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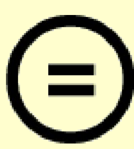
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Nephrotoxicity of Bisphenol A, Bisphenol S, and Aristolochic Acid in Zebrafish Larvae and Human Kidney-2 Cells

제브라피쉬(*Danio rerio*) 치어와
HK-2 세포주를 이용한
Bisphenol A, Bisphenol S, 그리고 Aristolochic aicd의 신장 독성 영향 연구

2020년 8월

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환경보건학과 환경보건 전공

임 소 영

Abstract

Nephrotoxicity of Bisphenol A, Bisphenol S, and Aristolochic acid in Zebrafish Larvae and Human Kidney-2 Cells

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Bisphenol A (BPA) and its replacement, bisphenol S (BPS) have been used in plastic bottles, thermal papers, food containers, and epoxy resins. While their adverse effects on thyroid hormone disruption have been reported, little is known about their potential and underlying mechanisms of kidney toxicity.

The purpose of this study is to understand kidney toxicity of BPA and BPS using zebrafish larvae and human kidney-2 cells (HK-2 cells). In addition, aristolochic acid (AA), a chemical that has been well known for its kidney toxicity, was also chosen and its toxicity

was compared with those obtained from both bisphenols.

As a result of *in vitro* experiments, Gene transcription level of *KIM-1*, a kidney damage marker, was down-regulated in BPA or BPS exposed HK-2 cells. *NGAL*, a kidney damage marker, was up-regulated in BPA exposed HK-2 cells. The indicators related to inflammation, oxidative damage, autophagy, and fibrosis were significantly increased in BPA exposed groups. Transcription levels of BPS exposed groups showed an increasing tendency, but not statistically significant, except *NRF2*. In AA exposed HK-2 cells, *KIM-1*, autophagy, and fibrosis-related markers were increased. *NGAL*, *AQP1*, or inflammation and oxidative-related markers showed different patterns with BPA or BPS exposed HK-2 cells.

In the experiment using zebrafish larvae, transcription levels of nephrogenesis markers (*nephrin*, *wt1a*, *etv4*, *sim1a*) were significantly increased in BPA exposed groups. In BPS exposed groups, *nephrin*, *sod1*, *gpx1a* were up-regulated. *col11a*, the fibrosis marker, was significantly decreased in BPA exposed groups, but there is no change in BPS exposed groups. In the case of exposure to AA, there is no significant change of nephrogenesis markers, but significant up-regulation was observed.

From these results, it was found that BPA and BPS change the gene transcription level patterns similarly in HK-2 cells and zebrafish larvae. In addition, the results showed that the transcription level of genetic markers was greater than that of BPS, suggesting that BPA might greater affect to HK-2 cells and zebrafish than BPS. It was investigated that the effects and mechanisms of renal toxicity caused by AA are different from BPA or BPS.

Many evidences suggested that BPA and BPS could be the nephrotoxicants in the present study. Therefore, when evaluating the toxicity of BPA and BPS, the effect of renal toxicity should also be considered.

Keywords: Bisphenol A (BPA), Bisphenol S (BPS), Aristolochic acid (AA), Nephrotoxicity, Zebrafish, HK-2 cell line

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Contents

1. Introduction ………… 1
2. Materials and Methods ………… 3
 2.1. Chemicals ………… 3
 2.2. HK-2 cell culture and exposure ………… 5
 2.3. Zebrafish culture and exposure ………… 6
 2.4. RT-PCR ………… 7
 2.5. Measurement of proteinuria ………… 10
 2.6. Statistical analysis ………… 11
3. Results ………… 12
 3.1. HK-2 cell line exposure ………… 12
  3.1.1. Cell viability of HK-2 cells ………… 12
  3.1.2. Transcriptional changes in HK-2 cells ………… 14
 3.2. Zebrafish larvae exposure ………… 16
  3.2.1. Transcriptional changes in zebrafish larvae ………… 16
  3.2.2. Measurement of proteinuria in zebrafish larvae ………… 18
4. Discussion ………… 19
 4.1. Transcriptional changes in HK-2 cells ………… 19
 4.2. Transcriptional changes in zebrafish larvae ………… 22
 4.3. Proteinuria changes in zebrafish larvae ………… 25
5. Conclusion ………… 26
6. References ………… 27
Abstract in Korean ………… 35

List of Tables

Table 1. Physicochemical properties of BPA, BPS and Aristolochic acid ········ 4

Table 2. Primer sequences of HK-2 cells and zebrafish ········ 8

List of Figures

Figure 1. Cell viability measured from BPA, BPS, or AA exposed HK-2 cells in WST-1 assay ··· 13

Figure 2. Transcription level changes of genes related to nephrotoxicity in HK-2 cells by BPA, BPS, or AA exposure ··· 15

Figure 3. Transcription level changes of genes related to nephrotoxicity in zebrafish larvae by BPA, BPS, or AA exposure ··· 17

Figure 4. Protein levels of BPA, BPS, or AA exposed zebrafish larvae ··· 18

1. Introduction

Bisphenol A (BPA) has been used in plastic bottles, food containers, thermal papers, and epoxy resins (Liao and Kannan., 2014). Bisphenol S (BPS) is a structural analog of BPA and used as its substitute. BPS has been used in thermal papers, medical devices, dental sealants, and epoxy resins (Rochester and Bolden, 2015; Xue et al., 2018). Accordingly, BPA and BPS have been detected in various water samples worldwide. In India, BPA and BPS were detected up to 272 ng/L and 42 ng/L, respectively in the Han river (Yamazaki et al., 2015). In India, BPA and BPS were detected up to 1950 ng/L and 7200 ng/L, respectively (Yamazaki et al., 2015). BPA and BPS have also been detected in human urine. In the USA, BPA and BPS were detected up to 187.96 μg/g creatinine and 20.38 μg/g creatinine in urinary samples of cashier, respectively (Thayer et al., 2016). In Chinese children, BPA and BPS were detected up to 31.1 μg/L and 0.96 μg/L, respectively (Chen et al., 2018).

Both BPA and BPS have been associated with endocrine disruption in both epidemiological and experimental studies (Ji et al., 2013; Lee et al., 2019; Lee et al., 2017; Moreman et al., 2017; Scinicariello and Buser, 2016). Several epidemiological studies suggest that BPA exposure is associated with functional markers of kidney like albumineuria or estimated filtration rate (eGFR) (Li et al., 2012; You et al., 2011). However, relatively limited experimental studies were conducted for their kidney toxicity (Kobroob et al., 2018; Yuan et al., 2019; Zhao et al., 2018). In BPA exposed rats, serum urea and creatinine were increased and creatinine clearance was decreased (Kobroob et al., 2018). Also, BPA-induced apoptosis, oxidative stress or DAN damage were occurred in monkey kidney cells or mouse

(Yuan et al., 2019; Zhao et al., 2018). Especially for BPS, little is known about its kidney toxicity and potential mechanisms.

The purpose of the present study is to understand the effects of BPA and BPS exposure on kidney damage and associated mechanisms using HK-2 cells and zebrafish (*Danio rerio*) larvae. HK-2 cells are derived from proximal tubule epithelial cells of normal adult human kidney (Ryan et al., 1994). HK-2 cells have been used to evaluate nephrotoxic effects of various compounds including cisplatin and aristolochic acid (Li et al., 2010; Li et al., 2018; Qi et al., 2007; Shon et al., 2013). BPA induced mitochondrial injury and increasing antioxidant markers were observed in HK-2 cells (Bosch-Panadero et al., 2018). In addition, zebrafish is a widely used freshwater fish model for environmental toxicity test. Because of ease of handling and simple kidney structure, zebrafish has a utility to be used as an animal model for kidney toxicity (Poureetezadi and Wingert, 2016).

In the present study, kidney toxicity of BPA and BPS was evaluated using both zebrafish larvae and HK-2 cells. For this purpose, three chemicals were exposed to zebrafish larvae and HK-2 cells. Transcription level changes in kidney damage or nephrogenesis markers were measured. Proteinuria, an indicator of renal function impairment, was also measured after the exposure. For comparison, aristolochic acid, a chemical derived from plants of *aristolochiaceae* and used as herbal medicine, was chosen, and its responses were compared with those observed from both bisphenols. The results of this study will help understand kidney toxicity potentials of major bisphenols, and associated mechanisms.

2. Materials and Methods

2.1. Chemicals

Bisphenol A (BPA, CAS no. 80-05-7, purity: 97 %), bisphenol S (BPS, CAS no. 80-09-1, purity: 98 %), and aristolochic acid (CAS no. 313-67-7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The physicochemical properties of BPA, BPS, and aristolochic acid were described in Table 1. Dimethyl sulfoxide (DMSO, purity: 99 %) was chosen as a solvent, and purchased from Junsei Chemical Co. (Tokyo, Japan). For *in vitro* studies, 0.1 % dimethyl sulfoxide (DMSO) was used as a solvent for all chemicals. For *in vivo* studies, 0.01 % for BPA exposure groups. 0.1 % for BPS and aristolochic acid were used.

Table 1. Physicochemical properties of BPA, BPS and Aristolochic acid

Compound	Bisphenol A (BPA)	Bisphenol S (BPS)	Aristolochic acid (AA)
CAS No.	80-05-7	80-09-1	313-67-7
Structure			
Usage	Plastic bottles, food containers, thermal papers and epoxy resins	Medical devices, dental sealants, water bottles	Herbal medicine
Molecular formulate	$C_{15}H_{16}O_2$	$C_{12}H_{10}O_4S$	$C_{17}H_{11}NO_7$
Molecular weight (g/mol)	228.29	250.27	341.28
LogKow	3.32[a]	1.65[a]	1.65[b]

[a] US EPA

[b] Tangtong et al., 2014

2.2. HK-2 cell culture and exposure

HK-2 cells were maintained at 37 °C with 5 % CO_2. For culture medium, a mixture of Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (Sigma-Aldrich, St.Louis, MO, USA) and 10% fetal bovine serum (Thermofisher scientific) were used and sodium bicarbonate (Sigma-Aldrich, St.Louis, MO, USA) 1.2 g was added. For exposure, reduced-serum medium which contained 0.2 % fetal bovine serum was used. HK-2 cells were seeded at 2.4×10^5 cells/well density in 6well plates and incubated for 24 hours using a reduced-serum medium. Based on the result of WST-1 cell proliferation assay, 0, 12.5, 25, and 50 mg/L for BPA, BPS, and AA were chosen as sub-lethal concentrations. Three replicates of each group were used. After the 24 hours-exposure, the harvested cells were homogenized for gene analysis. Three biological replicates were exposed to each chemical, and results were averaged.

2.3. Zebrafish culture and exposure

Adult wild-type zebrafish were obtained from a commercial supplier (Greenfish, Seoul, Korea) and in-house cultured in Environmental Toxicology laboratory at Seoul National University, Seoul, Korea. Embryos were obtained from adult male and female zebrafish (over six months old). Larvae hatched during 72-76 hours post fertilization (hpf) were collected and randomly divided into 50 mL beaker at 4 days post fertilization (dpf). In each beaker, 20-30 larvae were exposed to BPA, BPS, or AA, for 24 hours from 4 dpf to 5 dpf. Sub-lethal exposure concentration is 0, 3.75, 7.5, and 15 mg/L for BPA, 0, 6.25, 12.5, and 25 mg/L for BPS, 0, 2.5, 5, and 10 mg/L for AA in gene analysis. Preliminary experiments were conducted to determine the sub-lethal exposure concentrations. In the preliminary experiments, at the highest concentration of each chemical, zebrafish larvae showed over 10 % mortality during 48 hours exposure, but 0 % mortality during the first 24 hours exposure. At 5 dpf, each group of larvae was pooled for gene analysis. The zebrafish larvae whole-body samples were stored at -80 °C until the gene transcription level analysis. Three biological replicates were exposed to each chemical, and results were averaged.

For proteinuria measurement, concentrations of 0, 2.5, 5, and 10 mg/L for BPA, 0, 6.25, 12.5, and 25 mg/L for BPS, 0, 2.5, 5, and 10 mg/L for AA were chosen. Each group of larvae weight was measured. The protein level of each group of larvae was divided into dry weights of larvae for normalization. The experiments conducted at 27±1 °C under a photoperiod of 14:10 h light:dark. For the water quality, dissolved oxygen, conductivity, temperature, pH were measured and recorded.

2.4. RT-PCR

Following exposure, samples were homogenized with lysis buffer, and RNA was immediately extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). The quality and quantity of RNA were validated using Gen5 2.05 (BioTek, Winooski, USA). Complementary DNA was were synthesized by using cDNA synthesis kit (BioRad, Hercules, CA, USA). Quantitative real-time PCR was conducted using PCR premix containing 10 μL of Power SYBR™ Green PCR master mix (Thermo Fisher Scientific), 0.1 μL of forward and reverse primer (10 pmol), 6.8 μL nano pure water and 2 μL cDNA templates. The comparative thresholdcycle (Ct) methods ($2^{-\Delta\Delta Ct}$) were applied in gene transcription level analysis (Livak and Schmittgen, 2001). Each gene was normalized by the house-keeping gene (*GAPDH* for HK-2 cells, *rpl8* for zebrafish). Primer information for targeted genes in zebrafish larvae and HK-2 cells were shown in Table 2. Three independent experiments were conducted and analyzed in BPA, BPS, and AA exposed HK-2 cells and zebrafish larvae.

Table 2. Primer sequences of HK-2 cells and zebrafish.

Assay	Gene	Potential marker for	Accession No.		Sequence (5'-3')
in vitro HK-2	*GAPDH*	Reference gene	DQ403057.1	F	GTGGACCTGACCTGCCGTCT
				R	GGAGGAGTGGGTGTCGCTGT
	KIM-1	Kidney injury marker	NM_012206.3	F	GAACCAGTAGCCACTTCACCA
				R	CTGTCACGGTGTCATTCCCA
	NGAL	Kidney injury marker	X83006.1	F	GTTACCTCGTCCGAGTGGTG
				R	CAGGGAGGCCCAGAGATTTG
	AQP1	Water transportation	NM_198098	F	CTGGGCATCGAGATCATCGG
				R	ATCCCACAGCCAGTGTAGTCA
	IL-1β	Inflammation	BC008678	F	AACCTCTTCGAGGCACAAGG
				R	GGCGAGCTCAGGTACTTCTG
	IL-8	Inflammation	NM_000584	F	AAGGAACCATCTCACTGTGTGTAAAC
				R	ATCAGGAAGGCTGCCAAGAG
	NRF2	Oxidative stress	NM_001145412.3	F	GGTTCCAAGTCCAGAAGCCA
				R	GGTTGGGGTCTTCTGTGGAG
	CAT	Oxidative stress	AY028632.1	F	GCCACCTGAAGGATGCACAA
				R	TTGCCACCAAGTGAGATCCG
	LC3B	Autophagy	NM_022818.5	F	AAGGCGCTTACAGCTCAATG
				R	CTGGGAGGCATAGACCATGT
	a-SMA	Fibrosis	NM_001613.4	F	GACATCAGGGGGTGATGGTG
				R	CAGGGTGGGATGCTCTTCAG
in vivo zebrafis	*rpl8*	Reference gene	NM_200713	F	TTGTTGGTGTTGTTGCTGGT
				R	GGATGCTCAACAGGGTTCAT
	kim-1[a]	Kidney injury marker		F	ACAATTGGCCCCTACAACAG

				R	CGGACATCATCTGTGGTCAT
In vivo zebrafish	*nephrin*	Formation of podocyte	XM_017351014.2	F	TCCACTTATTATGAGGGAAGAGCA
				R	CCTAAACCTGACCTGAGCAGA
	wt1a	Podocyte differentiation	NM_131046.2	F	CAGCAAGCCAACCTCCAC
				R	GCAACCGTGCCGTAACCT
	etv4	Formation of nephric duct	AJ003200.1	F	GTGTGTTTCGTGAAGGTGCC
				R	TCATAGTTCATGGCTGGGCG
	sim1a	Formation of proximal tubule	NM_178222.3	F	CATAGCCTGCAAGTGAGGGG
				R	TGGCTCGGATTAGGGTCTCA
	sod1	Oxidative stress	NM_131294.1	F	TTCACTCTCTCACAACTTCTC
				R	GTCACCTTCACTGGCTTC
	gpx1a	Oxidative stress	NM_001007281.2	F	CTGCGTGTTGCCCTTTGAG
				R	GGTGTAATCCCTGACTGTTGTG
	col1a1	Fibrosis	NM_199214.1	F	GAGGATGGTTGTACGTCGCA
				R	GACTGGGCCAACTTCAATGC

[a]Yin et al., 2016

2.5. Measurement of proteinuria

The protocol of Wang et al (2016) was modified to measure the proteinuria of zebrafish larvae. Briefly, zebrafish larvae were exposed at 4 dpf to each chemical for 24 hours and rinsed with fresh medium and removed to 6 well plates with medium for additional 24 hours (Wang et al., 2016). At 6 dpf, 1.5 mL of medium was harvested to 2 mL tubes and add 300 μL 100 % trichloroacetic acid solution (Sigma-Aldrich, St. Louis, MO, USA) and kept at 4 °C for 1 hour. The samples were centrifuged at 13,000 rpm at 4 °C for 5 minutes, and the supernatant was discarded. The samples were washed with 100 % cold acetone and centrifuged at 13,000 rpm at 4 °C for 5 minutes twice. The remaining liquid was dry thoroughly at room temperature. After drying the samples, 200 μL of fresh medium water were added. Protein level of each sample was measured using the Micro BCA protein assay kit (Thermo Fisher scientific). For this purpose, 562 nm absorbance was measured using Microplate reader (Spark®, Tecan, Männedorf, Swiss). The protein level was normalized by dry weight of each group of larvae. Methods of measuring dry weights of larvae were followed Weber et al (1989). Each group of larvae were transferred to weighing boats and dried at 60 °C for 1 day. After 24 hours, weighing boats were removed to desiccator. The dry weight of each larvae group was recorded, and protein levels were adjusted using dry weights of larvae for normalization.

2.6. Statistical analysis

SPSS statistical software 25 (IBM Corporation, New York, USA) was used for data analysis. To compare the differences between each concentration group, one-way analysis of variance (ANOVA) with Dunnet's tests were conducted. Spearman's rank correlation analysis was conducted to analyze the trend of gene expression levels. In all statistical results, $p < 0.05$ was considered significant.

3. Results

3.1. HK-2 cell line exposure

3.1.1. Cell viability of HK-2 cells

The results of WST-1 assay showed that cell viability of HK-2 cells was greater than 80 % at the highest concentration of each chemical (Fig. 1).

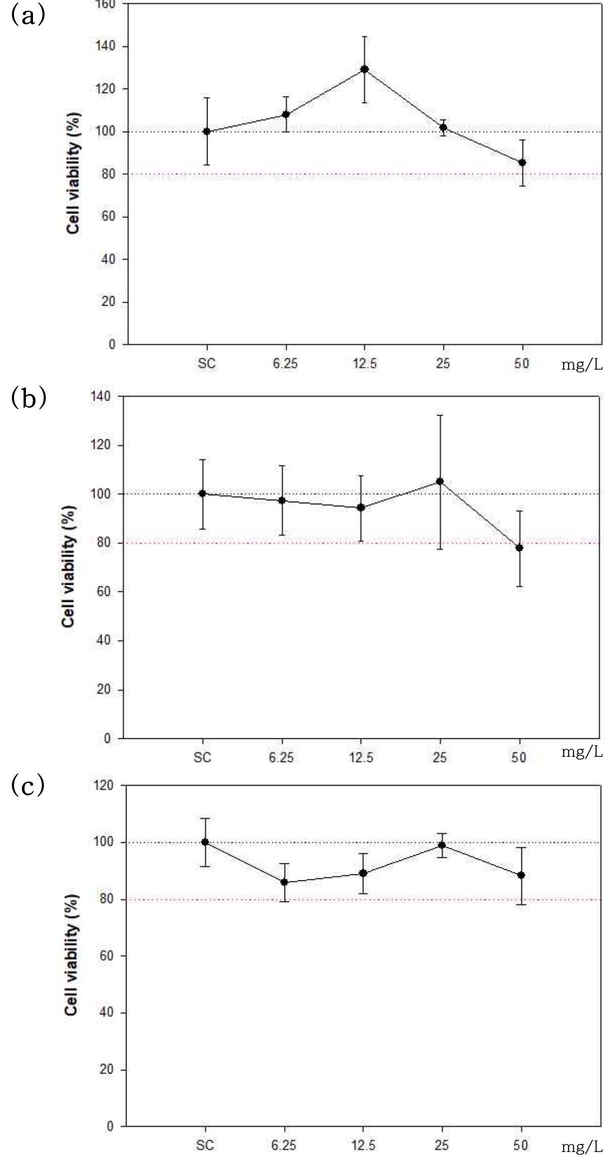

Figure 1. Cell viability measured from BPA, BPS, or AA exposed HK-2 cells in WST-1 assay. The cell viability was normalized by that of the solvent control group (0.1 % of DMSO). All data were shown as the mean ± SEM of triplicates.

3.1.2. Transcriptional changes in HK-2 cells

In HK-2 cells, both BPA and BPS showed similar directions of transcriptional changes of genes that are associated with kidney toxicity, but the responses from BPA exposure were more significant (Fig. 2). For BPA, while kidney injury molecule-1 (*KIM-1*) gene was significantly down-regulated (Fig. 2 (a)), most of the other genes including neutrophil gelatinase-associated lipocalin (*NGAL)*, interleukin-1β (*IL-1β*), interleukin-8 (*IL-8*), nuclear factor erythroid 2-related factor 2 (*NRF2*), catalase (*CAT*), microtubule-associated proteins 1A/1B light chain 3B (*LC3B*), and alpha-smooth muscle actin (*a-SMA*) were significantly up-regulated. Following exposure to BPS, *KIM-1* was significantly down-regulated, and *NRF2* gene showed up-regulating trend (Fig. 2 (b)). In contrast, following exposure to AA, genes such as AQP1, IL-1b, NRF2, and CAT showed different pattern of regulation, while KIM-1 showed the same significant down-regulation (Fig. 2 (c)).

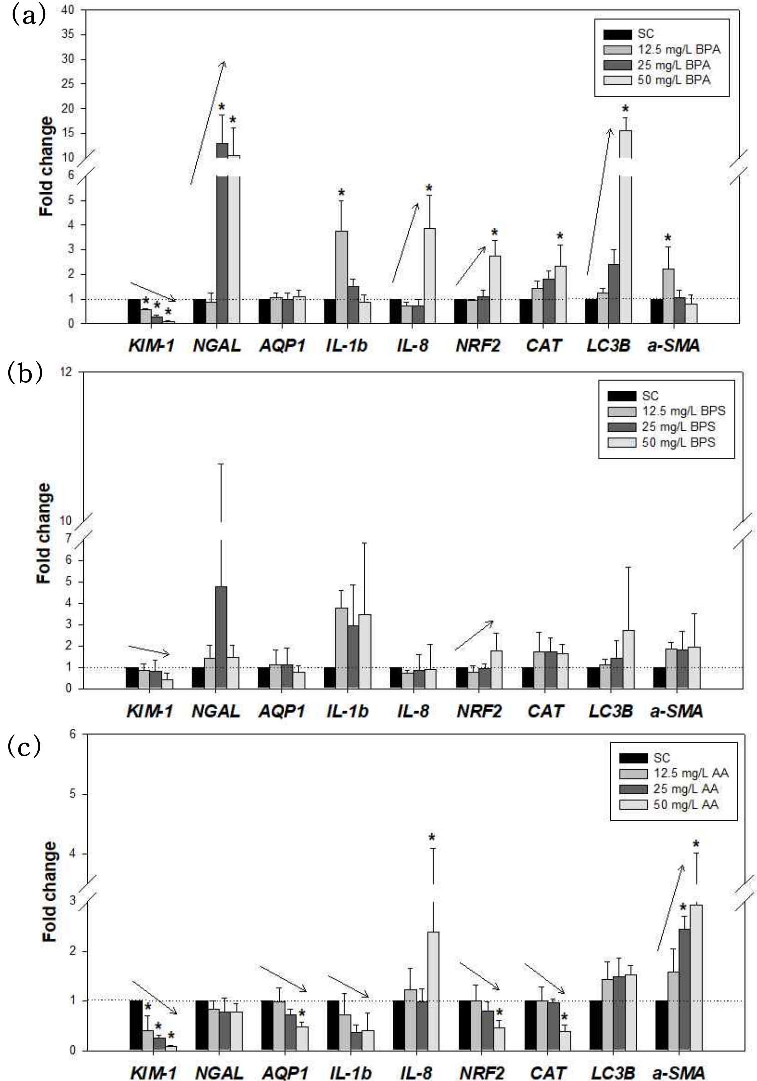

Figure 2. Transcription level changes of genes related to nephrotoxicity in HK-2 cells by (a) BPA, (b) BPS, or (c) AA exposure. Asterisks (*) indicate significant differences ($p<0.05$) from solvent control (0.1 % of DMSO). Arrows indicate significant trends ($p<0.05$) according to Spearman's rank correlation analysis. All data were shown as the mean ± SEM of triplicates.

3.2. Zebrafish larvae exposure

3.2.1 Transcriptional changes in zebrafish larvae

Transcription level changes of BPA, BPS, and AA in zebrafish larvae were measured (Fig. 3). BPA and BPS exposed zebrafish larvae showed similar gene transcription level changes. For BPA, significant kidney injury molecule-1 (*kim-1*) decrease was observed (Fig. 3 (a)). Nephrogenesis related genes like *nephrin*, wilms' tumor suppressor-1 (*wt1a*), ETS variant transcription factor 4 (*etv4*), single-minded homolog 1-A (*sim1a*) were significantly increased (Fig. 3 (a)). Fibrosis marker, collagen 1a1 (*co11a1*) was significantly down-regulated in BPA exposed zebrafish larvae (Fig. 3 (a)). For BPS exposure, *nephrin*, *sod1*, *gpx1a* showed up-regulation trends (Fig. 3 (b)). In AA exposed groups, transcription level change of gpx1a was similar with BPS exposed groups. Following exposure to AA, trends of up-regulation were significant to gpx1a (Fig. 3 (c)).

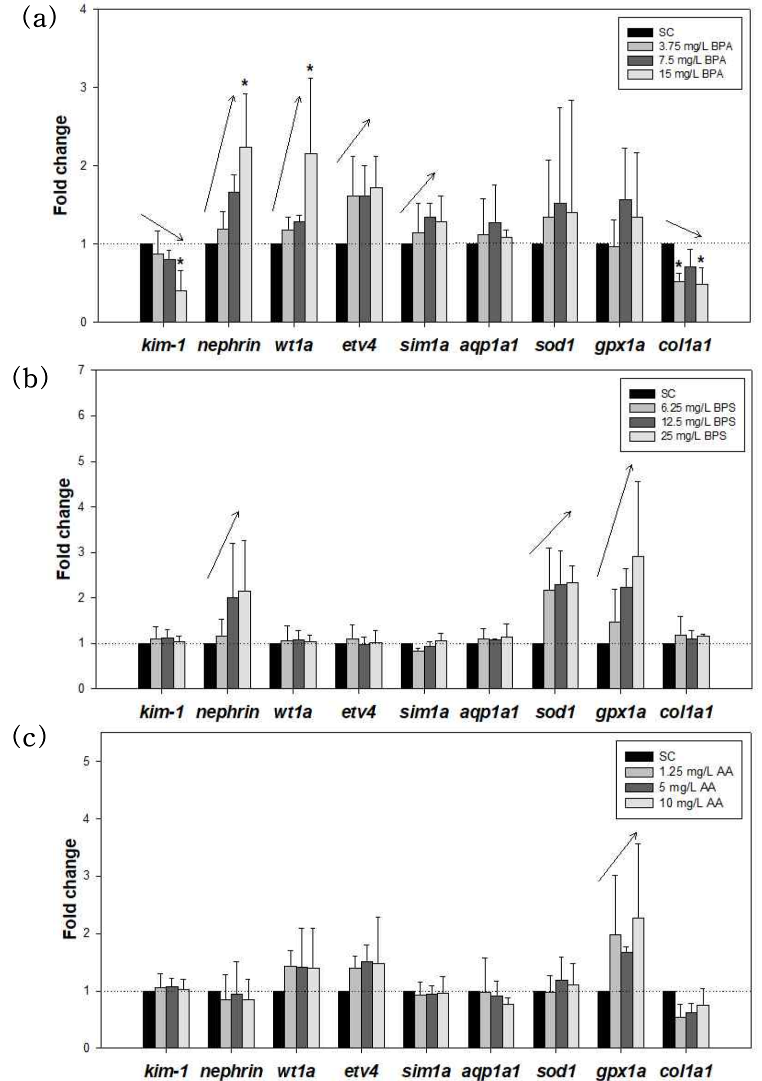

Figure 3. Transcription level changes of genes related to nephrotoxicity in zebrafish larvae by (a) BPA, (b) BPS, or (c) AA exposure. Asterisks (*) indicate significant differences ($p<0.05$) from solvent control (0.01 % or 0.1 % of DMSO). Arrows indicate significant trends ($p<0.05$) according to Spearman's rank correlation analysis. All data were shown as the mean ± SEM of triplicates.

3.2.2. Measurement of proteinuria in zebrafish larvae

At the highest concentration of AA, a significant increase of protein content was observed in medium water (Fig. 4). Following BPA exposure, the protein content showed an increase at the lowest experimental concentration, but statistical significance was not observed (Fig. 4). BPS exposure did not show changes in protein content (Fig. 4).

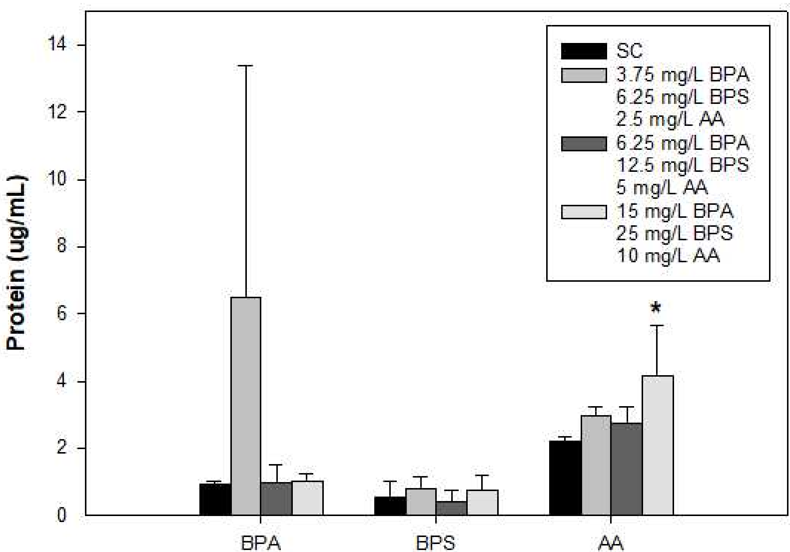

Figure 4. Protein content measured from the zebrafish media following exposure to BPA, BPS, or AA exposure. Asterisks (*) indicate significant trends ($p<0.05$) of each group in Spearman's rank correlation. All data were shown as the mean ± SEM.

4. Discussion

4.1. Transcriptional changes in HK-2 cells

Following exposure to BPA or BPS, while most of significant regulatory changes were observed for BPA, regulation of the measured genes showed generally similar pattern of change (Fig. 2). Up-regulation of *neutrophil gelatinase-associated lipocalin* (*NGAL*), a kidney damage marker in human urinary or serum samples (Han et al., 2002; Hauschke et al., 2017; Wheeler et al., 2008), by over 10-fold following the exposure to BPA supports that BPA has nephrotoxic potential (Fig. 2 (a)). Similarly, following BPS exposure, *NGAL* was up-regulated in HK-2 cells, although statistically insignificant (Fig. 2 (b)). These observations are comparable to epidemiological studies which reported significantly increased NGAL in urine or serum when kidney injury occurred (Hirsch et al., 2007; Mishra et al., 2005). Up-regulation of *NGAL* was also reported in HK-2 cells following exposure to gentamycin, an antibiotic with potential nephrotoxic side effect (Luo et al., 2016). On the other hand, observations on *kidney injury molecule-1* (*KIM-1*) gene, which showed significant down-regulation following exposure to both bisphenols (Fig. 2), warrant explanation, because the direction of change was different from those reported in epidemiological and experimental studies on nephrotoxicity. In epidemiological studies, *KIM-1* was up-regulated in human urinary samples when kidney injury occurred (Han et al., 2002; Tu et al., 2014). Similarly, in HK-2 cells, gentamicin exposure led to up-regulation of *KIM-1* gene dose-dependently (Luo et al., 2016). However, other observations are also available. Following exposure to cisplatin, a down-regulated pattern of *KIM-1* was observed in cisplatin exposed lysates of HK-2 cells (Sohn et al.,

2013). Sohn et al., (2013) suggested that the decrease of *KIM-1* is because cisplatin could induce the shedding of *KIM-1* ectodomain. Transcription level changes of aquaporin 1 (*AQP1*) was not observed in BPA or BPS exposed HK-2 cells (Fig. 2).

The major mechanisms of BPA and BPS-induced nephrotoxicity suggested as oxidative stress, fibrosis, autophagy, inflammation in the present study. Significant up-regulation of *nuclear factor erythroid 2-related factor 2* (*NRF2*) and *catalase* (*CAT*) following exposure to both bisphenols, suggest oxidative stress potential of these bisphenols (Fig. 2). *NRF2* and *CAT* genes are related to antioxidant activities (Aebi, 1974; Ma, 2013). In BPA exposed HK-2 cells, the gene expression level of *a-SMA* was significantly increased (Fig. 2). Fibrosis marker, *alpha-smooth muscle actin* (*a-SMA)*, is activated by kidney damage through fibrosis mechanism (Li et al., 2018). Up-regulation of *microtubule-associated proteins 1A/1B light chain 3B* (*LC3B*) gene following the exposure to both bisphenols (Fig. 2) suggests autophagy may be involved in the nephrotoxicity of bisphenols. *LC3B* is an autophagy marker and autophagy is suggested to play a protective role in acute kidney injury (Jiang et al., 2012). Moreover, significant up-regulation of *interleukin 1β* (*IL-1β*) and *interleukin 8* (*IL-8*) genes by BPA and up-regulation of *IL-1β* by BPS exposure (Fig. 2) suggest inflammation did also occur. *IL-1β* and *IL-8* are activated in inflammation responses (Harada et al., 1994; Watkins et al., 1995).

Transcriptional changes observed following exposure to AA suggest that the mode of nephrotoxicity for both bisphenols are different from that of AA. Different from the bisphenols, *NGAL* expression level did not change by AA exposure. Gene expression levels changes of *KIM-1* in AA exposed HK-2 cells were similar to BPA exposed

HK-2 cells. In AA exposed groups, *AQP1* was significantly decreased. In addition, regulation of oxidative stress-related genes such as *NRF2* and *CAT* showed different patterns from those observed following BPA or BPS exposure. Following exposure to AA, both *NRF2* and *CAT* were significantly down-regulated in HK-2 cells. Down-regulation of oxidative stress markers was also supported by a previous studies of AA-induced nephrotoxicity using HK-2 cells or rats (Yu et al., 2011; Zhao et al., 2015). The observations suggest that the major mechanisms of AA-induced nephrotoxicity in HK-2 cells include oxidative stress and fibrosis.

4.2. Transcriptional changes in zebrafish larvae

Up-regulation of several genes related to nephrogenesis (*nephrin*, *wt1a*, *etv4*, *sim1a*) that was observed following BPA exposure in zebrafish larvae (Fig. 3 (a)) suggests nephrotoxic potential of BPA. Similar pattern of regulatory changes following exposure to BPS, even though statistical significance was not as frequently detected, suggest that this BPA substitute may have similar nephrotoxic potential. Up-regulation of *nephrin* gene by both bisphenols is different from conventional response of nephrotoxic chemicals, which was down-regulation of *nephrin* gene (Wang et al., 2016), and therefore require discussion. The *nephrin* gene is mainly expressed in pronephric glomerulus of zebrafish and involves in formation of glomerulus foot process (Fukuyo et al., 2014). Studies suggested that inflammatory cytokines could up-regulate *NEPHRIN* in human embryonic kidney epithelial cells and podocytes (Huwiler et al., 2003; Ren et al., 2005). In Ren et al. (2005), transcription levels of *NEPHRIN* were significantly increased by IL-1β or tumor necrosis factor-α (TNF-α) in the presence of PPARα agonists in human kidney cells. While transcription levels of inflammation related markers were not investigated in zebrafish larvae, but HK-2 cell observations showed up-regulation of inflammation markers (Fig 2). *wt1a* acts as an activator of *nephrin* expression in mammals and zebrafish (Guo et al., 2004; Perner et al., 2007; Wagner et al., 2004; Zhang et al., 2018), and therefore up-regulation of both *wt1a* and *nephrin* genes in BPA exposed zebrafish larvae was deemed reasonable (Fig. 3 (a)). Transcription level changes of *e-twenty six transcription factors* (*etv4*) *and single-minded homolog 1-a* (*sim1a*) were significantly up-regulated in BPA exposed zebrafish larvae groups but not in BPS exposed zebrafish larvae groups (Fig. 3). The

up-regulation of *etv4* and *sim1a* genes were suggested as an abnormality of nephrogenesis (Chen et al., 2011; Cheng and Wingert, 2015). In zebrafish larvae experiments, the pattern of transcription level changes of *kim-1* was the same as the HK-2 cell experiment (Fig. 2 and 3). In BPA and BPS exposed zebrafish larvae, *kim-1* was significantly down-regulated (Fig. 3). While *kim-1* expression level was significantly increased in gentamicin exposed adult zebrafish kidney (Yin et al., 2016), regulatory changes of *kim-1* gene have not been reported in chemical-induced nephrotoxicity in larval zebrafish larvae previously. Additional studies are needed for *kim-1* expression changes in zebrafish larvae by nephrotoxic chemical exposure.

Mechanisms involved in BPA-induced nephrotoxicity appear to include oxidative stress and fibrosis. The transcription levels of antioxidants, *superoxide dismutase 1* (*sod1*) and *glutathione peroxidase 1-A* (*gpx1a*), were significantly increased in BPS exposed zebrafish larvae (Fig. 3). Similarly, the transcription levels of *sod1* and *gpx1a* in BPA exposed zebrafish larvae group were up-regulated although statistical significance was not observed (Fig. 3). The transcription level of *collagen 1a1* (*col1a1*) was significantly down-regulated in BPA exposed zebrafish larvae groups (Fig. 3). Collagen is deposed in the initial stage of fibrosis damage in mammals (Chablais et al., 2011). No changes of *col1a1* in BPS exposed zebrafish larvae groups (Fig. 3).

Mechanisms involved in AA-induced nephrotoxic effects appeared to be slightly different from those of BPA and BPS. In AA exposed zebrafish larvae, transcription level changes related to glomerulus formation and nephrogenesis related genes like *wt1a*, and *etv4* were up-regulated, in a way similar to those observed from BPA or BPS exposed zebrafish larvae, but without statistical significance (Fig. 3).

The transcription level of *nephrin* was did not change in AA exposed zebrafish larvae (Fig. 3 (c)), which is different from a previous study which reported down-regulation of *nephrin* in zebrafish larvae following AA exposure (Wang et al., 2016). This difference may be explained by the concentrations that were used for the exposure. In Wang et al. (2016), transcription level of *nephrin* was analyzed in 20 and 40 μmol/L of AA but these concentrations showed over 10% mortality rates (Wang et al., 2016). Interestingly, oxidative stress markers showed different patterns from those obtained from bisphenols exposure (Fig. 2 and 3). The different patterns of oxidative stress-related genes could be due to the difference between organ-specific and whole body responses. The transcription level of *gpx1a* in zebrafish larvae was observed in the whole body of zebrafish larvae. On the other hand, transcription level changes in HK-2 cells were specifically observed in the kidney.

4.3. Proteinuria changes in zebrafish larvae

Proteinuria used as a marker of glomerular barrier or tubular damage in the kidney (Haraldsson et al., 2008). Proteinuria of AA exposed zebrafish larvae were observed in the previous study (Wang et al., 2016). In this study, only AA exposed zebrafish larvae showed a significant increase in proteinuria. The reason of proteinuria level changes in AA treated groups suggests that the transcription level of *nephrin* is slightly down-regulated in AA exposed groups but not in BPA or BPS exposed groups (Fig. 4). According to previous and present studies, an increase of proteinuria in AA exposed zebrafish larvae suggest that AA could affect the glomerulus filtrate function of zebrafish larvae. BPA and BPS not significantly affect the glomerulus filtration function of zebrafish larvae. No changes of proteinuria were not inferred that BPA and BPS were not affect to the zebrafish kidney. Transcription levels related to nephrogenesis in zebrafish larvae were significantly changed in the present study, gene expression changes should also be considered.

5. Conclusion

Nephrotoxicity of bisphenol A (BPA) and bisphenol S (BPS) were limitedly studied compared to sex hormone or thyroid hormone disruption. In the present study, the nephrotoxic effects of BPA and BPS, a substitute for BPA, were compared for the first time in the same experimental models. In the *in vivo* and *in vitro* studies, studies results infer that the nephrotoxic effects of BPA was higher than that of BPS. It was also found that the transcription patterns of BPA exposed zebrafish larvae and HK-2 cells were similar to BPS exposed zebrafish and HK-2 cells. Aristolochic acid (AA) showed different nephrotoxic effects and nephrotoxicity related mechanisms than BPA and BPS. Further studies related to BPA-induced or BPS-induced nephrotoxicity using zebrafish larvae and HK-2 cells experimental models should be followed.

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국문 초록

제브라피쉬 치어와 HK-2 세포주를 이용한 비스페놀 A, 비스페놀 S, 그리고 아리스토로크산(Aristolochic acid)의 신장 독성 영향 및 기전 연구

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비스페놀 A (BPA)와 비스페놀 S (BPS)는 플라스틱 병, 영수증, 음식 포장지, 에폭시 레진 제조 등에 널리 사용되는 물질이다. BPA와 BPS는 기존에 성호르몬이나 갑상선호르몬 교란에 관해서는 많은 실험 연구들을 통해 그 영향 및 기전이 밝혀졌지만, 신장 독성에 관한 연구는 아직 많이 이루어지지 않아 추가적인 연구가 필요하다.

본 연구에서는 BPA 와 BPS 의 신장 독성 영향을 제브라피쉬 치어와 사람 신장 세포인 HK-2 세포주를 이용하여 확인하고자 하였고, 이를 기존에 신장 독성 영향이 잘 알려진 물질인 아리스토로크산(Aristolochic acid (AA))의 신장 독성 영향과 비교하고자 하였다.

HK-2 세포주를 이용한 실험에서는 BPA 와 BPS, 그리고 AA 를 HK-2 세포주에 노출시킨 뒤 신장 손상 지표, 산화적 손상 지표, 섬유화 지표, 자가포식 지표, 염증 지표들의 유전자 발현 변화를 대조군과 비교하여 관찰하였다. 제브라피쉬 치어를 이용한 실험에서는 유전자 발현 변화 비교와 단백뇨 관찰 실험을 진행하였다. 유전자 발현 변화의 경우

BPA, BPS, 그리고 AA 를 각각 제브라피쉬 치어에 노출시킨 후 신장 초기발달 지표, 신장 구성 지표, 산화적 손상 지표, 섬유화 지표들의 발현 변화를 대조군과 비교하여 관찰하였다. 단백뇨 관찰 실험의 경우 세 물질을 각각 노출 시킨 후 배양수에서의 단백질의 양의 변화를 BCA assays 로 측정하여 비교하였다.

사람 신장 세포주인 HK-2 세포주를 BPA, BPS 그리고 AA 에 각각 노출시킨 결과 BPA 노출군과 BPS 노출군에서 비슷한 경향성의 유전자 변화가 나타났다. 그러나 AA 노출군의 경우 일부 유전자 발현 변화 양상만이 비슷하게 나타났고 대부분의 유전자 발현 변화에서 두 물질을 노출한 경우와 다른 발현 변화가 나타났다. 제브라피쉬를 이용한 실험에서는 BPA 노출 결과 신장 손상 지표인 *kim-1* 이 유의하게 감소하였고, 신장 발달 및 구성에 관여하는 유전자들의 발현이 유의하게 변화함을 관찰했다. 산화적 손상 그리고 섬유화 손상과 관련된 지표들의 유전자 발현 변화도 관찰되었다. BPS 노출군에서는 산화적 손상 지표들의 뚜렷한 증가가 관찰되었다. 제브라피쉬 치어에서 단백뇨를 관찰한 결과 BPA 와 BPS 노출군의 경우 변화가 크게 나타나지 않았지만, AA 의 경우 농도 의존적으로 단백뇨가 증가했다.

이러한 실험 결과를 통해 BPA, BPS는 제브라피쉬 치어와 사람 신장 세포주에서 비슷한 양상으로 신장 발달 및 구성 관련 지표와 신장 손상 메커니즘 관련 지표들의 발현을 변화시킨다는 사실을 알 수 있었다. 또한 AA 로 인한 신장 독성 영향 및 기전은 BPA와 BPS와는 차이가 있다는 사실을 확인하였다. 따라서 BPA와 BPS의 독성 평가 시 신장 독성 영향 또한 고려되어야 하며 추후 비스페놀류 물질의 신장 독성 영향을 평가할 때 활용될 수 있을 것으로 기대된다.

주요어: 비스페놀 A (BPA), 비스페놀 S (BPS), 아르스토로크산 (Aristolochic acid), 신장 독성, 제브라피쉬, HK-2 세포주

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