.30	0.003
3 months	-0.13	-1.10	0.84	0.781	-0.13	-1.00	0.73	0.746
9 months	0.27	0.09	0.45	0.003	0.27	0.09	0.45	0.003
12 months	0.06	-0.14	0.27	0.540	0.02	-0.17	0.22	0.821
15 months	0.13	-1.19	0.99	0.843	0.13	-1.19	0.99	0.843
9 months ~ 15 months	0.16	0.02	0.31	0.029	0.16	0.02	0.31	0.029

[a] Pb and free cortisol remained the statistical significance at birth; MiBP at 3 months; All chemicals lost significance at 9, 15 months; MnBP and MiBP were remained the significance at 12 months; All chemicals lost significance from 9 to 15 months

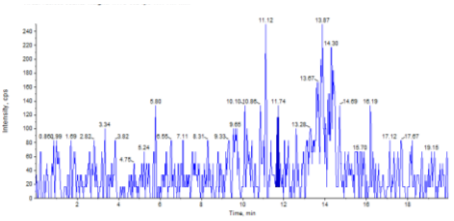
Acetonitrile



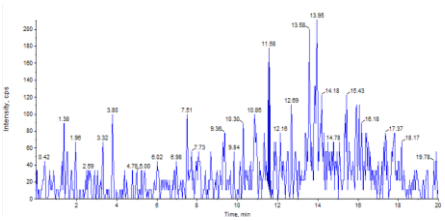
STD (1ng/mL)



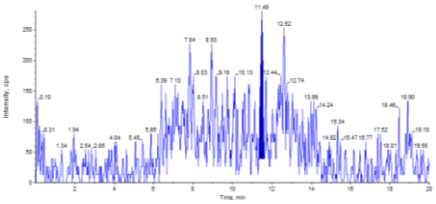
Urine blank



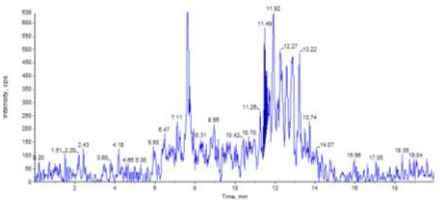
Serum blank



Placenta blank



Breast milk blank



Breast milk spiked blank (1ng/mL)

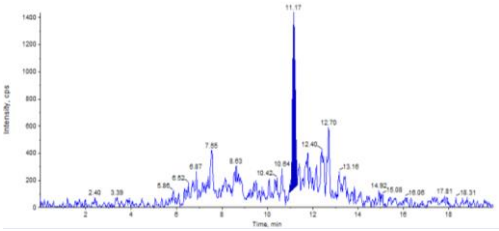


Figure S1. HPLC-MS/MS chromatograms of BPA in acetonitrile, standard, and procedural blank of each media.

국문초록

주산기를 포함한 영유아기의 BPA 노출 및 산화 손상과의 상관성

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이 장 우

Bisphenol A (BPA)는 흔히 볼 수 있는 젖병, 재사용 가능한 물병, 식품 용기, 스트레치 필름, 종이 및 판지를 비롯한 수많은 소비재 제품 등으로 많이 사용되는 화학 물질이다. 그러므로 BPA 는 다양한 환경 샘플 (예 : 물, 공기 및 먼지), 식품 샘플 (예 : 플라스틱 용기 및 캔 라이닝) 및 생물학적 샘플 (예 : 혈청, 소변, 태반, 모유 및 양수)에서 검출되고 있다.

또한 BPA 는 동물 및 인간을 대상으로 수행한 실험에서 에스트로겐 수용체에 결합하는 것 외에 조직 효소 및 호르몬 수용체의 변화를 가져오고 안드로겐 및 글루코코르티코이드 수용체 등 다른 호르몬 반응 시스템과 상호 작용한다는 연구 결과를 보고하였다. 그리고 BPA 는 미토콘드리아 기능, 뇌, 신장 및 고환에서 항산화 효소 조절과 같은 직접 또는 간접 경로를 통해 산화적 항상성을 방해 할 수 있다.

특히, 태아와 영아는 성인보다 체중을 기준으로 독성 물질 노출에 생물학적으로 더 민감 할 수 있다. 그러나 몇몇 역학연구에서 여성 및 성인을 대상으로 BPA 노출과 산화손상과의 상관성을 확인하였으며, 태아와 영유아의 BPA 노출수준에 대한 정보 역시 제한적이다. 그러므로 이러한 민감집단에서 BPA 노출 및 관련 건강영향을 조사 할 필요가 있다. 이러한 부족한 정보를 확인하기 위해 CHECK (Children's Health and Environmental Chemicals in Korea) 연구에서 영유아의 BPA 노출 및 건강영향 지표에 대한 자료를 사용하였다. CHECK 연구는 태중 및 영유아기 동안에 환경 오염 물질 (POPs, BPA, 프탈레이트 및 중금속)의 노출 그리고 건강 영향과 관련된 정보를 수집하기 위해 수행되었다.

CHECK 연구에서는 한국의 4 개 지역에 소재한 6 개 대학 병원에서 2011 년부터 2013 년까지 임산부와 영유아 쌍(n=355)을 모집하였다. 본 연구에서는 출생 시점에 모집된 참여자 중 임산부와 신생아 318 쌍으로부터 생체 시료 (산모 혈청, 산모 소변, 태반, 모유, 제대 혈청, 신생아 소변)를 채취 한 다음 영유아 소변 (n = 187) 및 이유식 (n = 210) 샘플을 후속 패널 (n = 173)로부터 출생 후 9, 12, 15 개월 째에 수집하여 BPA 노출 수준을 확인하였다. BPA 노출에 의한 산화 손상은 소변 샘플 (n=271)에서의 BPA 농도, 산화손상지표 농도, 그리고 공변량 자료가 있는 190 명의 영유아로부터 확인하였다. 생체 시료 및 이유식 샘플의 BPA 수준은 HPLC-MS / MS 및 GC-MS 를 이용하여 각각 측정되었다. Free cortisol 은 삼광 연구소에서 분석하였고 8OHdG (8-hydroxy-2'-deoxy-guanosine)는 EIA kit 와 분광광도계를 이용하여 측정하였다.

위와 같이 수집된 데이터를 이용하여 부족한 정보를 채우기 위한 이 연구의 구체적인 목적은 다음과 같다. 첫째, 산모-신생아 쌍 샘플에서

상관 관계와 농도 비율을 사용하여 주 산기 BPA 노출을 기술하고, 둘째, 소변 및 이유식 샘플의 오염 상태를 통해 이유 기간의 BPA 노출을 평가하고, 마지막으로 출생 후 15 개월까지 추적 수집 된 소변 샘플에서 BPA 농도와 산화 스트레스 마커의 상관 관계를 통해 BPA 노출에 대한 건강 영향을 추정하는 것이다.

2 장 연구에서는 임산부의 다양한 생체 시료에서 BPA 노출 수준을 확인하고 임산부 신생아 쌍 샘플 사이의 연관성과 BPA 비율을 기반으로 태아 및 영유아의 BPA 노출을 설명하였다. 임산부 및 신생아 표본의 79.5−100 %에서 BPA 가 검출되었다. 수집 샘플에서 BPA 의 중앙값을 신생아 소변 (4.75 ng / mL), 산모 소변 (2.86 ng / mL), 제대 혈청 (1.71 ng / mL), 산모 혈청 (1.56 ng / mL), 모유 (0.74 ng / mL), 태반 (0.53 ng / g)에서 보고하였다. 그리고 산모 소변과 제대 혈청, 산모 소변과 태반 간에는 유의 한 상관 관계가 있었다. 우리는 모체 혈청과 다른 샘플을 통해 BPA 수준의 비율을 추정하였다. BPA 가 두 표본에서 모두 발견 된 160 쌍의 모체 − 태아 쌍에 대한 제대혈청 / 산모 혈청 비율의 중앙값 (95 백분위 수)은 1.12 (15.2)였다. 태반, 산모 소변, 신생아 소변 및 모유 / 산모 혈청 비율은 각각 0.28 (5.31), 1.79 (29.9), 1.98 (28.2) 및 0.51 (10.5)이었다. 또한, 제대 혈청 − 태반 비의 중앙값 (95 백분위 수)은 4.03 (45.8)이었고 신생아 소변 − 제대 혈청 비는 1.95 (25.6)이었다. 95 백분위 수 값은 중간 값보다 14−20 배 더 높았다. 제대혈청과 신생아 소변의 BPA 검출은 태아가 엄마를 통해 BPA 에 노출되었음을 나타내고 있다. 그리고 이러한 BPA 비율의 큰 변이는 임산부가 반복적으로 지속적으로 BPA 에 노출되었을 때 영유아가 태반 및 모유 수유를 통해 BPA 가 높게 노출될 수 있음을 암시한다.

3 장에서 영유아의 생후 9, 12, 15 개월 이유식과 소변에서 BPA 수준을 확인하였다. 가정식 이유식에서 BPA 의 중앙값은 0.45 ng / g (IQR : ND−5.16 ng / g 습식 중량)이었으며 보정되지 않은 소변 중 BPA 는 0.93 μg / L (IQR : <LOD−2.66 μg / L) [비중을 보정한 값은 0.94 μg / L (IQR : <LOD−2.80 μg / L)]이었다. 15 개월 된 영유아 (중앙값 : 5.09 ng / g)의 이유식에서 BPA 농도는 9 개월 또는 12 개월 (중앙값 : 각각 <LOD 및 0.47 ng / g)에서 검출 된 것보다 유의하게 더 높았다. 그러나 소변 중 BPA 농도는 영유아의 연령에 따라 변화가 없었다. 육류, 유제품 및 스낵과 같은 포장 재료 및 / 또는 용기에 포장 된 식품의 섭취율이 증가했기 때문에 출산 후 15 개월 만에 이유식의 BPA 수치가 더 높았다. 그러나 BPA 수준의 소변과 이유식 샘플 간에는 상관 관계가 없었다. 따라서 본 연구의 결과는 영유아의 주요 BPA 노출원이 섭취 식이인 성인과 달리 다른 환경 요인이 신생아의 소변 내 BPA 에 영향을 줄 수 있다는 것을 의미한다.

4 장에서는 반복적으로 수집 한 소변 샘플에서 BPA, 8−OHdG 및 free cortisol 수치를 측정 한 다음, 소변 중 BPA 와 산화손상지표의 상관 관계를 각 개체에 대해 평가하였다. 모든 소변 샘플에서 비중 보정한 기하 평균 (기하 표준 편차)은 1.9 (4.9) ng / mL (중앙값과 95 백분위 수 : 0.8 과 27.5 ng / mL)이었으며 8−OHdG 와 free cortisol 은 63.7 (2.2) ng / mL (중앙값과 95thpercentile : 61.0 과 282 ng / mL), 15.6 (2.6) μg / dL (중앙값과 95thpercentile : 각각 16.7 과 66.8 μg / dL)이었다. BPA 는 비선형 관계 (p <.0001)에서 8−OHdG 와 free cortisol 모두와 유의한 상관관계가 있었다. 영유아의 성별, 연령 및 기타

화학 물질[프탈레이트 대사 물, 납 (Pb) 및 수은 (Hg)]을 보정 한 후 선형 혼합 효과 모델을 사용하여 8-OHdG 및 free cortisol 에 대한 BPA 노출의 영향을 평가하였다. BPA 와 free cortisol 과의 상관성은 나타나지 않았다. 그러나 BPA 수준과 8-OHdG 와의 연관성에서 MnBP는 또한 BPA 노출과 상호 작용하는 것으로 밝혀졌다. 두 물질간의 상호작용을 확인하기위해 BPA 수치를 25, 75 백분위 수를 사용하여 저, 중, 고 그룹으로 나누어 살펴보았다. 그 결과 고농도의 MnBP 그룹에서 BPA 고농도 그룹의 8-OHdG 수준은 저 및 중농도 그룹보다 높은 결과를 나타내었다. 따라서 두 화합물에 대한 노출은 8-OHdG 의 증가에 같이 영향을 주는 것으로 나타났다. 이러한 결과는 BPA 노출과 8-OHdG 의 상관성을 보고 한 이전 연구들에 의해 뒷받침되었지만 이전 연구는 임산부, 성인 및 다른 연구 설계를 통한 연구결과 였다. 또한 free cortisol 농도는 8-OHdG 농도와 유의한 상관 관계가 있었으며 (ρ = 0.42, p <.0001), 몇몇 연구는 cortisol 과 8-OHdG 사이의 양의 상관관계를 보고하였다. 이러한 결과는 free cortisol 이 산화 손상을 일으키는 또 다른 경로로서 산화 손상 평가 시 고려되어야 한다는 것을 의미한다. 따라서 본 연구에서 중요한 태아기 및 출생 후 발달 기간 동안 BPA 의 산화 스트레스 가능성을 확인하였다.

결론적으로, 우리 연구는 BPA 노출의 현재 수준이 유아기의 산화 손상을 일으킬 수 있음을 입증하였다. 다른 연구들과 비교했을 때, 제대혈과 신생아 소변의 BPA 수준은 높았으며 영유아 소변은 유사한 결과를 나타내었다. BPA 비율의 큰 변이는 산모의 생활 패턴과 신진 대사 기능의 차이로 인한 개인차에 의한 것으로 생각된다. 이유식의 BPA 는 소변의 BPA 와 유의 한 상관 관계를 나타내지 않았으며 이는

영유아의 생활 환경과 행동에 따른 노출과 같은 이유식 이외에 다른 노출 요인이 있음을 의미한다. 또한 영유아의 반복 수집된 소변 샘플에서 8-OHdG 와 free cortisol 에 대한 BPA 노출의 양의 상관관계를 통해 태아기 그리고 출생 후 BPA 노출이 태아, 영유아의 발달에 영향을 줄 수 있다는 것을 알 수 있었다. 그러므로 임신기와 생애 초기단계에서의 BPA 노출이 산화 스트레스에 미치는 영향을 고려한다면 앞으로 산모와 영유아의 BPA 노출에 대한 지속적인 관찰을 통해 노출원을 파악하고 산화손상에 대한 BPA 의 작용기전을 확인하는 추가 연구가 필요하다.

주요어 : 비스페놀 에이, 신생아와 영유아, 소변, 비스페놀 농도 비율, 영유아 이유식, 산화 손상