ABSTRACT

DNA methylation alterations in an association between bisphenol A and endometrial condition

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Bisphenol A is one of the endocrine disrupting chemicals and extensively used as protective coatings on food-storage containers, baby bottles, bottle tops, water pipes, and medical equipment. Bisphenol A binds to estrogen receptors, and acts as an estrogen agonist. Bisphenol A also induces epigenetic modifications. Endometrium expressed estrogen receptors, thus it could be a target tissue for bisphenol A. Several studies explored relationship between bisphenol A and gynecologic disorders. However, there are few studies about cytotoxic and epigenetic effects of bisphenol A on endometrium. Therefore, it is necessary to evaluate the relationships between bisphenol A and endometrial disorders.

This study evaluated cytotoxicity and DNA methylation changes in bovine endometrial cells induced by bisphenol A. And urinary bisphenol A concentrations were assessed from women with endometrial disorders, then the relationship was explored between urinary bisphenol A levels and methylation levels of endometrial tissue DNA.

When bovine endometrial cells were treated with 100 μM of bisphenol A for 3 h, the cell viability was significantly decreased. Also, apoptotic cells were significantly induced after 24 h treatment of bisphenol A at 100 μM. S phase arrest was observed after 3 h treatment, and G2/M cell cycle arrest was observed after 24 h treatment. After 1 h treatment, intracellular ROS was significantly increased. DNA damage was induced after 3 h treatment of bisphenol A. After 3 h treatment, global DNA methylation and HOXA 10 methylation levels were decreased. Meanwhile, the methylation level of RASSF1A, one of tumor suppressor genes, was increased after 24 h treatment of 100 μM bisphenol A.

Endometrial tissue DNAs were obtained from 44 women with endometriosis, myoma, or adenomyosis. The relationships between urinary bisphenol A and DNA methylation levels, and the diseases and DNA methylation status were evaluated. There were no significant differences about urinary bisphenol A level among the diseases, also the relationships between urinary bisphenol A levels and DNA methylation status were not observed. However, global DNA methylation level in proliferative phase was significantly higher than that in secretory phase. Sat2 methylation level was significantly higher in adenomyosis than that in myoma. Also, HOXA 10 methylation was significantly associated with endometrial polyps.

In conclusion, bisphenol A can induce cytotoxicity and modify DNA methylation level in bovine endometrial cells. The relations between urinary bisphenol A concentration and endometrial disorders, or urinary bisphenol A

concentration and DNA methylation could not find in the epidemiologic study. On the other hand, global DNA hypomethylation was associated with endometrium phase, and endometrial polyps showed HOXA 10 hypermethylation. Therefore, HOXA 10 methylation can be used as a biomarker for endometrial polyp.

Keywords: bisphenol A, endometrium, endometrial cells, endometriosis, myoma, adenomyosis, endometrial polyp, DNA methylation, HOXA 10

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Introduction

1. Background

Humans are exposed to a wide range of chemicals in commonly used products, and exposure to some of these chemicals is potentially associated with numerous toxic effects, including disturbances in the endocrine system (Kim et al. 2011). Endocrine disrupting chemicals (EDCs) are exogenous chemicals that interfere with hormonal homeostasis such as alteration in estrogen signaling (Louis et al. 2013). EDCs can be separated into classes of chemicals including pesticides (DDT), fungicides (vinclozolin), herbicides (atrazine), industrial chemicals (PCBs, dioxins), plastics (phthalates, bisphenol A, alkylphenols) and plant hormones (phytoestrogens) (Skinner et al. 2011). As shown Table 1, there are lots of studies to determine the relationship between EDCs and adverse effects in cell lines, animal or human (modified from Casals-Casas and Desvergne, 2011). However, the impact of EDCs is not yet fully understood and there still remain data gaps for human exposure and fecundity endpoints such as gynecologic disorders.

Table 1 Health effects of EDCs in animal/cell lines and human studies

Substance	In vitro/animal studies	Human epidemiologic studies	Legal status	Reference
DDT	Malformations of reproductive tract	Reduced birth weight	1970s: DDT banned in most developed countries	Farhang et al. (2005); Aneck-Hahn et al. (2007); Lee et al. (2007)
		Decreased semen quality		
		Associated with MetS and diabetes		
Dioxin	Altered estrus cycle	Endometriosis	2000s: PCB banned and other dioxins restricted by the Stockholm Convention	Pauwels et al. (2001); Bruner et al. (2011); Mocarelli et al. (2008); Cimafranca et al. (2004)
	Sexual precocity	Reduced fertility		
	Adipogenesis inhibition	Reduced sperm concentration and motility		
		Associated with MetS, obesity, and diabetes		
Phthalates	Inhibited testosterone synthesis	Affect thyroid activity	Restricted in children's toys in the EU (1999) and the United States (2009)	Parks et al. (2000); Huang et al. (2007); Feige et al. (2007); Stahlhut et al. (2007)
	Adipogenesis induction in cells	Associated with obesity and insulin resistance		
PBDE	Glucose oxidation decrease	Associated with MetS and diabetes	Banned in the EU and some U.S. states. 2009: some BFRs banned by the Stockholm convention	Swedenborg et al.(2009); Hoppe et al. (2007)

Bisphenol A (BPA, 2, 2- bis(4-hydroxyphenyl)propane) is one of the EDCs and an estrogenic monomer with two unsaturated phenol rings. BPA is one of the highest-volume chemicals produced worldwide; global BPA production was 5.5 million tons (Zhang et al. 2013). It is extensively used in the production of polycarbonate plastic and epoxy resins, which are used as protective coatings on food-storage containers, baby bottles, bottle tops, water pipes, medical equipment, and dental sealants (Hiroi et al. 2004). People also can contact daily at home and in workplaces with coating of CDs, DVDs, electrical and electronic equipment, automobiles, sports safety equipment, recycled paper, and register receipts (Rubin, 2011). The extensive use of BPA is indicated that increasing its potential exposure to humans through water, air, soil, and food contamination. Among these routes, oral exposures with food are the major source for BPA exposure in all age groups of non-occupationally exposed general population (EFSA 2006; EU 2003; Dekant and Völkel 2008). Especially, BPA leaches from cans to contained food when exposed to high temperature, to acidic or basic substances (Vom Saal and Hughes, 2005). As a result, human exposures are most likely through the oral route (Kang et al. 2006; Vandenberg et al. 2007). BPA was considered as an environmental estrogen in 1936. The structure of BPA is not similar with estrogen, but BPA can bind to estrogen receptors (ER) α and β and acting as a weak estrogen agonist (Hiroi et al. 1999). Although BPA can be 10,000 to 100,000-fold less potent than E_2, BPA competes with endogenous E_2 for ER binding and activation of estrogen-responsive genes (Gore, 2007).

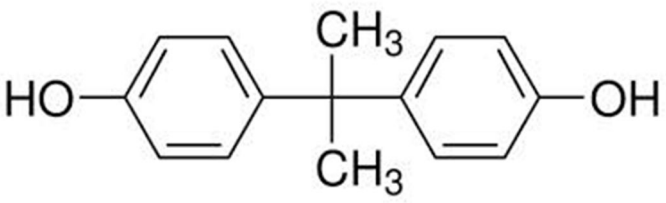


Figure 1 Chemical structure of BPA

There are many studies about biomonitoring of BPA levels, and BPA are found in the serum, breast milk, saliva, and urine of humans, especially can detect in more than 95 % of human urine (Calafat et al. 2005). It means that human exposure to BPA continuously happened at low dose and it is unavoidable, thus this can affect human health. Therefore the effects of BPA on human health have become of growing global concern, and it should be assessed for the toxicity of BPA in humans.

Several studies have suggested adverse effects of BPA on human health (Table 2). Human studies about the effects of BPA exposure are limited. BPA levels have been associated with a variety of disease, such as polycystic ovary syndrome (PCOS), obesity, recurrent miscarriage, cardiovascular disease, diabetes, and heart disease. Two studies suggested that women with PCOS had higher BPA levels than women without PCOS (Takeuchi and Tsutsumi, 2002; Takeuchi et al. 2004). While BPA levels were negatively associated with serum estradiol levels, total T4 and TSH, and semen quality, levels of BPA were positively correlated with androgen levels (Meeker and Ferguson, 2011; Li et al. 2011; Takeuchi et al. 2004). Sugiura-Ogasawara et al. (2005) reported an association between recurrent miscarriage and serum BPA levels. The mean values for BPA in patients were 2.59 ng/ml, it was significantly higher than the 0.77 ng/ml BPA levels in controls. Additionally, in a

cross-sectional analysis of NHANES (Melzer et al. 2010), higher BPA levels were associated with coronary heart disease in 2005/06 population (OR=1.33, 95 % CI: 1.01-1.75, p=0.043) and in pooled data (OR=1.42, 95 % CI: 1.17-1.72, p=0.001). Finally, the study based on NHANES (2003-2008) was demonstrated that the association between BPA and BMI-based obesity and waist circumference-based obesity was significant across gender and race-ethnic groups (Shankar et al. 2012).

According to the previous studies, World Health Organization (WHO, 2009) and the European Food Safety Authority (EFSA, 2006) concluded that the no-observed-adverse-effect level (NOAEL) for BPA is 5 mg/kg bw/day, and the temporary tolerable daily intake (TDI) is 50 μg/kg bw/day.

Table 2 The adverse effects of BPA on human health

Study	Heath related outcome	Relationship	Reference
14 healthy women, 16 PCOS, 11 healthy men	PCOS	PCOS women have higher BPA levels than healthy women.	Takeuchi and Tsutsumi (2002)
7 hyperprolactinemia, 21 hypothalamic amenorrhea, 13 non-obese PCOS, 19 non-obese controls, 7 obese controls	Obesity and PCOS, Androgen level	Compared to non-obese women, BPA was higher in obese women, non-obese PCOS, obese PCOS women. BPA levels were associated with androgen levels.	Takeuchi et al. (2004)
45 cases (recurrent miscarriage), 32 controls	Recurrent miscarriage	Compared to controls, BPA was higher in women with recurrent miscarriage.	Sugiura-Ogasawara et al. (2005)
1455 adults, Cross-sectional study	Cardiovascular diagnosis, diabetes	Higher urinary BPA concentrations were associated with cardiovascular diagnoses and diabetes.	Lang et al. (2008)
84 women	Oocytes retrieved per cycle and estradiol levels	Urinary BPA concentrations were inversely associated with number of oocytes retrieved per cycle. Inverse relationship between BPA and peak serum estradiol levels.	Mok-Lin et al. (2009)
Cross-sectional study, 1455 adults(2003/04), 1493 adults (2005/06)	Heart disease	Higher BPA exposure is consistently associated with heart disease.	Melzer et al. (2010)

218 workers	Semen quality	Inverse relationship between BPA levels and semen quality.	Li et al. (2011)
1346 adults	Thyroid levels	Inverse relationship between urinary BPA and total T_4 and TSH.	Meeker and Ferguson (2011)
4792 adults	Obesity	Higher urinary BPA levels are positively associated with obesity.	Shankar et al. (2012)
1904 Korean adults	E levels	High BPA levels are associated with higher E levels. Especially 4-hydroxyestrogens was activated by BPA.	Kim et al. (2014)

Although there are several epidemiological studies about the relationship between BPA exposure and health-related end points, there are few studies about evaluate the effects of BPA exposure on estrogen-dependent diseases, such as adenomyosis or endometriosis. In addition, the association between exposure BPA and endometrial disorders has not shown consistent.

Endometrial cells are critical components of female reproductive tissues and function when they grow in their appropriate environment, endometrium. However endometrial cells grow inappropriate places, they lack the normal growth control and continue to respond to hormonal signals (Birnbaum and Cummings, 2002). Endometrial cells are important for the endometrial function and several conditions, such as menstrual bleeding and endometriosis. Therefore the investigation of possible effects of BPA exposure on endometrial cells is important.

Endometriosis and adenomyosis are common and benign gynecological disorders, affecting at least 15 and 19.5 % of women of reproductive age, respectively (Cobellis et al. 2009; Birnbaum and Cummings, 2002; Nie et al. 2010; Devlieger et al. 2003). Both diseases are characterized by the presence of endometrial glands and stroma outside the uterus cavity (Juo et al. 2006). Endometriosis is the presence and growth of functional endometrial-like tissues, endometrial glands and stroma, outside the uterus cavity. Adenomyosis is defined as the presence of ectopic endometrial glands and stroma within the myometrium (Fischer et al. 2010). Endometriosis often causes dyspareunia, dysmenorrhea, pelvic pain and subfertility. It can be severely alter quality of life, so there is a need to find appropriate solutions to improve women's life quality. The symptoms of adenomyosis are a soft and diffusely enlarged uterus with menorrhagia, dysmenorrhea, and subfertility (Farquhar and Brosens, 2006). Besides symptoms, there are molecular aberrations in adenomyosis and endometriosis; impaired apoptosis (Dmowski et al. 2002) and accelerated proliferation (Meresman et al. 2002) of the eutopic endometrium. Although the pathophysiology of endometriosis

and adenomyosis is poorly understood, several theories have been proposed to explain. Sampson proposed that shed endometrial cells flow with retrograde menstrual through the fallopian tubes and implant on the peritoneal surfaces or ovaries, and then endometriosis occurs (Sampson, 1940; Guo, 2009). Endometriosis and adenomyosis require estrogen (Birnbaum and Cummings 2002). Estrogen plays a major role in growth of endometrial cells, wherever the cells are in. Therefore endometriosis and adenomyosis have been regarded as an ultimate hormonal disease, especially estrogen-depend disease. Recently, one emerging hypothesis suggests a putative role for environmental contaminants, EDCs in endometrial disorders. Especially there were few studies assessed effects of BPA exposure on endometriosis or adenomyosis. Given the previous studies, we hypothesized that higher urinary BPA concentrations would be associated with endometrial disorders, such as endometriosis or adenomyosis.

Epigenetic change is defined as stable and heritable changes in gene expression without changes in the nucleotide sequence. These include DNA methylation, histone modification and transcription factor network (Guo, 2009). DNA methylation is one of the most widely studied epigenetic modifications. It occurs that a methyl group can covalently bound at the 5' carbon of a cytosine on a CpG palindromic sequence, especially short stretches of C: G-rich DNA, called CpG islands. The CpG islands are methylated in a promoter of a particular gene, the gene may become silenced or less expressed. And the CpG islands remain unmethylated, the gene may be more expressed. CpG islands are usually unmethylated in adult human tissues, but CpG islands have been found to become aberrantly methylated, and activation of the wrong gene may lead to the onset of several types of diseases, such as cancers (Trinh et al. 2001). DNA methylation is an important mechanism in the effect of environmental chemicals to influence human diseases (Singh et al. 2012). And DNA methylation plays an important role in developmental processes, imprinting, cell proliferation, and the maintenance of

genome stability (Jones and Baylin, 2007). Aberrant DNA methylation may result in loss of genomic stability, and have emerged as one of the most consistent molecular alterations in carcinogenesis (Jones and Baylin, 2007). A global loss of DNA methylation is often associated with genomic instability and tumors (Sutherland and Costa, 2003). Promoter hypermethylation is the major mechanisms to inactivate this gene, while loss of function mutation is an infrequent event (Liu et al. 2002). And aberrant hypermethylation of tumor suppressor genes is associated with the lack of gene transcription, thus, can contribute to the formation and progression of cancer by providing a loss of tumor suppressor gene function (Shen et al. 2002).

There are a number of studies assessed DNA methylation effects from BPA exposure (Esterik et al. 2015; Table 3). Maternal BPA exposure decreases offspring methylation at Agouti promoter (Dolinoy et al. 2007; Anderson et al. 2012). While global DNA methylation of DNA isolated from tail of agouti mouse increased, bone marrow derived mast cells (BMMC) from perinatal mouse exposure of BPA decreased level of global DNA methylation (Anderson et al. 2012; O'Brien et al. 2014). Ma et al. (2013) and Li et al. (2014) showed that BPA exposure induced global DNA hypomethylation in sperm and liver of rat, and in placenta of mouse (Susiarjo et al. 2013). BPA exposure induced global DNA hypermethylation in male mouse, but global DNA hypomethylation in female mouse (Patel et al. 2013). Fernandez et al. (2012) showed whether BPA exposure induced alteration DNA methylation in human breast epithelial cells. On the other hand, there are few epidemiologic studies in human. Blood BPA levels were associated with DNA hypomethylation at TSP50 (testes-specific protease 50) in women with In vitro fertilization (IVF) (Hanna et al. 2012). Additionally, Kim et al. (2013) showed BPA levels associated with hypomethylation of DNA involved in immune function, transport activity, metabolism, and caspase activity.

Endometriosis has been regarded as a genetic disease. There are numerous gene expression studies in endometriosis. The abnormally high levels of expression

of aromatase in endometriosis compared with those of normal women (Hudelist et al. 2007). Taylor et al. (1999) showed that HOX gene expression significantly lower in women with endometriosis than normal women. Aberrant expression of Bcl-2, Bax, and Cyclooxygenase-2 (COX-2) in endometrium from women with endometriosis was found (Meresman et al. 2000; Chishima et al. 2002). To maintain cellular identity, the gene expression must be iterated through cell divisions in a heritable fashion by epigenetic processes (Guo, 2009). Recent studies demonstrated a role for aberrant of DNA methylation in the pathogenesis of endometriosis. Wu et al. (2005) and Andersson et al. (2014) demonstrated that HOXA 10 methylation levels in women with endometiosis were significantly higher than control group, and Lee et al. (2009) found that HOXA 10 hypermethylated in mouse with endometiosis. Wu et al. (2006) analyzed the methylation level of progesterone receptor isoform B (PR-B) in endometriosis. The promoter region of PR-B was hypermethylated in endometriosis. Recently, the analysis of genome-wide methylation was performed using endometrial biopsies from women with endometriosis. There were aberrant DNA methylations of various genes, such as cell division cycle associated DNA, immunoglobulin superfamily, and tumor necrosis factor receptor, this may contribute to abnormal regulation of endometrial cell proliferation (Naqvi et al. 2014). In light of these, endometriosis is an epigenetic disease. However, it has been unknown why the epigenetic change occurred in endometriosis. And there's no information about the BPA effects of epigenetic changes on endometriosis.

Taken together, BPA is extensively used and human exposure is common. BPA has estrogenic properties and effects of DNA modification. Therefore the effect of BPA on human health have become of growing global concern. There are few studies about the relationship between BPA and reproductive disorder, especially endometriosis or adenomyosis. This study investigated the relationship between endometriosis or adenomyosis and BPA levels. Also this study explored the

modification effects of BPA on DNA methylation on endometrial cells.

Table 3 Studies on DNA methylation changes induced by BPA [a]

Subject	Tissue/Cell type	Dose (μg/kg bw/d, μM)	Exposure route	Assay	Methylation result	Reference
Agouti mouse	Tail	10,000	Diet	Bisulfite sequencing	Hypomethylation at Agouti promoter	Dolinoy et al. (2007)
Agouti mouse	Tail	0.01 10 10,000	Diet	LUMA; bisulfite sequencing	Global DNA hypermethylation, hypomethylation at Agouti promoter	Anderson et al. (2012)
Agouti mouse	Bone marrow derived mast cells	0.01 10 10,000	Diet	Enzyme immunoassay kit	Global DNA hypomethylation	O'Brien et al. (2014)
CD-1 mouse	Uterus	5000	Intraperitoneal	Bisulfite sequencing	Hypomethylation at HOXA 10 promoter, intron	Bromer et al. (2010)
Wistar rat	Liver	50	Oral gavage	HPLC; bisulfite sequencing	Global DNA hypomethylation	Ma et al. (2013)
SD rat	Sperm, Liver	40	Oral gavage	Methylamp™ Global Quantification kit; bisulfite sequencing	Global DNA hypomethylation	Li et al. (2014)
C57BL/6n mouse	Ventricle heart	0.5 5 200	Drinking water	Methylamp™ Global Quantification kit; bisulfite sequencing	Male; Global DNA hypermethylation Female; Global DNA hypomethylation	Patel et al. (2013)
C57BL/6xC7 mouse	Placenta	10-10,000	Diet	LUMA	Global DNA hypomethylation	Susiarjo et al. (2013)

Human	Breast epithelial cells	1, 10 μM	-	MeDIP-on-chip	Hypermethylation at BCL2L11, PARD6G, FOXP1 and SFRS11 Hypomethylation at NUP98, RBBP8	Fernandez et al. (2012)
Human	Blood from women with IVF	-	-	llumina GoldenGate Cancer Panel I bead array	Hypomethylation at TSP50_P137 (testes-specific protease 50)	Hanna et al. (2012)
Human	Prepubescent girls	-	-	Infinium HumanMethylation27 BeadChip	Hypomethylation at involved in immune function, transport activity, metabolism, and caspase activity	Kim et al. (2013)

[a], modified from Esterik et al. (2015)

2. Objectives

Bisphenol A, frequently exposed to people in their daily life, thus it can affect human health, especially endometrium. Therefore, this study was attempted to evaluate the cytotoxicity and DNA methylation alteration induced by bisphenol A in endometrium; normal bovine endometrial cell line and endometrial tissue of women with endometrial disorders.

The details of objectives in this study are followings;

1. In order to evaluate the cytotoxicity and DNA damage induced by bisphenol A in normal bovine endometrial cell lines
2. In order to evaluate the changes of DNA methylation in bisphenol A exposed normal bovine endometrial cell lines
3. In order to evaluate the change of DNA methylation in human endometrial cells exposed to bisphenol A
4. In order to examine the DNA methylation level of endometrial tissue DNA of women with endometrial disorders in relation to urinary bisphenol A concentration

Chapter 2.

Cytotoxicity and DNA methylation changes in bovine endometrial cells induced by bisphenol A

1. Introduction

Bisphenol A (BPA) is widely used in the production of polycarbonate plastic and epoxy resins, which are used as protective coatings on food-storage containers, baby bottles, bottle tops, water pipes, medical equipment, and dental sealants (Hiroi et al. 2004). BPA is one of the highest-volume chemicals produced worldwide; global BPA production was 5.5 million tons (Zhang et al. 2013). The extensive use of BPA is indicated that increasing its potential exposure to humans through water, air, soil, and food contamination. Since BPA was detected in 95 % of the urine samples in the USA (Calafat et al. 2005), human exposure of BPA is continuous and unavoidable. Therefore the effects of BPA on human health have become of growing global concern and it became necessary to assess should be assessed for the toxicity of BPA in humans. Recent epidemiological studies indicate that exposure BPA is associated with early childhood behavior, obesity, polycystic ovary syndrome (PCOS), recurrent miscarriage, and reduction of sperm production (Braun et al. 2009; Carwile and Michels, 2011; Kandaraki et al. 2011; Sugiura-Ogasawara et al. 2005; Meeker et al. 2010).

There are many studies about the effects of BPA *in vitro*. Table 2.1 summarizes the results from published studies. Breast cancer models, embryonic models, and reproductive models were used to investigate adverse effects of BPA. Breast cancer cells were induced DNA damage, altered gene expressions such as ER and PR (Buterin et al. 2006; Cappelletti et al. 2003; Iso et al. 2006; Singleton et al. 2006). And the levels of methylation and gene expression were influenced by 50 μM of BPA in normal mammary epithelial cells (Qin et al. 2012). BPA inhibited the proliferation in human endometrium endothelial cells, induced apoptotic cells and G2/M arrest in primary granulosa cells (Lee et al. 2003; Xu et al. 2002). And BPA suppresses differentiation and survival of bone marrow cells (Hwang et al. 2013),

and induces apoptotic cells of testes (Iida et al. 2003).

The mechanism of BPA has been proposed that BPA act as hormone mimics via ER mechanisms; interfere with the endocrine hormonal systems (Welshons et al. 2003). BPA binds to ER, with 1000-10,000 fold lower affinity than estradiol (Kuiper et al. 1998). But BPA can effect on some cellular responses at very low concentrations (Alonso-Magdalena et al. 2005).

Table 2.1 Effects of BPA *in vitro* (modified from Wetherill et al. 2007)

Reference	Species	Cell line	Exposure time	Doses (M)	Endpoints
Buterin el al. (2006)	Human breast	T47D/Luc, MCF 7	3-24 h	10^{-13}-10^{-5}	Estrogen regulated gene promoter activation, cell proliferation
Cappelletii et al. (2003)	Human breast	T47D, BT20	6 days	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}	Promote of cell growth, up-regulation of ER, PR expression
Iso et al. (2006)	Human breast	MCF7, MDA-MB-231	1, 3, 24 h	10^{-8}, 10^{-6}, 10^{-4}	Induce DNA damage (comet assay)
Singleton et al. (2006)	Human breast	MCF7-C4-12	3 h	10^{-6}	Differential mRNA expression
Lee et al. (2003)	Human endometrium	Female primary endothelial cells	24, 48, 72 h	10^{-8}, 10^{-6}, 10^{-4}	Inhibit the proliferation
Xu et al. (2002)	Mice B6C3F1	Primary granulosa cells	72 h	10^{-10}	Decrease of cell viability, induce apoptosis and G2/M arrest
Tsutsui et al. (2000)	Hamster (Syrian)	Primary embryo cells	24, 48, 72 h	5×10^{-5}, 10^{-4}, 2×10^{-5}	Cell growth, morphological, DNA adduct formation, aneuploidy
Tabuchi et al. (2006)	Mice	TTE3 Sertoli cells (testis)	3, 6, 12 h	2×10^{-4}	mRNA expression
Hwang et al. (2013)	BKW mice	Bone marrow cells, RAW 264.7	24 h	5×10^{-7}, 1.25×10^{-5}	Inhibit cell viability, up-regulated Bax, Bcl-2 protein
Bredhult et al. (2009)	Human endometrium	Female primary endothelial cells	24 h	5×10^{-5}	Altered various gene expression
Qin et al. (2012)	Human breast	Mammary epithelial cells	1 week	10^{-7}	Epigenetic change and altered gene expression

Endometrial cells are critical components of the reproductive tissue and function when they grow in endometrium. However endometrial cells grow inappropriate places, they lack the normal growth control and continue to respond to hormonal signals (Biornbaum and Cummings, 2002). Estrogen and progesterone may control the menstrual cycle and pregnancy (Kimmins et al. 2001). Endometrial cells express ER and PR, and therefore endometrial cells are an important target of BPA. However, studies have shown inconsistent data about the effects of BPA in endometrial cells. Bredhult et al. (2007) evaluated the effects of BPA on the proliferation and viability of human endometrial endothelial cells. They found the proliferation of human endometrial endothelial cells was significantly decreased after 24 h for treatment of 0.01, 1, and 100 μM BPA. While the proportion of apoptotic cells was not increased at 100 μM BPA, BPA significantly induced necrotic cells. Aghajanova and Giudice (2011) showed the proliferation of endometrial cells significantly decreased at 50 and 100 μM BPA after 48 h treatment. But there was no difference in cell death upon treatment with any BPA concentration used. Meanwhile, accumulating evidence indicates that BPA can alter gene expression, and may alter DNA methylation status. BPA may alter gene expression and DNA methylation in breast epithelial cells (Weng et al. 2010). And BPA exposure increases susceptibility for adverse phenotypic outcomes via epigenetic mechanisms (Anderson et al. 2012).

There are few studies about the BPA effects on endometrial cells, but they only analyzed cytotoxic effects or changes of gene expression after BPA treatment. Also it remains unclear that what makes to change expression of various genes. In this study, we speculated that BPA induced the alterations of DNA methylation status, which might be the mechanism of the changes of gene expression.

2. Materials and methods

2.1. Materials

Bisphenol A was purchased from Sigma-Aldrich (St. Louis, MO). BPA was dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration in the various media was 0.5 % (v/v), a concentration that had no effect on survival of the cells.

2.2. Cell culture

Bovine endometrial epithelial cells (BEND cells; CRL-2398) were obtained from the American Type Culture Collection (ATCC, USA). BEND cells were cultured in complete medium (CM) consisting of 1:1 mixture of Eagle's Minimal Essential medium (EMEM) and Ham's F12 medium, 10 % fetal bovine serum, 10 % horse serum, antibiotic-antimycotic (ABAM, Gibco) supplemented with D-valine (0.034 g/L) and insulin (200 U/L) in a humidified atmosphere with 5% CO_2 at 37°C. For all experiments, attached cells in plates were cultured overnight with phenol red-free DMEM prior to the onset of treatment to avoid hormonal effects, including by estrogen in FBS (Iso et al. 2006). We then added BPA or DMSO (as control) at different concentrations (1, 10, or 100 μM). Although the selected BPA doses in this study are higher than the physiological levels in the general population (0.1 ~ 10 μM; Schonfelder et al. 2002; Aghajanova and Giudice, 2011), it is necessary to use higher doses in order to obtain a response in cell culture systems.

2.3. Evaluation of cell viability

The effect of BPA on the viability of BEND cells were examined using the MTT (3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyl-tetrazolium bromide) assay. The cells were seeded in a 96-well plate at a density of 1×10^4 cells/mL. After 24 h, the cells were incubated with phenol red free DMEM containing various concentration of BPA (DMSO, 1, 10, or 100 μM) for 3, 6, 12, 24, 48 or 72 h. Then, the cells were treated with 0.5 mg/mL MTT solution (Sigma Aldrich, USA) in medium and the plate was incubated for 4 h. Thereafter, violet formazan crystals were dissolved in DMSO and the absorbance was measured at 560 nm by using a microplate reader.

2.4. Single-cell gel electrophoresis (comet assay)

Single-cell gel electrophoresis was used to assess DNA strand break and abasic sites. The assay was performed as described by Singh et al. (1988). The cells were treated with BPA (DMSO, 1, 10, or 100 μM) for 3 h, which is the optimal duration of treatment for DNA damage induced by BPA. After 3 h, cells were harvested and maintained 4 °C to prevent DNA repair. Cells were mixed with 0.5 % low-melting agarose and placed on agarose-layered slides. Another 0.5 % low-melting agarose were layered on the top. The slides were transferred to the lysis solution (2.5 μM NaCl, 0.1 M Na_2-EDTA, 0.01 M Tris-HCl, 1% Triton X-100, and 10 % DMSO, pH 10) for 1 h at 4 °C, and were rinsed with distilled water. The slides placed in the electrophoresis solution (300 mM NaOH and 1 mM Na_2-EDTA, pH 13) for 20 min, and then electrophoresis was done at 0.78 V/cm and 300 mA for an additional 25 min. The cells were neutralized with 0.4 M Tris-HCl buffer (pH 7.5) and were fixed with absolute ethanol. Sixty randomly selected cells per slide were

analyzed using a KOMET 5.5 image analysis system (Kinetic imaging, Nottingham, UK). Olive tail moment, an indicator of the degree of DNA damage, was measured under a microscope (Nikon, Tokyo, Japan) equipped with an excitation filter of 515 ~ 560 nm and a barrier filter of 590 nm.

2.5. Analysis of apoptosis and cell cycle

The cells were harvested and fixed with 1 mL of ice-cold 70 % ethanol in phosphate-buffered saline (PBS). Then the cells were washed with PBS, and stained with a solution containing propidium iodide (50 μg/mL; Sigma) and RNase A (50 μg/mL; Sigma-Aldrich). The mixture was incubated for 30 min in the dark at room temperature. Apoptotic cells were analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, CA, USA). The cells were identified by the sub-G1 DNA content. The data were analyzed using the Cell Quest Pro software (Becton-Dickinson, CA, USA), and then cell cycle distribution was determined using ModFit LT™ software (Becton Dickinson, CA, USA).

2.6. Measurement of ROS

The level of intracellular reactive oxygen species (ROS) generation was determined using 5-(and-6)-carboxy-2', 7'-dichlorofluorescein diacetate (DCFDA; Molecular Probes, USA), as reported by Wang et al (1999). BEND cells were treated with 10mM N-acetylcysteine (NAC) for 2 h, prior to treatment with BPA. After the cells were treated to BPA for 1 h or 3 h, the samples were harvested and incubated with fresh medium containing 20 μM DCFDA for 20 min at 37 °C in the

dark. Then the cells were analyzed for DCF fluorescence by flow cytometry.

2.7. Extract of DNA and bisulfite modification

Genomic DNA from the cells was isolated using the Wizard DNA extraction kit (Promega, Madison, WI, USA) according to the protocol. The quality and quantity of the isolated genomic DNA was measured by the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technology, Cambridge, UK). The genomic DNA was modified with sodium bisulfite treatment using the EZ methylation Kit (Zymo Research, Irvine, CA, USA).

2.8. Global DNA methylation

Global DNA methylation status was measured by using a commercially available Methylflash Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer's protocols. Briefly, the genomic DNA was bound to high DNA affinity wells. Methylated DNA was recognized using capture and detection antibodies to 5-methylcytocine (5-mc) and then quantified by reading the absorbance at 450 nm in a microplate reader. The amount and percentage of methylated DNA was proportional to the OD intensity measured.

2.9. Methylation-Specific PCR

Bisulfite-treated DNA was amplified by PCR using well-designed primer sets which included multiple cytosine residues flanking the targeted CpG islands for unmethylated and methylated sequences. PCR was done in a final volume of 20 μL containing template DNA, 10 μM of each primers, and AccuPower PCR Premix (Bioneer, Seoul, Korea). PCR amplification was initiated at 95 °C for 5 min, cycled 45 times at 95 °C for 1 min, followed by the specific annealing temperature for each gene (Table 2.2) for 1 min, with a final extension at 72 °C for 10 min. The PCR products were then electrophoresed on 3 % agarose gel and visualized with ethidium bromide (EtBr). The EtBr stained agarose gels were analyzed using the public domain NIH ImageJ gel analysis program (developed at the U.S. National Institutes of Health).

2.10. Statistical analysis

Statistical analysis was performed using SAS 9.3 (SAS Institute, Cary, NC). All experiments were performed in triplicate, and the results were expressed as means ± standard deviation (SD). Differences in cell viability were analyzed using Student's t-test. Differences between the control and treatment groups of Olive tail moments (OTM) were evaluated using the Mann–Whitney U-test. A p-value < 0.05 was deemed to indicate statistical significance.

Table 2. 2 The sequences of HOXA 10 and RASSF1A for MSP

Gene	Forward primer sequence (5'- 3')	Reverse primer sequence (5'- 3')	Annealing temperature
HOXA 10	u GGGTTAGGTTTTTTATTAGTTATGT	CAATCCTAAACCAAAATTTCCAC	59 °C
	m AGGGGTTAGGTTTTTTATTAGTTACGT	CAATCCTAAACCAAAATTTCCG	
RASSF1A	u AATGTTTTGATAGTTGATTTTTTGG	TATAAAACTTATACACATATACACCAACAT	65 °C
	m TAAACGTTTTGATAGTCGATTTTTC	ACTTATACGCATATACACCAACGTA	

3. Results

3.1. Cell viability

BENDs were treated for 3, 6, 12, 24, 48, and 72 h with 100 μM of BPA (Figure 2.1). Cell viability was significantly decreased over times. After 3 h treatment, BEND was significantly decreased by 62.5 %. The viability of BEND was decreased by 65.1, 59.8, 49.9, 49.3, and 43.0 % at 6, 12, 24, 48, and 72 h, respectively. Since the cell viability was significantly decreased after 3 h BPA treatment, the cell viability assay was performed after 3 h treatment with 1, 10, or 100 μM of BPA.

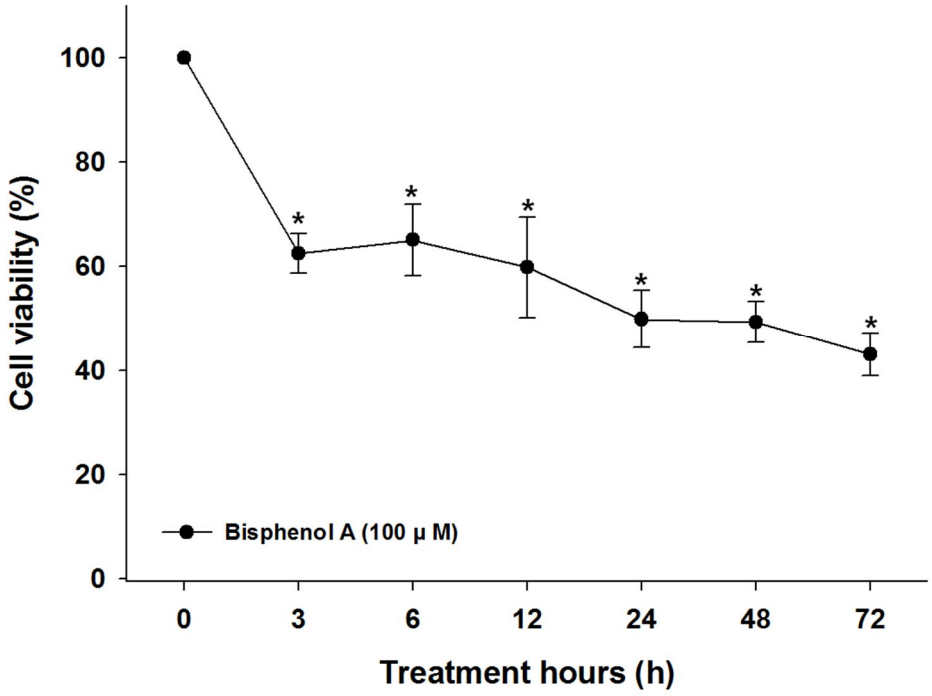


Figure 2.1 Effects of BPA at 100 µM on the cell viability over time. Data are means ± SD (n = 3). *p < 0.05 compared with DMSO controls.

3.2. Effect of BPA on the viability of BEND cells

Cell viability assay performed time course using 100 μM BPA, which dose decreased most markedly cell viability (Figure 2.2). 100 μM BPA decreased cell viability in time-dependent manner, and the decrease rate was significant from 3 h. Therefore the effect of various concentration of BPA on the viability of BEND cells was assessed for 3 h. The BEND cell viability was significantly decreased after 3 h treatment of 100 μM BPA. At concentration of 100 μM, BPA significantly inhibited the survival of BEND cells by 57.1 %.

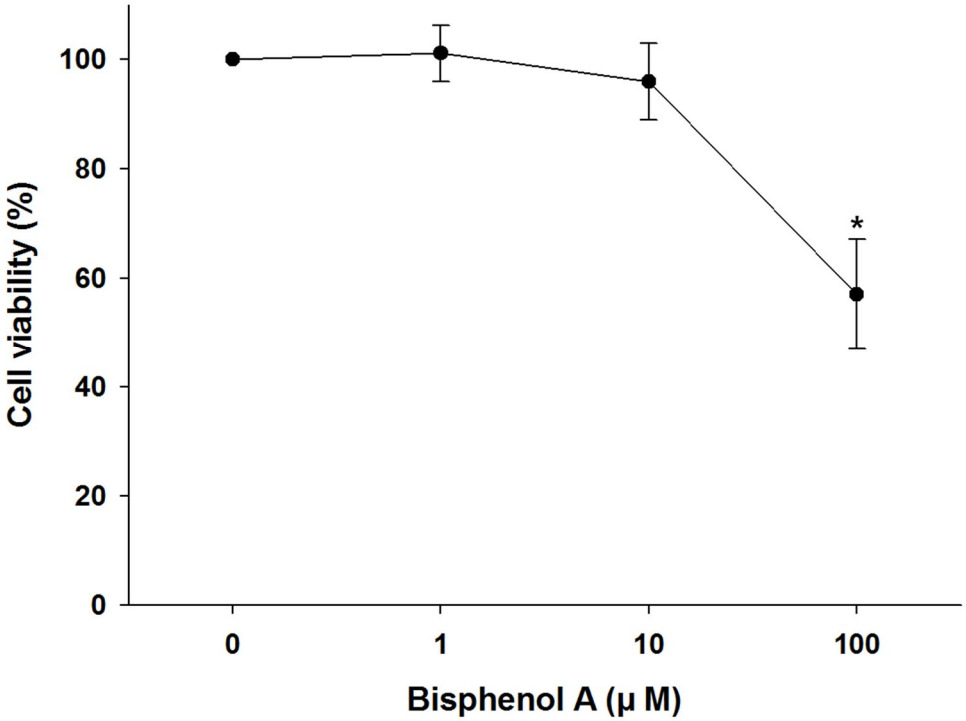


Figure 2.2 Effects of BPA on the viability of BEND cells. Cells were exposed to BPA for 3 h. The viability of cells was measured by MTT assay. Data are means ± SD (n = 3). *p < 0.05 compared with DMSO controls.

3.3. Induction of cellular apoptosis by BPA

The effect of BPA on the cellular apoptosis was evaluated using flow cytometry (Figure 2.3). While BPA didn't induce apoptosis for 3 h treatment of BPA, there was a significant induction of apoptotic cells for 24 h treatment of BPA. In 24 h exposed cells, there were 17.1, 19.4, 20.9 and 35.7 % of cells treated with control, 1, 10, and 100 μM of BPA, respectively.

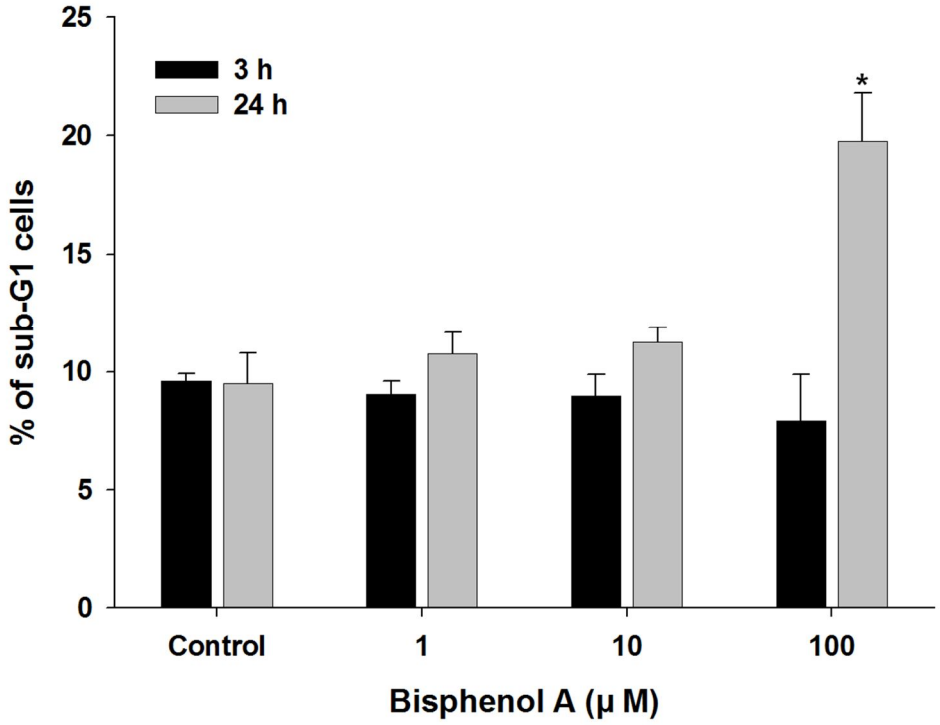
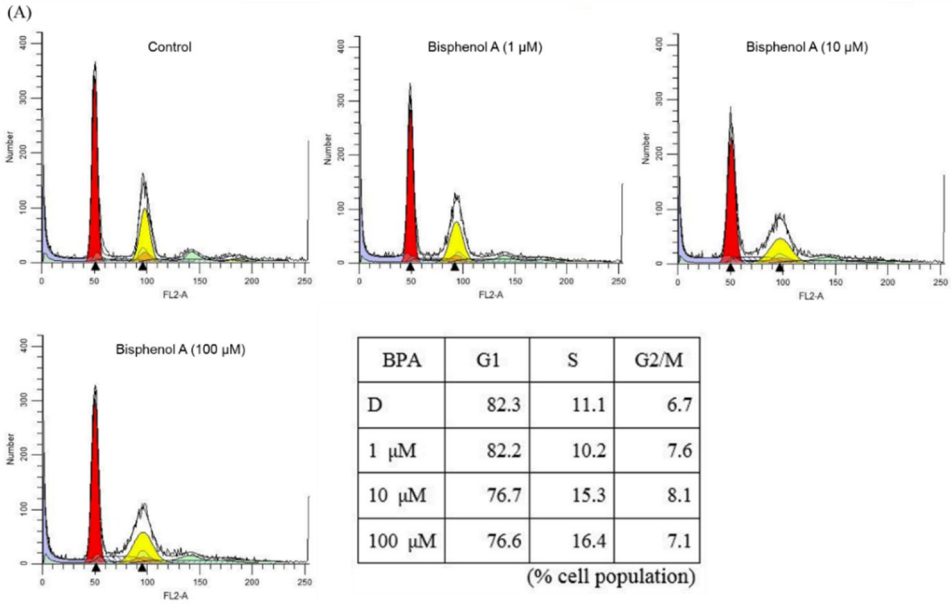


Figure 2.3 Effects of BPA on the induction of apoptotic cell death after 3 or 24 h of treatment, as determined by flow cytometry. Data are means ± SD (n = 3). *p < 0.05 compared with DMSO controls.

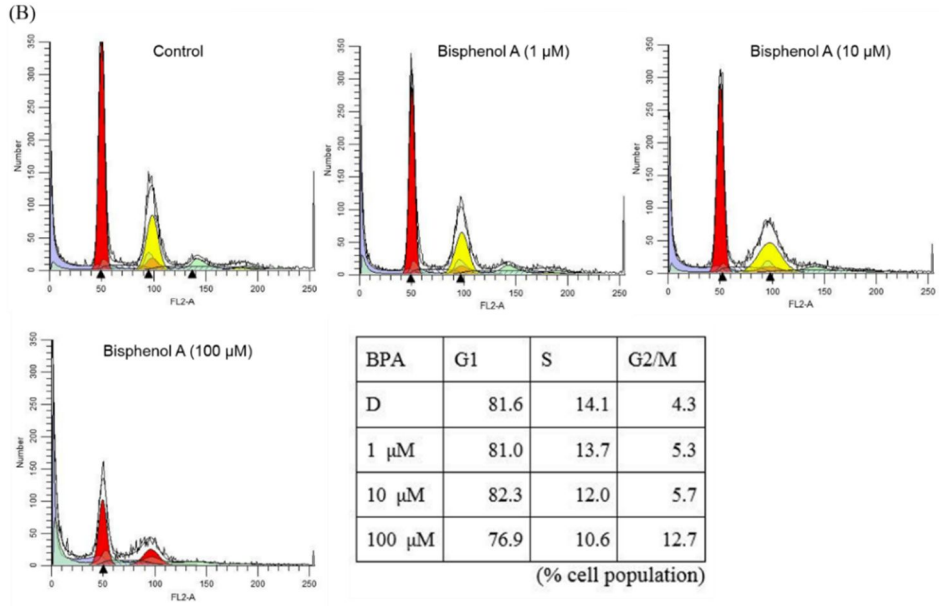
3.4. Cell cycle arrest after treatment with BPA

The effect of BPA on cell cycle was evaluated using flow cytometry (Figure 2.4). A dose-dependent effect on the cell cycle was observed after 3 h treatment of BPA. After 3 h of 100 μM BPA treatment, cells at the S phase showed increases of 16.4 % compared to control (1.5-fold increases compared to DMSO treated cells). Additionally, an exposure of BPA for 24 h induced marked G2/M cell cycle arrest as revealed by increasing to 5.3, 5.7, and 12.7 % (1.2-, 1.3-, and 3.0-fold increases compared with DMSO control) in treated with 1, 10, and 100 μM BPA, respectively.



BPA	G1	S	G2/M
D	82.3	11.1	6.7
1 μM	82.2	10.2	7.6
10 μM	76.7	15.3	8.1
100 μM	76.6	16.4	7.1

(% cell population)



BPA	G1	S	G2/M
D	81.6	14.1	4.3
1 μM	81.0	13.7	5.3
10 μM	82.3	12.0	5.7
100 μM	76.9	10.6	12.7

(% cell population)

Figure 2. 4 Effects of BPA on the cell cycle progression. Data are expressed as percentage of cells in each phage (means ± SD (n = 3)). (a) BEND cells treated with BPA for 3 h. (b) BEND cells treated with BPA for 24 h. Graphs of one representative experiment were shown. *$p < 0.05$ compared with DMSO controls.

3.5. Increase of intracellular ROS

Intracellular ROS generation by BPA treatment was examined using DCFDA fluorescent dye (Figure 2.5). A significant increase of the fluorescent intensity was observed in treated with BPA for 1 h. And the fluorescent intensity in 3 h treated cells was higher than that seen in 1 h treated cells. The fluorescent intensity markedly increased from 100 % (control) to 120.6, 126.5, and 139.7 %, respectively, by 1, 10, and 100 μM of BPA. To verify ROS origin, we pretreated with NAC for 2 h and then treated with 100 μM BPA. The fluorescent intensity in cells pretreated with NAC became lower compared to only BPA treated cells.

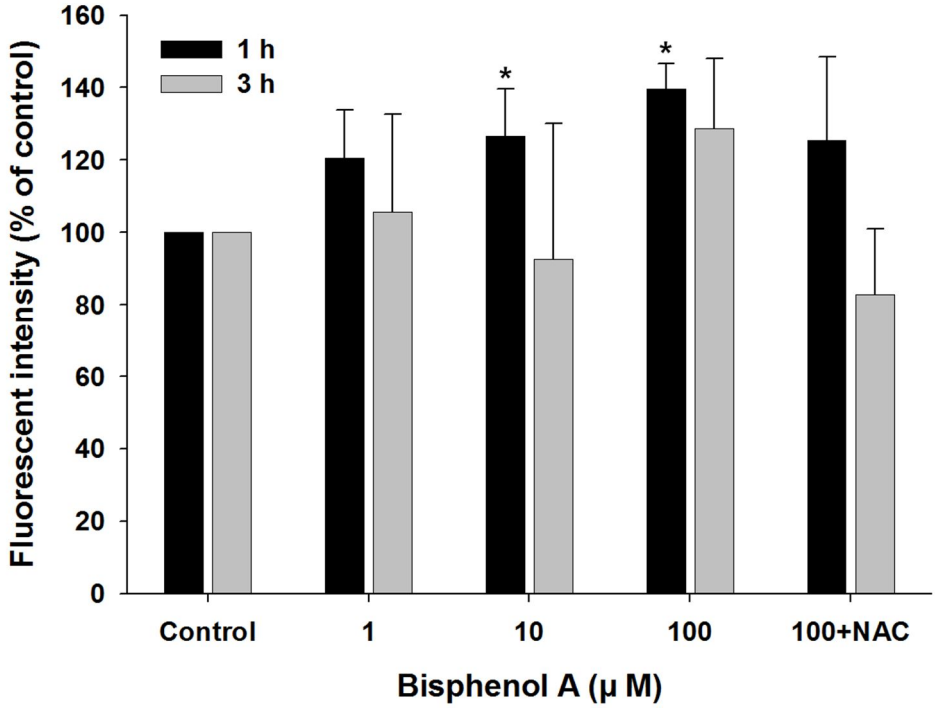


Figure 2.5 Influence of BPA on the intracellular ROS production after 1 or 3 h treatment. Data are means ± SD (n = 3). *p < 0.05 compared with DMSO controls.

3.6. Extent of DNA damage as indicated by the comet assay

The comet assay was used to determine DNA damage, especially DNA single-strand breakage. The level of DNA damage was represented as OTM, defined as the fraction of total DNA in the tail and the mean distance of migration in the tail (Olive and Banath, 1990). Figure 2.6 shows that the OTM was significantly increased with BPA dose. These data indicates that BPA significantly induced DNA damage in BEND cells.

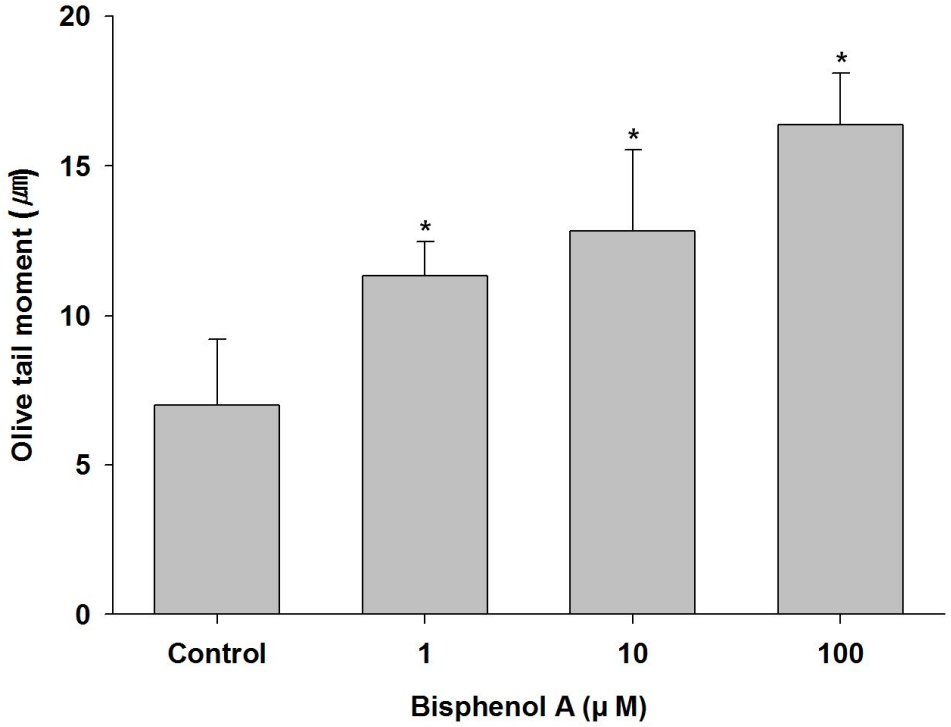


Figure 2.6 DNA damage induced by BPA in BEND cells. After treatment for 3 h, single-strand DNA breaks were evaluated by means of OTM. The results are mean ± SD. *$p < 0.05$ compared with DMSO controls.

3.7. Effect of BPA on global DNA methylation level

The change of global methylation level induced by BPA treatment was evaluated (Figure 2.7). The global methylation level was decreased with BPA doses. Increasing concentration of BPA was associated with a decrease in the global DNA methylation level. Global DNA methylation level was significantly decreased at 1 or 100 μM BPA after 3 or 24 h treatment, respectively.

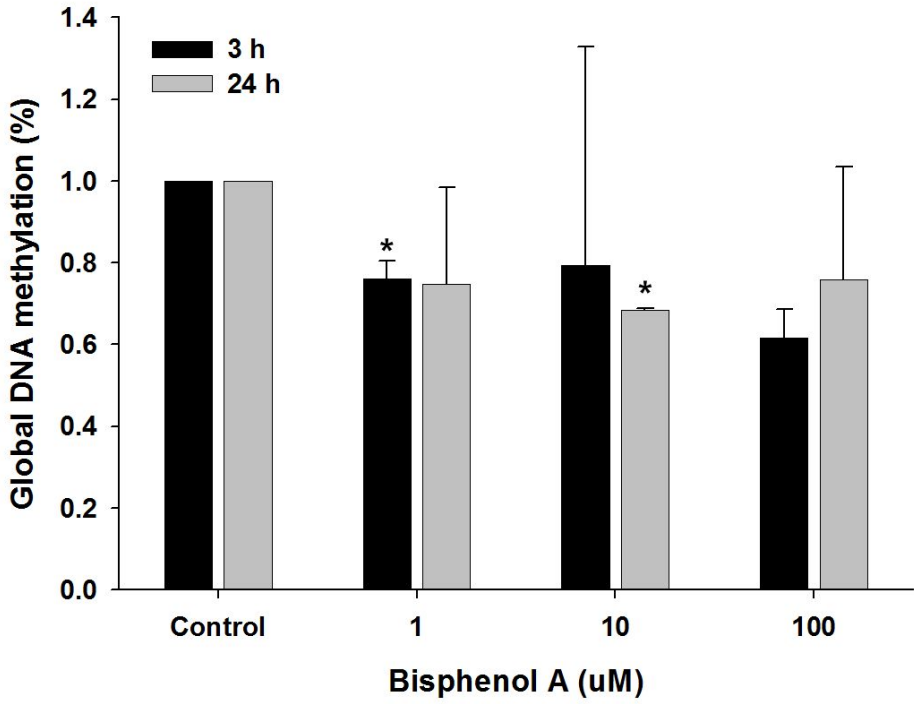


Figure 2.7 The level of global DNA methylation after 3 h treatment of BPA. Global methylation was analyzed using Methylflash™ Methylated DNA Quantification Kit (Epigentek). Data are mean ± SD. *$p < 0.05$ compared with DMSO controls.

3.8. Specific locus DNA methylation

3.8.1. HOXA 10 (Homeobox 10)

The effects of BPA on HOXA 10 methylation were examined (Figure 2.8). BPA exposure for 3 h resulted in a decrease of methylated HOXA 10. The relative density of band was 81.9, 63.3, and 72.5 % at 1, 10, and 100 μM of BPA, respectively. This result showed the inverse linear dose-response relationship.

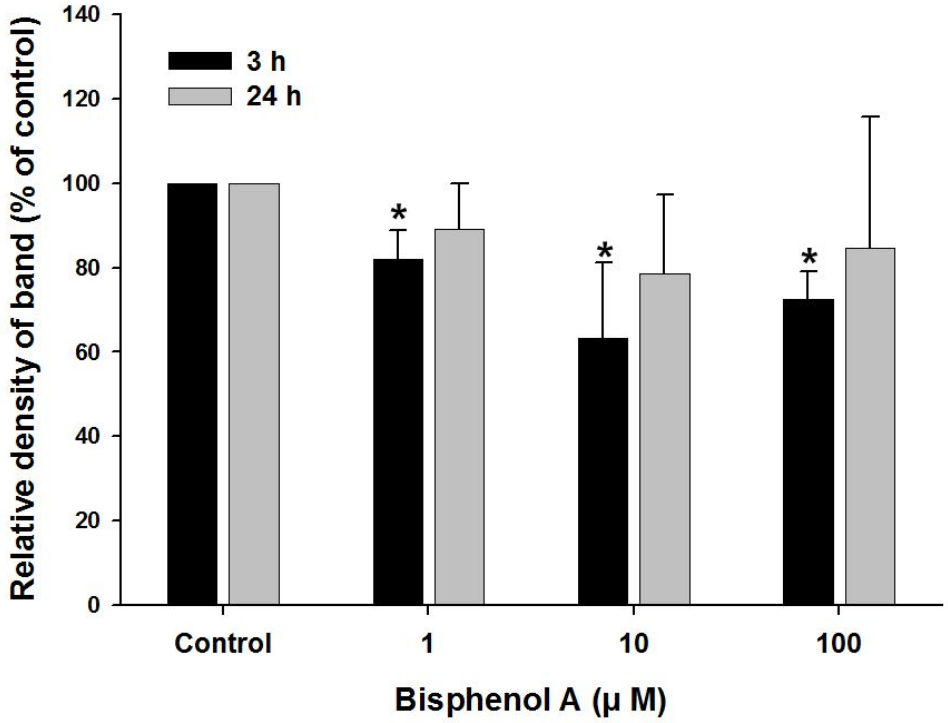


Figure 2.8 The level of HOXA 10 methylation after 3 h treatment of BPA. The products of PCR were analyzed using NIH ImageJ program. Representative agarose gel photograph showing EtBr stained PCR products. Data are mean ± SD. *$p < 0.05$ compared with DMSO controls.

3.8.2. RASSF1A (Ras-association domain family 1, isoform A)

The changes of RASSF1A methylation were analyzed after 3 or 24 h treatment of BPA. Figure 2.9 shows that the level of RASSF1A methylation was slightly increased in 3 h and significantly increased in 24 h. After 3 h treatment, the relative density of band was 95.9, 111.1, and 113.4 % at 1, 10, and 100 μM of BPA. And the relative density of band was 94.0, 91.7, and 188.4 at 1, 10, and 100 μM of BPA after 24 h treatment. At 100 μM BPA, the RASSF1A methylation was increased compared to control, especially in 24 h BPA treatment cells.

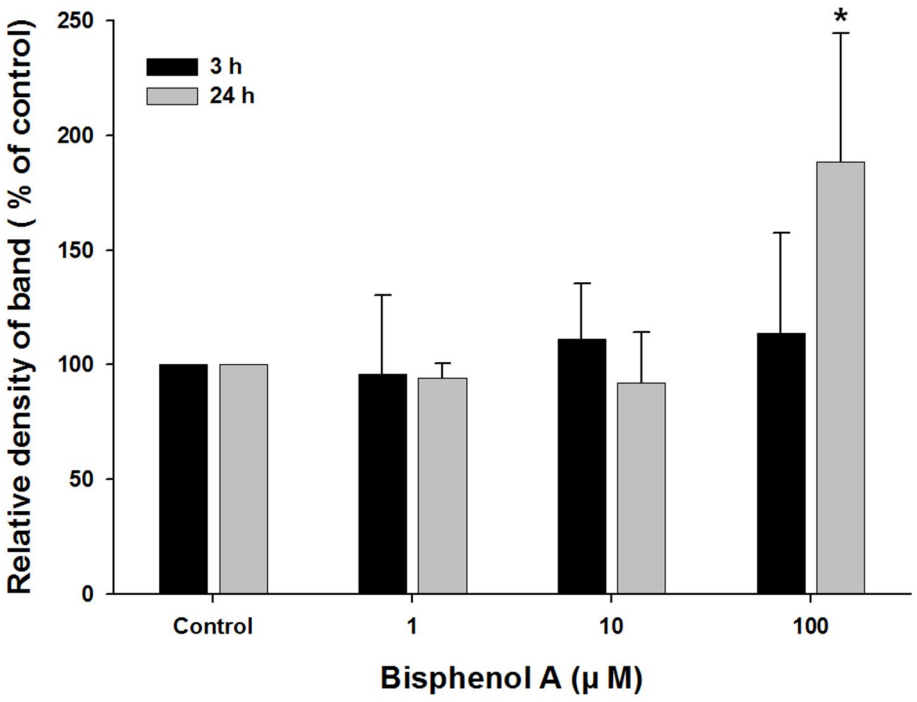


Figure 2.9 The level of RASSF1A methylation after 3 or 24 h treatment of BPA. The products of RASSF1A PCR were analyzed using NIH ImageJ program. Representative agarose gel photograph showing EtBr stained PCR products.

4. Discussion

In this study, BPA significantly reduced BEND cell viability after 3 h treatment. The apoptotic cells were increased dose-dependent manner after 24 h treatment. Also there were S arrest and G2/M arrest for 3 and 24 h treatment, respectively. The intracellular ROS was significantly increased after 1 h treatment, and DNA damage was also induced after 3 h treatment of BPA. DNA methylation status was different with BPA treatment. The level of global DNA methylation was decreased according to BPA treatment. The status of HOXA methylation was significantly decreased in 3 h BPA treatment. And the methylation level of RASSF1A was significantly increased at 100 μM BPA after 24 h treatment.

BPA is one of the endocrine disrupting chemicals, and it is widely used as protective coatings on food-storage containers, baby bottles, bottle tops, water pipes, medical equipment, and dental sealants. Increasing evidence has shown that BPA may adversely affect fetal or neonatal health. In contrast, there were a few studies about the effects of BPA exposure on adults, especially in reproductive tract. In this study, the effects of BPA on endometrial cells were analyzed. Several studies found that BPA induced apoptosis, but the results were inconsistent. Naciff et al. (2010) did not observe an effect of BPA on Ishikawa epithelial cell apoptosis, and Aghajanova and Giudice (2011) also didn't observe an effect of BPA on human endometrial stromal fibroblasts apoptosis for 48 h treatment. However, the proliferation of human endometrial endothelial cells was significantly decreased according to BPA concentration after 24 h treatment (Bredhult et al. 2007). And there were no induction of apoptotic cells, but BPA significantly induced necrotic cells. In this study, BPA significantly affected the viability of BEND cells after 3 h treatment, but BPA did not induced apoptosis at 3 h. Because cell death generally occurs either through apoptosis or necrosis (Leist et al. 1997), necrosis also assessed

for 3 h treatment, but BPA did not induced necrosis (data not shown). After 24 h treatment, BPA significantly induced apoptosis, but not necrosis (data not shown). Interestingly, S arrest and G2/M arrest were observed at 3 and 24 h, respectively. Therefore, BPA promotes cell-cycle arrest and growth inhibition by forcing S phase arrests, and BPA induced apoptosis in BEND cells along with a strong G2/M phase arrest at 24 h. By promoting cell cycle arrest and subsequent growth inhibition, cells contacting damaged DNA are allowed to be checked and possibly undergo apoptosis (Schwartz and Shah 2005). MTT assay is reportedly for assaying cell survival and proliferation. MTT assay is cleaved in active mitochondria, so the reaction occurs only in living cells, and activated cells produce more formazan than resting cells, which could allow the measurement of activation even in the absence of proliferation (Zhang et al. 1998). BPA may arrest S phase, inhibit of growth of BPA treated cells, therefore the cell viability was decreased, though apoptotic cells were not increased after 3 h treatment. To verify these results, there is needed to evaluate the proliferation of BEND and the level of expression of cyclin A or B, which is S or G2/M checkpoint, respectively.

BPA can initiate DNA modification by generating ROS, if it can't repair DNA, can lead to cell transformation. BPA was significantly induced intracellular ROS in rat insulinoma INS-1 cells (Xin et al. 2014), and in epididiymal sperm of rats (Chitra et al. 2003). However, there were no studies to assess oxidative stress induced by BPA on endometrial cells. In this study, BPA significantly induced intracellular ROS after 1 h treatment. The generation of ROS by BPA could elicit DNA damage. Also ROS arrests cell cycles at S or G2/M (Piret et al. 1999). Oxidative DNA lesions may result in DNA damage (Ziech et al. 2011). The mechanism of DNA damage caused by BPA may work through the induction of ROS (Meeker et al. 2010; Chitra et al. 2003). DNA strand breaks are strongly linked with the initiation stage of carcinogenesis (Kim et al. 2006). DNA damage was analyzed using comet assay. After 3 h treatment, OTM was significantly increased

according to BPA concentration. These findings are in agreement with prior studies. BPA significantly induced DNA damage in MCF-7 cells after 3 h treatment (Iso et al. 2006). And BPA induced DNA breakage in germ cells of male rats (Wu et al. 2013). These results indicate that BPA can cause intracellular ROS and DNA damage in endometrial cells. And the ROS and DNA damage can cause reduction of cell viability, and induction of apoptosis (Moon et al. 2010).

BPA has been shown to change the expression of genes in various tissues. While these effects are typically attributed to ER-mediated action of BPA, it is unclear how the low potency at ERs could account for the strong effects observed in many tissues after low dose BPA exposure. In addition, there is evidence suggesting that in utero BPA exposure results in changes in gene expression that persist into adulthood (Ho et al. 2006). These long-term effects could be explained by epigenetic pathways, which can lead to persistent changes in gene expression (Kundakovic and Champagne 2011). Single strand breakage (hydroxyl radical induced lesions) have been shown to contribute to decreased DNA methylation by means of interfering with the ability of the DNA methyltransferases (DNMTs) and thus resulting in global hypomethylation (Franco et al. 2008). In addition, ROS may induce epigenetic alterations that affect the genome and play a key role in the carcinogenesis (Campos et al. 2007). Oxidative stress can contribute to gene silencing by mechanisms that involve aberrant hypermethylation of tumor suppressor gene promoter and thus lead to progress malignancy (Ziech et al. 2011).

There were few studies about the relationship between BPA exposure and global DNA methylation. Most of the previous studies were limited to specific locus of DNA. Global DNA methylation was significantly decreased in female mice fed BPA-containing diet (Patel et al. 2013). And O'Brien et al. (2013) showed a decrease in global DNA methylation levels in bone marrow-derived mast cells (BMMCs) from BPA-exposed mice. This current study also found the global DNA methylation was decreased according to BPA concentration. The observed global

hypomethylation in BPA exposed cells suggests an overall increase in transcription of genes which promoter regions were less methylated after BPA exposure (Anderson et al. 2012; Dolinoy et al. 2007; O'Brien et al. 2014). Global DNA hypomethylation is commonly found in most types of cancer (Guerrero-preston et al. 2010). The global loss of DNA methylation tends to get repackaged in a more open configuration (Pogribny et al. 2005) and can cause carcinogenesis by reactivating retrotransposable elements inducing genomic instability and activating proto-oncogenes (Robertson 2002; Weidman et al. 2007). However, the changes of specific DNA may or may not follow the global progression.

HOX genes act as transcription factors essential to development of embryo and uterine. HOX gene expression regulates endometrial development during the course of the menstrual cycle and is necessary for implantation (Browne et al. 2006). Alteration in expression of HOX, especially HOXA 10, is associated with implantation and infertility. HOXA 10 is necessary for normal decidualization and pregnancy (Smith and Taylor, 2007). Cheng et al. (2010) found that the level of HOXA 10 methylation was decreased according to BPA concentration. The pregnant CD-1 mice were treated with intraperitoneal injection of BPA, and then the HOXA 10 mRNA in uteri of female offspring was significantly increased compared to control. Also HOXA 10 methylation level was decreased and mRNA expression was increased than control (Bromer et al. 2010). These results indicate that the changes of DNA methylation status caused by BPA exposure may be significantly affected during developmental window. There were few studies assessed changes of HOXA 10 methylation level cause by BPA exposure on non-developmental stage. In this study, the HOXA 10 methylation level was analyzed in non-developmental cells, BEND. HOXA 10 methylation was decreased according to BPA levels. This result was agreed with previous studies. Hypomethylated HOXA 10 is linked overexpress of HOXA 10 gene. Increased HOXA 10 expression results in altered endometrial pinopods and microvillie as well as increased litter size (Daftary and Taylor, 2004;

Bagot et al. 2001; Smith and Taylor, 2007). Also elevated HOXA 10 expression is associated with ectopic pregnancy (Salih and Taylor, 2002).

RASSF1A is known as a tumor suppressor gene. Epigenetic inactivation of RASSF1A is one of the most common changes in cancer. RASSF1A hypermethylation induced silence gene expression in many cancers; lung, breast, prostate, and ovarian tumors (Agathanggelou et al. 2001; Fackler et al. 2003; Liu et al. 2002). RASSF1A were hypermethylated of promoter region and reduced expression in endometrial carcinoma (Pallarés et al. 2008). There were no studies about assess the methylation level of RASSF1A caused by BPA exposure. In this study, BPA induced hypermethylation of RASSF1A after 24 h treatment. It means that exposure of BPA may alter RASSF1A methylation and gene expression in endometrial cells, so it can lead to cancer. However, RASSF1A gene expression was not analyzed and further studies are needed. Future work will be needed to identify the more various specific gene methylation closely related to effects of BPA on endometrial cells.

This study has some limitations. The bovine endometrial cells were used in this study for evaluating the effects of BPA. Bovine endometrial cell is as a source of non-cancerous epithelial cells from bovine uterus. These bovine endometrial cells have functional characteristics similar to those of human endometrial cells (Austin et al. 1999; Parent et al. 2003; Khan et al. 2005). Human endometrial epithelial cells (hEECs) had limited for evaluating all experiments in this study, since hEECs has limit to grow and it grown *in vitro* express ER and PR at least up to passage 5 (Iruela-Arispe et al. 1999). And if hEECs are used for experiments, the susceptibility to BPA must be considered. Also commercial human endometrial cell lines were cancerous cell lines. For such reasons, bovine endometrial cells were chosen for evaluating the BPA effects. Unfortunately, there are few studies about evaluating DNA methylation in bovine endometrial cells. There was no evidence about primer and probe sequences for MethyLight assay, so DNA methylation status

was evaluated using MSP. The PCR products are analyzed by agarose gel electrophoresis and EtBr staining. MSP method is needed careful design of the primers, and is considered only qualitative, and the results should be further validated using quantitative method.

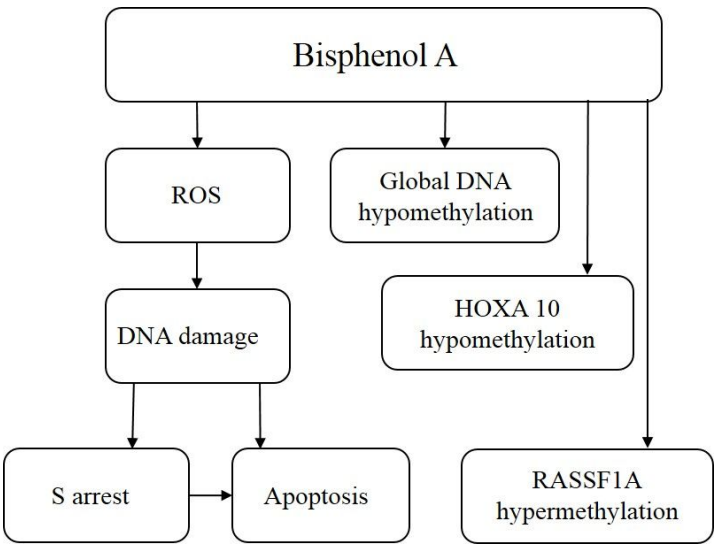


Figure 2.10 Scheme of the effects of BPA on cytotoxicity and DNA methylation changes in bovine endometrial cells.

Based on the results, the effects of BPA on BENDs were summarized as in Figure 2.10. BPA induced intracellular ROS, and then induced DNA damage. Excessive ROS can cause S arrest and apoptosis. If DNA damages can't repair during S arrest, the cells undergo apoptosis. Also ROS and DNA damage can induce aberrant DNA methylation; global DNA methylation, HOXA 10, RASSF1A methylation. This study suggests that exposure to BPA could induce the cytotoxicity on BEND cells, and BPA might be involved in the alterations of DNA methylation pattern.

Chapter 3.

DNA methylation changes in human endometrial cells after bisphenol A exposure

1. Introduction

Bisphenol A (BPA) is one of the endocrine disrupting chemicals (EDCs). It is extensively used as a coating of food containers, medical equipment, and dental sealants. Calafat et al. (2005) detected BPA in 95 % of 397 adults in NHANES. It means human is exposed to BPA continuously and commonly. Therefore, evaluations about effects of BPA on human are needed. BPA exposure is associated with reproductive disorders; endometrial hyperplasia, recurrent miscarriage, polycystic ovary syndrome (PCOS), and reduction of oocyte retrieved (Hiroi et al. 2004; Sugiura-Ogasawara et al. 2005; Takeuchi and Tsutsumi, 2002; Mok-Lin et al. 2009).

Several studies evaluated the effects of BPA on human endometrial cells. BPA significantly inhibited proliferation of human endometrial endothelial cells, and markedly induced necrotic cells (Bredhult et al. 2007). BPA altered expression of several genes; differentiation, angiogenesis, and mitotic process involved gene (Aghajanova and Giudice, 2011; Helmestam et al. 2014; Bredhult et al. 2009). These aberrant gene expressions might cause by DNA methylation. Epigenetics defines meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself (Egger et al. 2004). No data was found about assess the DNA methylation changes of BPA exposure in human endometrial cells.

The effects of BPA were investigated in bovine endometrial cells in chapter 2. But some differences may exist between different species and cell/tissue types. Therefore, in this chapter, the methylation changes caused by BPA in human endometrial epithelial cells (hEECs) were analyzed.

2. Materials and methods

2.1. Materials

BPA was purchased from Sigma-Aldrich (St. Louis, MO). All compounds were dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration in the various media was 0.5 % (v/v), a concentration that had no effect on survival of the cells. This study was approved by the Institutional Review Board of Ilsan Paik Hospital, Inje University, Korea, and written informed consent was obtained before tissue sampling. Endometrial biopsies were obtained from 2 women of undergoing hysterectomy. They had myoma or tubo ovarian abscess. They did not receive any hormone therapy during a period of at least three months prior to the surgery.

2.2. Cell culture

The endometrial biopsies were cut into smaller pieces and enzymatically digested by Trypsin-EDTA (Sigma Aldrich) for 30 min with gently vortex. The isolated cells were suspended in Dulbecco's Modified Eagle Medium (DMEM) / F12 (Gibco, Invitrogen, Carlsbad, CA, USA), 10 % fetal bovine serum and 100 U/mL of penicillin (Gibco). Cells were seeded in culture flasks (25 cm^2) and incubated in a humidified atmosphere with 5 % CO_2 at 37 °C.

2.3. Extract of DNA and bisulfite modification

Genomic DNA from the cells was isolated using the Wizard DNA extraction kit (Promega, Madison, WI, USA) according to the protocol. The quality and quantity of the isolated genomic DNA was measured by the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technology, Cambridge, UK). The genomic DNA was modified with sodium bisulfite treatment using the EZ methylation Kit (Zymo Research, Irvine, CA, USA).

2.4. Repetitive element DNA methylation assay

After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight technique as described previously (Weisenberger et al. 2005). Briefly, MethyLight was carried out using the following forward and reverse primers and probes (LINE1, Sat 2, and AluC4) as previously described (Weisenberger et al. 2005). PCR was performed in a 10 μL reaction volume with 0.3 μM each PCR primers, 0.1 μM probe, 1X TaqMan Universal PCR Master Mix, using the following PCR conditions: 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 55 °C for 1 min. AluC4 was used as a control reaction to normalize for the amount of input DNA. Assays were run on an ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Percent of methylated reference (PMR) values as index of global DNA methylation was calculated by dividing the LINE1(or Sat2):AluC 4 ratio of a sample by the LINE1(or Sat2):AluC4 ratio from CpGenome ™ Universal methylated DNA and multiplying by 100.

2.5. Specific locus DNA methylation assay

After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight technique as describes previously (Eads et al. 2001; Widschwendter et al. 2004). Briefly, DNA was PCR-amplified in a ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA), using TaqMan Universal PCR Master Mix (Applied Biosystems) under the following conditions; 95 °C 10 min, followed by 45 cycles at 95 °C for 15 s and 55 °C for 1 min. The primers and probes for HOXA 10, RASSF1A, PR and ACTB were previously described (Widschwendter et al. 2004; Widschwendter et al. 2009). ACTB was used to normalize for the amount of input DNA.

2.6. Statistical analysis

Statistical analysis was performed using SAS 9.3 (SAS Institute, Cary, NC). The results were expressed as mean ± standard deviation (SD). In all statistical analyses, each described in the relevant paragraph, p values < 0.05 were considered significant.

3. Results

3.1. The methylation of repetitive elements after BPA exposure

3.1.1. The level of LINE-1 methylation

LINE-1 methylation changes were performed using MethyLight assay (Figure 3.1). BPA induced slightly hypermethylated by 108.8 % of the methylation of LINE-1 after 3 h, and the methylation level was increased by 136.5 % compared to control.

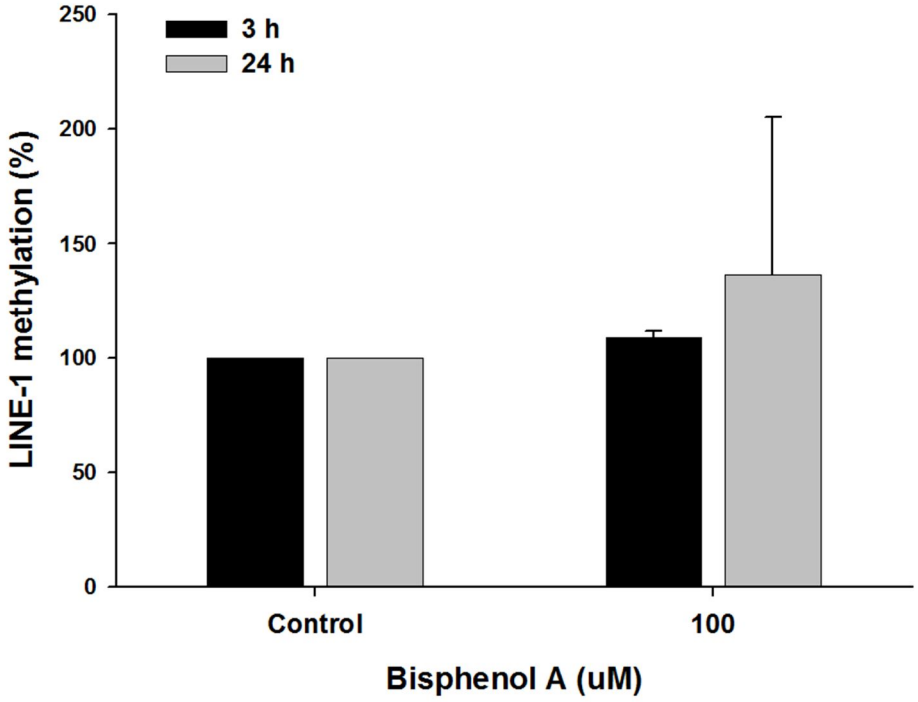


Figure 3.1 The changes of LINE-1 methylation were assessed in hEECs. BPA induced hypermethylation after 3 or 24 h treatment.

3.1.2. The level of Sat2 methylation

The change of Sat2 methylation level by BPA treatment was analyzed (Figure 3.2). After 3 h treatment of 100 μM BPA, Sat2 methylation level was decreased by 93.8 % compared to 100 % of control. And after 24 h treatment, Sat2 methylation level was decreased by 95.9 % compare to control.

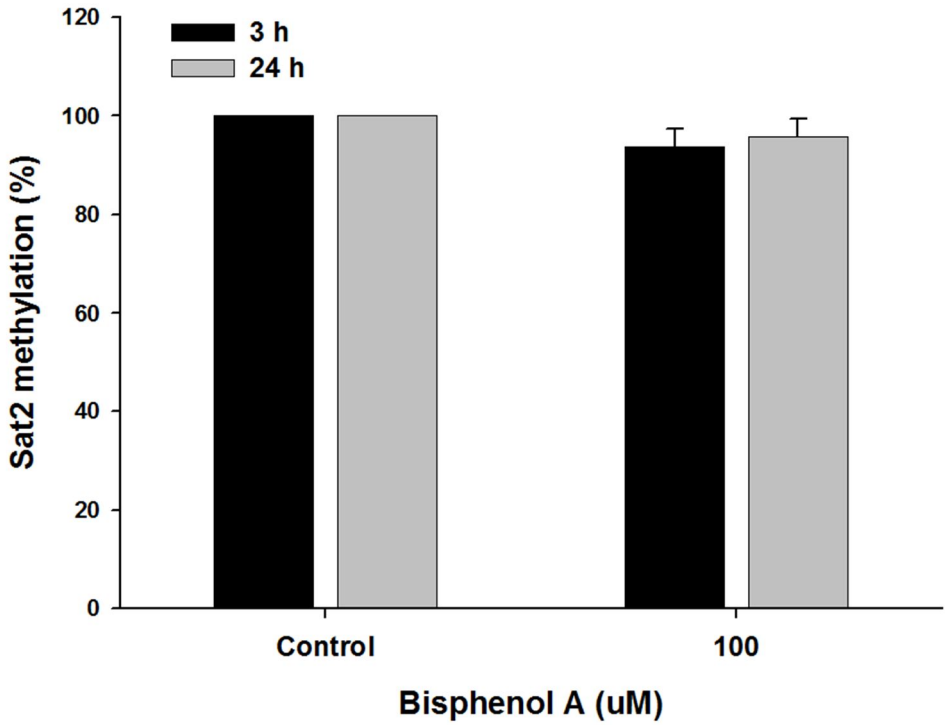


Figure 3.2 The changes of Sat2 methylation were assessed in hEECs. BPA induced hypomethylation after 3 or 24 h treatment.

3.2. Specific locus DNA methylation

3.2.1. HOXA 10 methylation level

The changes of HOXA 10 methylation was assessed (Figure 3.3). After 3 h treatment, HOXA 10 methylation showed opposite pattern between subjects. However, HOXA 10 methylation level was decreased at 89.9 % after 3 h treatment, and it was decreased at 77.2 % after 24 h treatment.

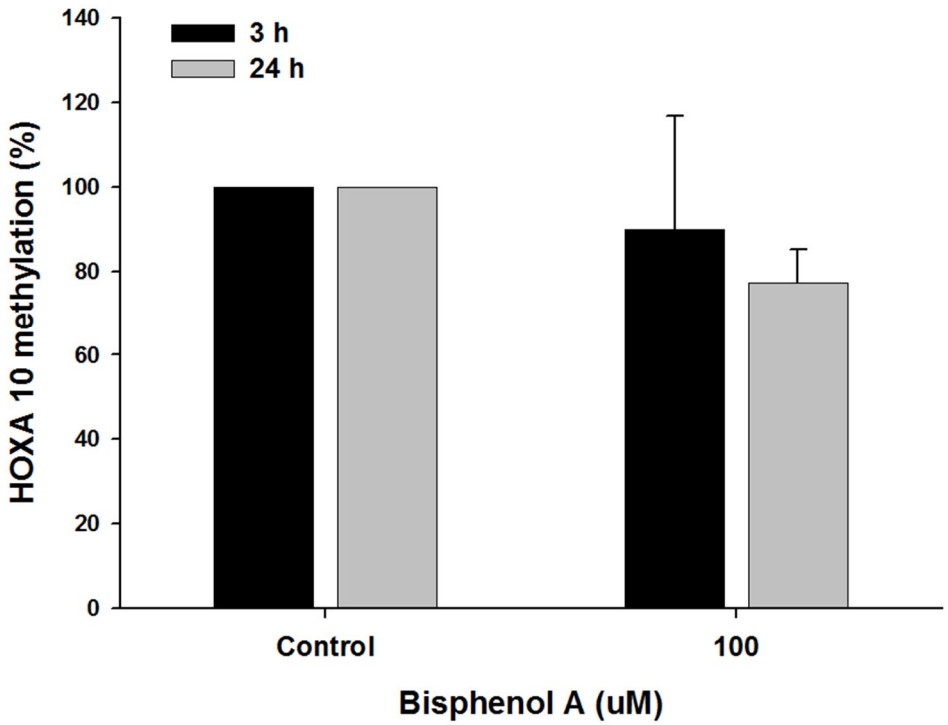


Figure 3. 3 The methylation level of HOXA 10 was assessed after 3 or 24 h treatment. The hEECs were hypomethylated compared to controls.

3.2.2. Progesterone receptor (PR) methylation level

The PR methylation level was showed different pattern between treatment times (Figure 3.4). After 3 h treatment, the level of PR methylation was increased at 102.9 %, but it was decreased at 86.1 % after treatment 24 h of 100 μM BPA.

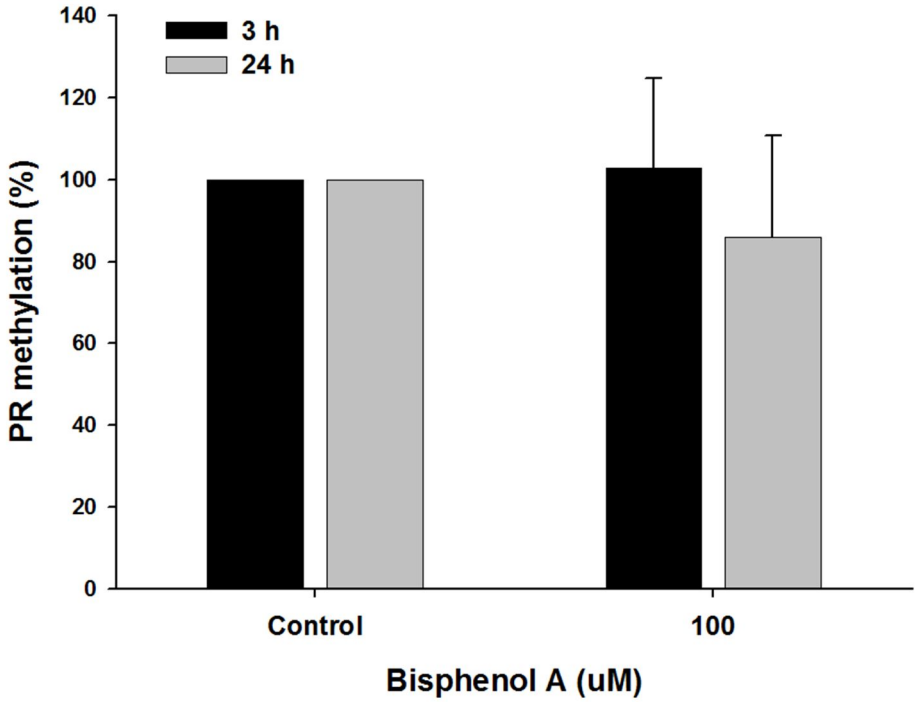


Figure 3.4 The PR methylation level was changed by 100 μM BPA. The methylation level was slightly increased after 3 h treatment, but the level was decreased after 24 h treatment.

4. Discussion

In this part, DNA methylation changes caused by BPA in hEECs were analyzed. Because there might be some gaps between species, the effects of BPA in human cells were analyzed in chapter 3. As a result, BPA altered LINE-1, Sat2, HOXA 10, and PR methylation level, but those were not significant.

Previous studies found that BPA can induce the changes of DNA methylation. The most of the studies were performed on development stages *in vivo*. The effects of BPA may differ between different species and cell/tissue types. In this part, the changes of DNA methylation level caused by BPA exposure in human endometrial cells were analyzed. Meanwhile, the most of normal cells have a limited number of passages. It is determined by senescence which is determined by cell cycle regulators, such as retinoblastoma protein (Rb) and p53 (Freshiney, 2006). Since the passage limits of the normal cells, it was not able to assess the cytotoxicity and changes of DNA methylation caused by BPA on hEECs. Therefore, the changes of DNA methylation were analyzed with only one BPA dose, 100 μM, in hEECs for 3 or 24 h.

Global DNA methylation was assessed in repetitive elements such as LINE-1 and Sat2. These sequences consist of interspersed repeats and tandem repeats comprise approximately 45 % of the human genome and they are normally highly methylated (Lander et al. 2001; Jordan et al. 2003). Aberrant repeat element methylation is associated with carcinogenic process or environmental exposures (Estecio et al. 2007). Pilsner et al. (2007) found arsenic induced increase of global DNA methylation, and Pavanello et al. (2009) showed polycyclic aromatic hydrocarbons (PAHs) increased Alu and LINE-1 methylation levels in human blood. However, global DNA hypomethylation was associated with benzene, lead, arsenic, diethylstilbestrol (DES), and cadmium (Zhao et al. 1997; Li et al. 2003; Takiguchi

et al. 2003; Bollati et al. 2007; Pilsner et al. 2009). These studies were different about evaluated tissues and genes. Also the effects on DNA methylation was different despite same environmental exposures; Pavanello et al. (2009) showed PAHs induced LINE-1 hypermethylation, but Duan et al. (2013) showed PAHs induced LINE-1 hypomethylation in human blood. There were few studies assessed global DNA methylation caused by BPA. Anderson et al. (2012) found the increase of global DNA methylation using Luminometric Methylation Assay (LUMA) in mouse tails. The other study found that BPA was associated with LINE-1 hypermethylation in placental tissues (Nahar et al. 2015). This study also found that the LINE-1 methylation level was increased compared to control. And the present study found a small decrease in Sat2 methylation by BPA exposure. There were no studies evaluated the relationship between Sat2 methylation and BPA exposure. Sat2 DNA sequences are located as tandem repeats in several chromosomes (Jeanpierre, 1994). Widschwendter et al. (2004) found ovarian carcinogenesis was significant relation with Sat2 hypomethylation. Also, Sat2 hypomethylation and global DNA hypomethylation was associated with ovarian tumor. In addition, Sat2 or global hypomethylation was associated with degree with malignancy (Ehrlich et al. 2006). In this study, the sample size was too small to find relationship between Sat2 methylation and BPA exposure. But this result implies BPA exposure may induce Sat2 hypomethylation.

BPA influenced methylation in specific locus such as HOXA 10, and ER (Bromer et al. 2010; Doshi et al. 2011). In this study, HOXA 10, PR, and RASSF1A methylation status was analyzed by using MethyLight assay. RASSF1A methylation could not analyze, because the methylation level of this gene was low. HOXA 10 is necessary for implantation, continues to be expressed dynamically in the adult endometrium (Bromer et al. 2010). The methylation level of HOXA 10 was decreased in the BPA exposed cells, but not significant. These results agree with previous studies. BPA exposure in utero induced HOXA 10 hypomethylation

(Bromer et al. 2010). This altered methylation affects aberrant DNA expression, cause female infertility (Lu et al. 2013).

Progesterone is required for maintain pregnancy. Progesterone prevents endometrial hyperplasia, is a key inhibitor of endometrial carcinogenesis (Yang et al. 2011; Aldad et al. 2011). Aberrant PR expression is leading to inadequate response to progesterone, results in infertility, pregnancy loss, or endometrial hyperplasia (Aldad et al. 2011). This study found PR methylation level was slightly increased for 3 h treatment, and decreased after 24 h treatment. It is hard to conclude about the changes of PR methylation. Markey et al. (2005) found that long-term fetal exposure of BPA induced overexpression of PR. The expression of PR was significantly increased in 100 nM of BPA exposed human endometrial carcinoma cell line (Bergeron et al. 1999).

The methylation levels on hEECs from only two subjects were examined. It is hard to conclude the pattern of methylation induced by BPA exposure. Further studies are needed to explore the changes of DNA methylation level caused by BPA exposure using more hEECs. However, DNA methylation level was evaluated using MethyLight assay. The MethyLight technique has several pros. It is a highly sensitive assay, and is a quantitative method allows accurate information about the fraction of DNA methylation. And the risk of PCR contamination is significantly reduced because no manual transfer of PCR products is required. Although there was only one gene, HOXA 10, which is a common gene evaluated in chapter 2 and chapter 3, the change of methylation pattern was coincide. Therefore the gaps between different species can be ignored.

Chapter 4.

Urinary bisphenol A concentrations and DNA methylation alterations in women with endometrial disorders

1. Introduction

BPA is one of the EDCs and extensively used in the production of polycarbonate plastic and epoxy resins, which are used as protective coatings on food-storage containers, baby bottles, bottle tops, water pipes, medical equipment, and dental sealants (Hiroi et al. 2004). The extensive use of BPA is indicated that increasing its potential exposure to humans through water, air, soil, and food contamination. Therefore, human exposure is unavoidable and BPA can affect human health. Human exposures are most likely through the oral route such as ingestion of food containing BPA. BPA leaches from cans into foods under normal conditions, especially the rate of leaching of BPA increases under heating of cans to sterilize food (Vom Saal and Hughes, 2005). Recent studies indicated that transdermal exposure is also a possible route such as handling of receipt papers (Ehrlich et al. 2014). Therefore the effects of BPA on human health have become of growing global concern and it became necessary to assess for the toxicity of BPA in humans.

BPA is metabolized by phase Ⅰ and Ⅱ enzymes, such as sulfotransferase (SULTs) and uridine diphosphate glucuronosyltransferases (UGTs), and entirely excreted in urine within 24 h (Figure 4.1; Yang et al. 2006). So BPA has been mainly measured in human urine. The half-life of BPA is < 6 h and urinary concentrations reflect recent BPA exposure.

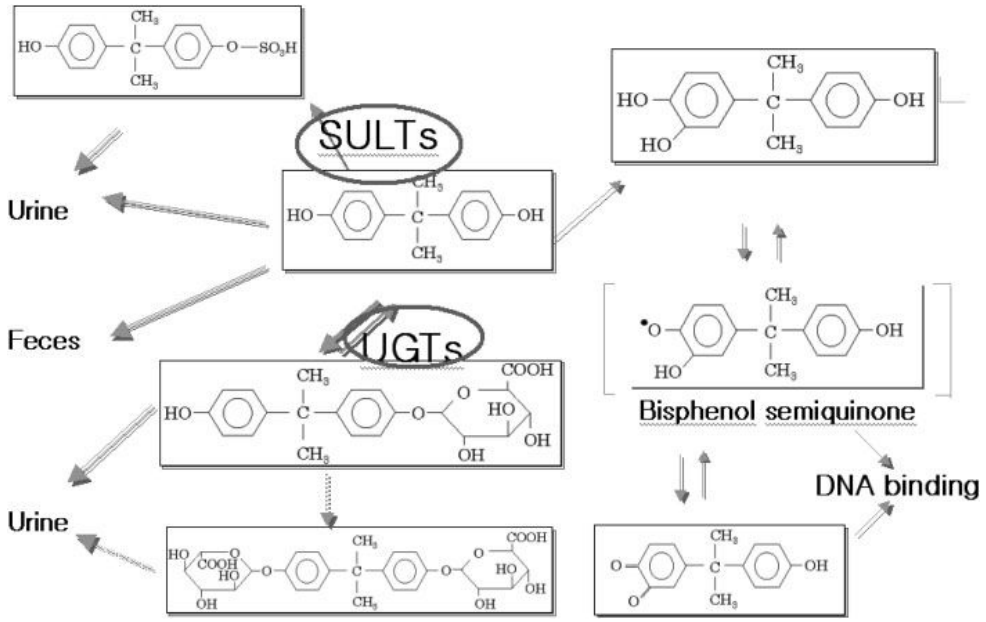


Figure 4. 1 Metabolism of BPA (Kawamoto and Matsumoto A, 2003)

Table 4.1 showed about the BPA levels in human urine. The US Center for Disease Control and Prevention (CDC) detected that BPA was detected in 95 % of the urine samples in the USA, and the range of BPA concentration was 0.4 - 149 ng/ml (Calafat et al. 2005). The GM of urinary concentrations were 8.91 μg/g creatinine in 73 Koreans (Yang et al. 2003), 1.79 μg/g creatinine in 1870 Koreans (Kim et al. 2011), and 2.2 μg/g creatinine in 1852 Koreans (Kim et al. 2014). As shown in previous studies, human exposed to chronic low-level of BPA, and it has raised serious concerns about effects on human reproductive health (Vom Saal, 2007).

BPA has estrogenic properties and can bind ER α and β. At even low levels of BPA can bind to the ERs and acts as an environmental estrogen (Kim et al. 2014).

Endometrium is the main target for estrogen besides the breast, the pituitary, and the hypothalamus (Lee et al. 2012). Therefore endometrium could affect by BPA through ER. Endometrial tissues have important roles for implantation and embryonic to blastocyst development, and disturbance in the function of this organ can lead to reduced fertility. There are a few studies about the relation between BPA level and endometrial disorders (Table 4.2). Cobellis et al. (2009) analyzed women with and without endometriosis for serum BPA levels. BPA was not detected in controls (n=11), but was detected in 52.7 % of women with endometriosis (n=58). They suggested the relation between BPA levels and endometriosis. In a population based case-control study, Upson et al. (2014) used data from the Women's Risk of Endometriosis (WREN) study and tested the relation between endometriosis and BPA. They did not observe a significant association between urinary BPA and endometriosis overall. But they found a significant relation between urinary BPA and non-ovarian pelvic endometriosis (0.36 - 0.86 μg/L vs. ≤ 0.36: OR 3.0, CI 1.2-7.3; 0.86 - 2.01 μg/L vs. ≤ 0.36: OR 3.0, CI 1.1-7.6). Hiroi et al. (2014) also carried out a case-control study. The study composed of simple endometrial hyperplasia (n=10), complex endometrial hyperplasia (n=9), endometrial cancer (n=7), and controls (n=11). The results show a significant association between BPA levels and endometrial hyperplasia and endometrial cancer, but the relationship was surprisingly inversed. They concluded that the mode of action of BPA may be more complex. Based on the above mentioned studies, there were a few studies about evaluate the relationship between BPA and endometrial disorders, but there remains inconsistencies. In addition, the existing data of relationship between BPA exposure and DNA methylation alterations in endometrial disorders are limited.

Endometriosis and adenomyosis are complex and benign, estrogen-dependent disorders, can lead to pelvic pain, dysmenorrhea, and infertility. But their pathophysiology are not fully understood yet (Giudice and Kao, 2004). Recently, endometriosis is regarded as an epigenetic disease. Epigenetic modification is a

possible mechanism about alteration of gene expressions and maintenance of altered expressions. BPA induced epigenetic changes *in vitro* and *in vivo* (Weng et al. 2010; Fernandez et al. 2012; van Esterik et al. 2015). Wu et al. (2005) firstly showed that putative promoter HOXA 10 in endometriosis patient's endometrium is hypermethylated as compared with that without endometriosis. And promoter of PR-B was hypermethylated in endometriosis, ER β was hypomethylated in ectopic endometrium (Wu et al. 2006; Xue et al. 2007). Also, HOXA 10 expression was decreased in endometrium from women with adenomyosis (Fischer et al. 2011). Nie et al. (2010) found firstly that adenomyosis has epigenetic aberration, and they suggest adenomyosis may be an epigenetic disease. Ectopic endometrial tissues from adenomyosis showed hypermethylation of PR-B promoter concomitant with reduced gene and protein expression, compared to control endometrial tissues from without endometriosis, adenomyosis, or myoma. In addition, the immunoreactivity to methyltransferases (DNMTs) in adenomyosis significantly increased compared in normal endometrium (Liu and Guo, 2012). This means that adenomyosis may be an epigenetic disease. Endometriosis and adenomyosis has something in common. These diseases are estrogen-dependent, and it is similar that endometrial tissues move to ectopic sites. However, adenomyosis and endometriosis are different from each other; endometrial tissues move to ovary, fallopian tube in endometriosis, and muscular layer of the uterus in adenomyosis. Myoma is a benign smooth muscle neoplasm in uterus, and found in 77 % premenopausal women (Hodges et al. 2000). The symptoms are abnormal uterine bleeding, pelvic pain, reduced fertility, miscarriage (Haney, 2000). The etiology of myoma is unknown, but estrogen plays a major role in the pathogenesis of myoma (Hodges et al. 2000). There was no information about the effects of BPA on myoma. Myoma also referred as an epigenetic disease. Li et al. (2003) found aberrant DNMT expression and DNA hypomethylation in myoma compared to normal myometrial tissue. Also hypomethylation of ER α and increased ER α expression in myoma was observed (Asada et al. 2008). Navarro et al. (2012) evaluated genome-wide DNA methylation

and mRNA expression in myoma, and they found aberrantly methylated or expressed 55 genes in myoma compared to normal myometrium. Based on these studies, DNA methylation might be a major role in the pathogenesis of myoma (Navarro et al. 2012).

Besides above diseases, there are tumors in the inner lining of the uterus, called as endometrial polyps. The endometrial tissue thickens and thins at different phases of the menstrual cycle. In each cycle, the endometrium grows and changes to prepare the endometrium for pregnancy. But endometrial tissue will grow too much, then creating a polyp. Polyps are attached to the wall of the uterus by a stalk and grow outward into the endometrial cavity. Endometrial polyps are common, affecting 10-24 % of women. They are commonly seen in women 40-50 year and are rare after menopause. Several studies found endometrial polyps are at increased risk of malignancy, and the presence of and endometrial polyp has been described as a risk factor for future endometrial cancer (Ben-Arie et al. 2004; Torres et al. 2012). The reason is because hyperplastic and neoplastic lesions can be found in context of polyps (Savelli et al. 2003). Although the causes of endometrial polyps are not known, the endometrial polyps seem to be related to excess estrogen levels. Maia et al. (2006) found a significant higher expression of aromatase in endometrial polyps compared with normal endometrium. Also Lopes et al. (2007) found the higher expression of ER and PR in endometrial polyp than normal endometrium. Because BPA can act like an estrogen, BPA might influence on creating polyps.

Taken together, BPA has an epigenetic modification activity, and described endometrial disorders are epigenetic diseases. Also the hypothesis is emerging that EDCs have a putative role in endometrial disorders. Therefore, it is possible to speculate the epigenetic modification of endometrial disorders caused by BPA exposure. In this chapter, the methylation levels in endometrial biopsies from endometriosis, adenomyosis, or myoma were examined, and the relationship between urinary BPA levels and DNA methylation levels was analyzed.

Table 4.1 BPA levels in human urine

Authors	Detection method	Subjects	LOD	Total BPA
Kim et al. (2003)	HPLC/FD	15 men	1 ng/ml	Mean 2.82 ng/ml
Kim et al. (2003)	HPLC/FD	15 women	0.28 ng/ml	Mean 2.76 ng/ml
Yang et al. (2003)	HPLC/FD	73 Korean	0.012 ng/ml	GM 8.91 μg/g cr
Yang et al. (2006)	HPLC/FD	172 Korean	0.026 ng/ml	Median 7.86 ng/ml Range 0.03-62.4 ng/ml
Calafat et al. (2007)	SPE-HPLC-MS/MS	2517 NHANES	0.10 ng/ml	Range 0.4-149 ng/ml
Itoh et al. (2007)	SPE-HPLC-MS/MS	140 Japanese women	0.30-0.55ng/ml	Median 0.80 μg/g cr Range 0.45-1.3 μg/g cr
Mahalingaiah et al. (2008)	SPE-HPLC-MS/MS	82 American	0.36 ng/ml	GM 1.31 ng/ml
Lang et al. (2008)	SPE-HPLC-MS/MS	1455 American	0.30 ng/ml	Mean-men 4.53 ng/ml Mean-women 4.66 ng/ml
Ye et al. (2008)	GC-MS/MS	100 women, Netherlands	0.26 ng/ml	GM 1.1 ng/ml
He et al. (2009)	HPLC	922 workers, China	0.31 ng/ml	GM 0.38 μg/g cr
Nepomnaschy et al. (2009)	HPLC-MS/MS	180 women, USA	0.18 ng/ml	GM 1.79 μg/g cr

Yang et al. (2009)	HPLC-MS/MS	368 Korean	0.063 ng/ml	GM 0.56 µg/g cr
Meeker et al. (2010)	SPE-HPLC-MS/MS	190 men, USA	0.4 ng/ml	Median 1.3 ng/ml Range 0.8-2.5 ng/ml
Mok-Lin et al. (2010)	SPE-HPLC-MS/MS	84 women, USA	0.4 ng/ml	GM 2.52 ng/ml Range 0.4-25.5 ng/ml
Kim et al. (2011)	GC/MS	1870 Korean	0.05 ng/ml	GM 1.79 µg/g cr
Li et al. (2011)	HPLC	218 men	0.31 ng/ml	Median-control 1.4 µg/g cr Median-case 38.7 µg/g cr
Zhang et al. (2013)	HPLC-MS/MS	50 Chinese	0.10 ng/ml	GM 0.48 µg/g cr Range 0.10-4.33 µg/g cr
Miao et al. (2013)	HPLC	149 Chinese	0.31 ng/ml	Median-control 0.89 µg/g cr Median-case 26.31 µg/g cr
Battal et al. (2014)	HPLC-MS/MS	80 Turkish	0.03 ng/ml	Range 0.24-615µg/g cr
Kim et al. (2014)	GC/MS	1852 Korean	0.10 ng/ml	GM 2.2 µg/g cr

Aberration: LOD; Limit of detection, GM; Geometric mean, HPLC; High performance liquid chromatography, FD; Fluorescence detection, SPE; Solid phase extraction, MS/MS; Tandem mass spectrometry, GC; Gas chromatography

Table 4.2 Epidemiologic studies about the relation between BPA and endometrial disorders

Authors	Subjects	Collection	BPA conc.	Result
Hiroi et al. (2004)	26 patients with endometrial hyperplasia, 11 controls	Serum	Mean-case 2.5 ng/ml Mean-control 2.2 ng/ml	Serum BPA was significantly lower in endometrial hyperplasia complex and endometrial cancer than controls.
Itoh et al. (2007)	131 endometriosis cases	Single spot urine sample	Media 0.80 μg/g cr	No significant monotonic association of endometriosis with urinary BPA concentration was observed.
Cobellis et al. (2009)	58 endometriosis cases, 11 controls	Serum	Mean-case 2.91 ng/ml	BPA was significantly more likely to be detected in the serum of women with endometriosis, compared to controls.
Louis et al. (2013)	127 population (ENDO study)	Single spot urine sample	GM-case 4.19 ng/ml GM-control 1.65 ng/ml	The relation was significant when adjusting for parity along with other relevant covariates (OR=1.68).
Upson et al. (2014)	143 endometriosis cases, 289 controls	Single spot urine sample	Median-case 1.32 μg/g cr Median-control 1.24 μg/g cr	Urinary BPA levels in relation to non-ovarian pelvic endometriosis, but not in relation to ovarian endometriosis

Aberration: GM; Geometric mean

2. Materials and methods

2.1. Study design and populations

This study was approved by the Institutional Review Board of Ilsan Paik Hospital, Inje University, Korea, and written informed consent was obtained before their inclusion in the study (IRB number: IB-1211-039). A structured questionnaire, including information on age, height, weight, smoking status, education, exercise frequency, parity, and medical history, was administered by the interviewers.

The study population came to the outpatient clinic in Ilsan Paik Hospital, Inje University. The subjects were composed of 44 women, who had myoma (n=16), adenomyosis (n=13), or endometriosis (n=15). The subjects underwent hysterectomy, and endometrial biopsies were obtained from all subjects. The subjects had received no hormone treatment for 3 months prior to surgery, and had been fasting for 24 h. Myoma is common benign tumor, arising from the smooth muscle of the uterus (Goodman et al. 2015). Adenomyosis is defined as the presence of heterotopic endometrial glands and stroma in the myometrium (Fischer et al. 2011). Endometriosis is characterized by the presence and growth of functional endometrial-like tissues outside the uterine cavity (Guo, 2009). Adenomyosis and endometriosis has something in common, and eutopic endometrial tissues of the subjects were used for DNA extraction. Thus adenomyosis and endometriosis can be treated as a case group altogether. But endometriosis and adenomyosis are clearly different diseases from each other. Therefore, endometriosis and adenomyosis are treated as separate disease groups. On the other hand, myoma is not associated with move of endometrial cells. Thus, myoma regarded as a control group compared to endometriosis or adenomyosis.

2.2. Urine sample collection and total BPA measurements

All subjects provided single spot urine samples, and the samples were collected in BPA-free polypropylene containers. The samples were divided into aliquots in BPA-free 2 ml tubes and immediately refrigerated at -80 °C until analysis. Urine samples were thawed at room temperature, and spiked with 20 μL of BPA-d16 1 μg/ml. The samples were added to glucuronidase/sulfatase solution and proceed at 37 °C for 2 h. After then, the samples were sonicated for 10 min and added to 0.1 % acetic acid. Finally, total urinary BPA concentration was quantified using a Liquid Chromatography-tandem mass spectrometry (AB SCIEX API 4000 LC/MS-MS systems, Applied Biosystems, USA). Internal laboratory control procedures included analyzing quality control samples at two concentration levels, positive control and blank in each batch of samples. The limit of detection (LOD) was 0.61 ng/ml. Urinary BPA concentration was adjusted to a creatinine. Creatinine-corrected total urinary BPA concentration was estimated by dividing the total urinary BPA concentration (ng/ml) by the creatinine concentration (mg/dl) and multiplying by 100.

Urine sample collection procedures resulted in minimal external contamination of samples and degradation of BPA during storage. The urine samples were froze immediately after surgery, avoiding the use of preservatives in urine, and using samples which had not undergone a prior freeze/thaw cycle or laboratory analysis.

2.3. Extract of DNA and bisulfite modification

Genomic DNA from the endometrial biopsies was isolated using the

DNeasy Blood & Tissue kit (Qiagen, CA, USA) according to the protocol. The quality and quantity of the isolated genomic DNA was measured by the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technology, Cambridge, UK). The genomic DNA was modified with sodium bisulfite treatment using the EZ methylation Kit (Zymo Research, Irvine, CA, USA).

2.4. Global DNA methylation

Global DNA methylation status was measured by using a commercially available Methylflash Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer's protocols. Briefly, the genomic DNA was bound to high DNA affinity wells. Methylated DNA was recognized using capture and detection antibodies to 5-methylcytocine (5-mc) and then quantified by reading the absorbance at 450 nm in a microplate reader. The amount and percentage of methylated DNA was proportional to the OD intensity measured.

2.5. Repetitive element DNA methylation assay

After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight technique as described previously (Weisenberger et al. 2005). Briefly, MethyLight was carried out using the following forward and reverse primers and probes (LINE1, Sat 2, and AluC4) as previously described (Weisenberger et al. 2005). PCR was performed in a 10 μL reaction volume with 0.3 μM each PCR primers, 0.1 μM probe, 1X TaqMan Universal PCR Master Mix, using the following PCR conditions: 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and

55 °C for 1 min. AluC4 was used as a control reaction to normalize for the amount of input DNA. Assays were run on an ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Percent of methylated reference (PMR) values as index of global DNA methylation was calculated by dividing the LINE1(or Sat2):AluC4 ratio of a sample by the LINE1(or Sat2):AluC4 ratio from CpGenome ™ Universal methylated DNA and multiplying by 100.

2.6. Specific locus DNA methylation assay

After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight technique as describes previously (Eads et al. 2001; Widschwendter et al. 2004). Briefly, DNA was PCR-amplified in a ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA), using TaqMan Universal PCR Master Mix (Applied Biosystems) under the following conditions; 95 °C 10 min, followed by 45 cycles at 95 °C for 15 s and 55 °C for 1 min. The primers and probes for HOXA 10, RASSF1A, PR, ER1, ER2, MLH and ACTB were previously described (Widschwendter et al. 2004; Widschwendter et al. 2009). ACTB was used to normalize for the amount of input DNA.

2.7. Statistical analysis

Statistical analysis was performed using SAS 9.3 (SAS Institute, Cary, NC). Urinary BPA concentration was square-root transformed, and PMR values of Sat2, HOXA 10, and RASSF1A were log-transformed, to meet assumptions of normality. The differences between case and control for general characteristics were

tested using Mann-Whitney U test and χ^2 test. Mann-Whitney U test was used to compare the methylation levels in case with control. The relationship between urinary BPA level and DNA methylation level was tested by Kendall rank correlation. Multiple linear regressions with adjustment age and smoking status were used. All statistical analyses were two-sided, and p-values <0.05 were considered significant.

3. Results

3.1. General characteristics of study population

The population characteristics are shown in Table 4.3. The study subjects were composed of women with myoma, adenomyosis, and endometiosis. The mean ± standard deviation (SD) of age was 47.3 ± 4.2, 49.4 ± 4.7 and 46.3 ± 14.9 years in myoma, adenomyosis, and endometriosis, respectively. There were significantly differences in BMI and parity. The distribution of age and BMI group was significantly different among the groups. Education, smoking status, regular exercise, endometrial phase, and endometrial pathology were not difference among the disease groups.

Table 4.3 General characteristics of study population

Variables	Myoma (n=16)	Adenomyosis (n=13)	Endometriosis (n=15)	p-value
Age (years)				
<40	0	0	4 (26.7)	0.06[b]
40-49	11 (68.8)	8 (61.5)	6 (40)	
50<	5 (31.3)	5 (38.5)	5 (33.3)	
Mean ± S.D.	47.3 ± 4.2	49.4 ± 4.7	46.3 ± 14.9	0.53[a]
BMI (kg/m^2, %)				
< 18.5	0	0	5 (33.3)	0.01[b]
18.5-23	6 (37.5)	8 (61.5)	6 (40)	
≥ 23	10 (62.5)	5 (38.5)	4 (26.7)	
Mean ± S.D.	24.6 ± 4.1	22.9 ± 3.4	20.7 ± 2.4	0.02 [a]
Education (%)				
≤ Middle school	1 (6.3)	2 (15.4)	-	0.18[b]
High school	12 (75.0)	7 (53.9)	7 (46.7)	
≥ College	3 (18.8)	4 (30.8)	8 (53.3)	
Parity				
0	1 (6.3)	1 (7.7)	6 (40)	0.002[b]
1	1 (6.3)	2 (15.4)	6 (40)	
≥ 2	14 (87.5)	10 (76.9)	3 (20)	
Mean ± S.D.	1.8 ± 0.5	1.7 ± 0.6	0.8 ± 0.8	0.0003[a]
Smoking status (%)				0.40 [b]
No	14 (87.5)	13 (100)	13 (86.7)	
Yes	2 (12.5)	0	2 (13.3)	
Regular exercise				0.59[b]
No	1 (6.3)	0	1 (6.7)	
Yes	14 (93.8)	13 (100)	14 (93.3)	
Endometrial phase				0.63[b]
Proliferative	9 (56.3)	5 (38.5)	7 (46.7)	
Secretory	7 (43.8)	8 (61.5)	8 (53.3)	
Endometrial pathology				
Normal	13 (81.3)	11 (84.6)	11 (73.3)	0.74 [b]
Polyp	3 (18.8)	2 (15.4)	4 (26.7)	

[a]; Kruskal-wallis test
[b]; χ^2 test

3.2. Geometric means and quartiles of urinary BPA concentrations

3.2.1. Distribution of urinary BPA concentrations

Figure 4.3 showed about distribution of urinary BPA levels in a total subjects. Total BPA was detected in 98 % of samples. The urinary BPA showed left-skewed distribution. Also the mean of urinary BPA concentration of each group was described in Table 4.4 and Figure 4.4. Single spot urine samples were obtained from 44 women. The geometric median of endometriosis was lower at 27.98 μg/g cr than adenomyosis at 43.83 μg/g cr or myoma at 39.61 μg/g cr. But there was no significant difference among the groups (p=0.34, Kruskal-Wallis test). Also the differences of urinary BPA were not significant between myoma and endometriosis or adenomyosis (Mann-whitney test). When adenomyosis and endometriosis group are merged, there were no significant difference compared to myoma group (p=0.70, Mann-whitney test).

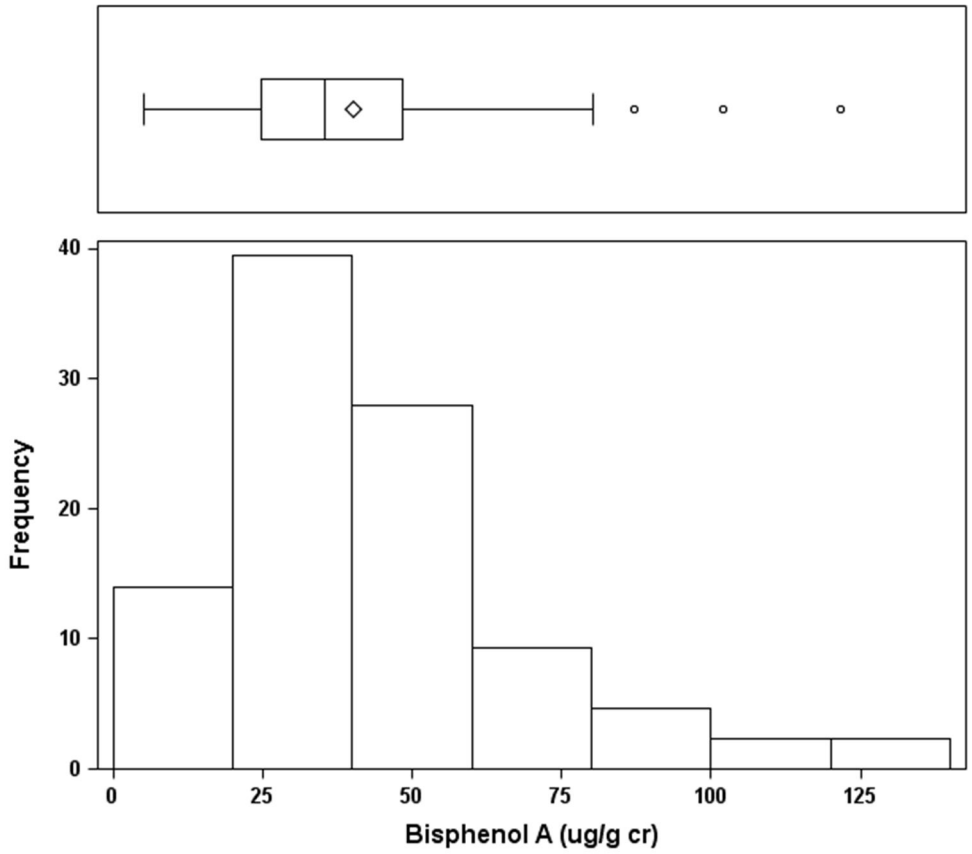


Figure 4. 2 Distribution of urinary BPA level in total subjects. The distribution was left skewed.

Table 4.4 Urinary concentration of bisphenol A in study population (unit: μg/g creatinine)

	Adenomyosis (n=13)	Endometriosis (n=15)	Myoma (n=16)
GM	43.83	27.98	39.61
Range	27.23-109.37	5.57-93.49	15.15-130.46
95% CI	33.67-57.05	17.95-43.62	29.36-53.43

Aberration: GM, Geometric mean; CI, Confidence interval

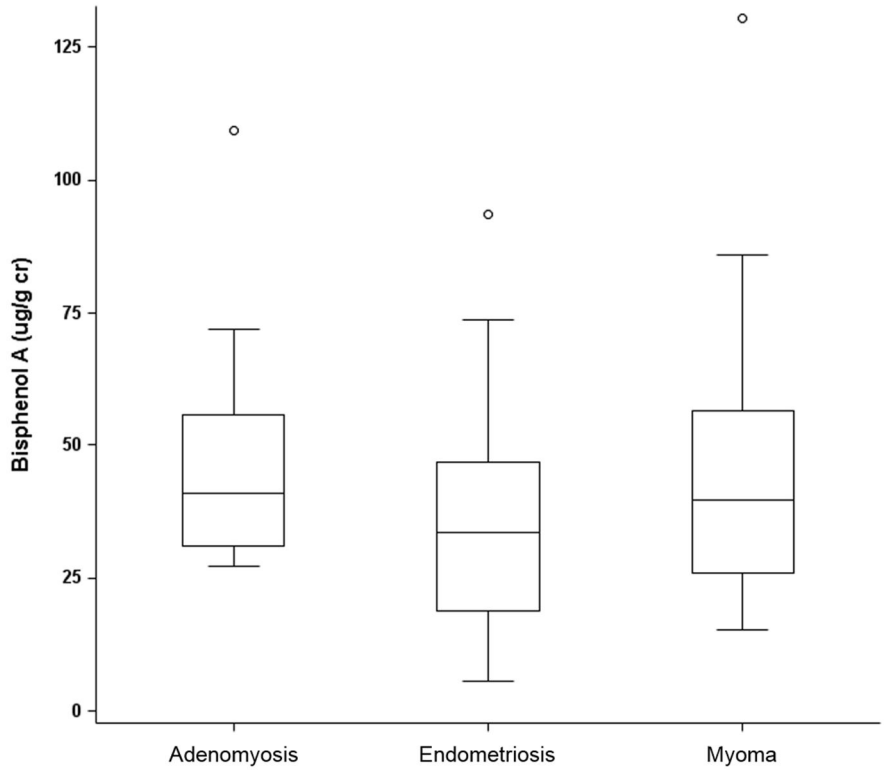


Figure 4.3 The mean ± SD values for BPA in the groups. The concentration of BPA in endometriosis was lower than in adenomyosis or myoma, but it was not significant (p=0.34, Kruskal-Wallis test).

3.2.2. Association between BMI and urinary BPA concentration

Association between BMI and urinary BPA concentration is described in Figure 4.5. The BPA concentration was decreased according to BMI level, but there was no significant difference. Table 4.5 shows the effects of various independent variables on BPA levels. In endometriosis, parity was significantly positive associated with BPA levels (p= 0.04, Kendall's τ).

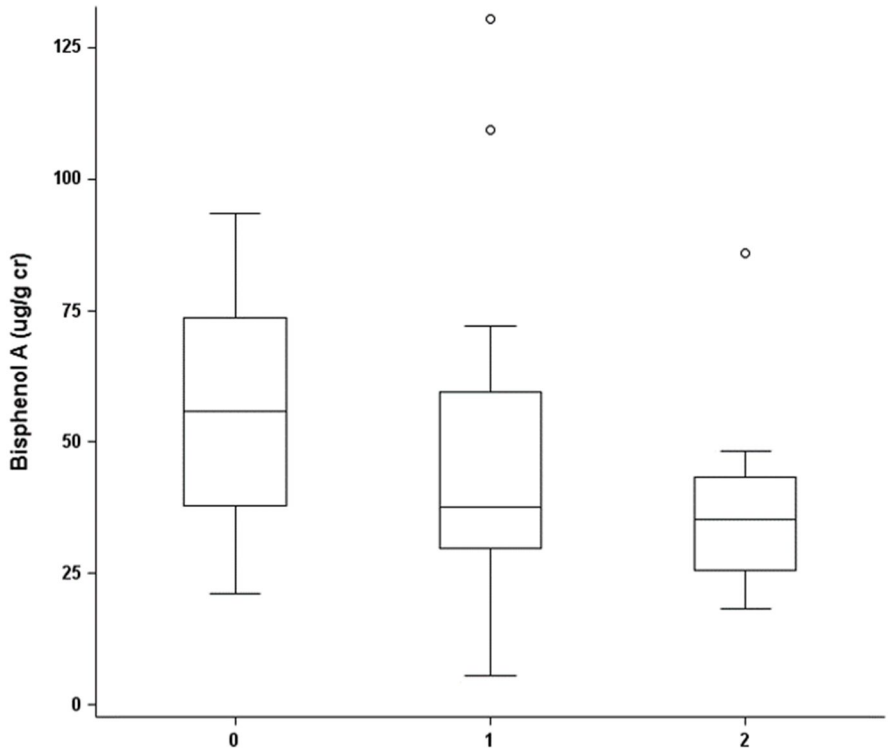


Figure 4.4 The mean ± SD values for BPA according to BMI. There were no significant differences among the BMI groups. 0, <18.5, 1, 18.5-23, 2, 23<. Circles represent outliers.

Table 4.5 The effects of age, BMI, parity, smoking status, endometrial phase, and endometrial pathology on the concentration of BPA

		Total subjects		Adenomyosis		Myoma		Endometriosis	
		N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)
Age (years)	<40	4	16.7 (4.7, 59.7)	-	-	-	-	4	16.7 (4.7, 59.7)
	40-49	25	38.5 (31.0, 47.8)	8	45.6 (28.6, 72.6)	11	33.7 (25.1, 45.4)	6	40.2 (20.2, 80.2)
	50<	15	40.0 (27.4, 58.5)	5	41.5 (28.8, 59.7)	5	56.4 (24, 131.8)	5	27.4 (9.3, 80.9)
	Kendall's τ	0.21		0.02		0.28		0.18	
	p	0.09		0.94		0.19		0.40	
BMI (kg/m^2)	< 18.5	5	49.8 (24.0, 103.3)	-	-	-	-	5	49.8 (24.0, 103.3)
	18.5-23	20	35.7 (24.4, 52.4)	8	48.6 (32.7, 72.1)	6	51.2 (24.5, 107.1)	6	16.6 (6.8, 40.1)
	≥ 23	19	33.4 (27.6, 40.4)	5	35.6 (27.7, 45.8)	10	34.0 (25.0, 46.0)	4	23.0 (13.7, 65.5)
	Kendall's τ	-0.19		-0.26		-0.38		-0.27	
	p	0.12		0.31		0.08		0.20	
Parity	0	8	24.8 (12.1, 50.8)	1	109.4	1	44.0	6	17.6 (9.1, 34.0)
	1	9	38.2 (21.0, 69.5)	2	43.8 (20.4, 94.2)	1	38.3	6	36.5 (13.1, 102.0)
	2	27	39.7 (32.7, 48.2)	10	39.6 (30.3, 51.7)	14	39.4 (27.8, 55.8)	3	41.5 (27.2, 63.4)
	Kendall's τ	0.13		-0.39		-0.08		0.44	
	p	0.28		0.12		0.69		0.04	
Smoking status	No	40	35.0 (28.4, 43.1)	13	43.8 (33.7, 57.1)	14	37.0 (26.6, 51.5)	13	26.8 (16.4, 43.7)
	Yes	4	48.7 (17.7, 133.8)	-	-	2	63.5 (41.8, 96.6)	2	37.3 (0, 213429)

	Kendall's τ	0.16		-		0.35		0.08		
	p	0.21		-		0.11		0.73		
Endometrial phase	Proliferative	21	38.4 (28.4, 52.1)	5	51.6 (29.6, 89.8)	9	34.5 (22.4, 53.1)	7	37.4 (16.5, 84.5)	
	Secretory	23	34.2 (25.9, 45.1)	8	40.4 (27.9, 58.5)	7	47.3 (28.4, 78.9)	8	21.7 (12.3, 38.3)	
	Kendall's τ	-0.13		-0.35		0.26		-0.39		
	p	0.31		0.17		0.22		0.08		
Endometrial pathology	Normal	35	35.3 (27.9, 44.7)	11	44.6 (33.1, 60.1)	13	39.2 (27.2, 56.4)	11	25.3 (14.4, 44.6)	
	Polyp	9	39.1 (26.3, 58.3)	2	40.2 (0.3, 5681.4)	3	41.6 (14.6, 118.4)	4	36.9 (12.1, 112.5)	
	Kendall's τ	0.02		-0.11		0.10		0.09		
	p	0.88		0.67		0.64		0.70		

Aberrations: GM, Geometric means; CI, Confidence interval
p, Tested by Kendall rank correlation coefficient (τ)

Multiple linear regression analysis was performed to evaluate the association between BMI and urinary BPA concentration independently of the variables such as age and smoking status (Table 4.6). The relationship between BMI and urinary BPA level was inversed, but not significant.

Table 4.6 Multiple linear regression analysis of the effect of the BMI level on the urinary BPA concentration

	β (95 % CI)	p-value
BMI (kg/m^2)		
Model 1 [a]	-0.15 (-0.30, 0.01)	0.06
Model 2 [b]	-0.14 (-0.30, 0.02)	0.09

[a] Model 1: Adjusted for age (years)
[b] Model 2: Adjusted for age and smoking status (yes or no).

3.3. Effect of BPA on global DNA methylation

3.3.1. Levels of global DNA methylation in the disease groups

Global DNA methylation levels (5-mc (%)) were assessed in adenomyosis, myoma, and endometriosis (Figure 4.6). The GM ± SE of global methylation were 6.38 ± 3.58 in adenomyosis, 3.57 ± 1.72 in myoma, and 6.23 ± 3.10 in endometriosis. There was no significant difference among the diseases (p=0.46, Kruskal-wallis test). Also there was no significant difference between myoma and endometriosis (p=0.24, Mann-whitney test) or between adenomyosis and myoma (p=0.42, Mann-whitney test).

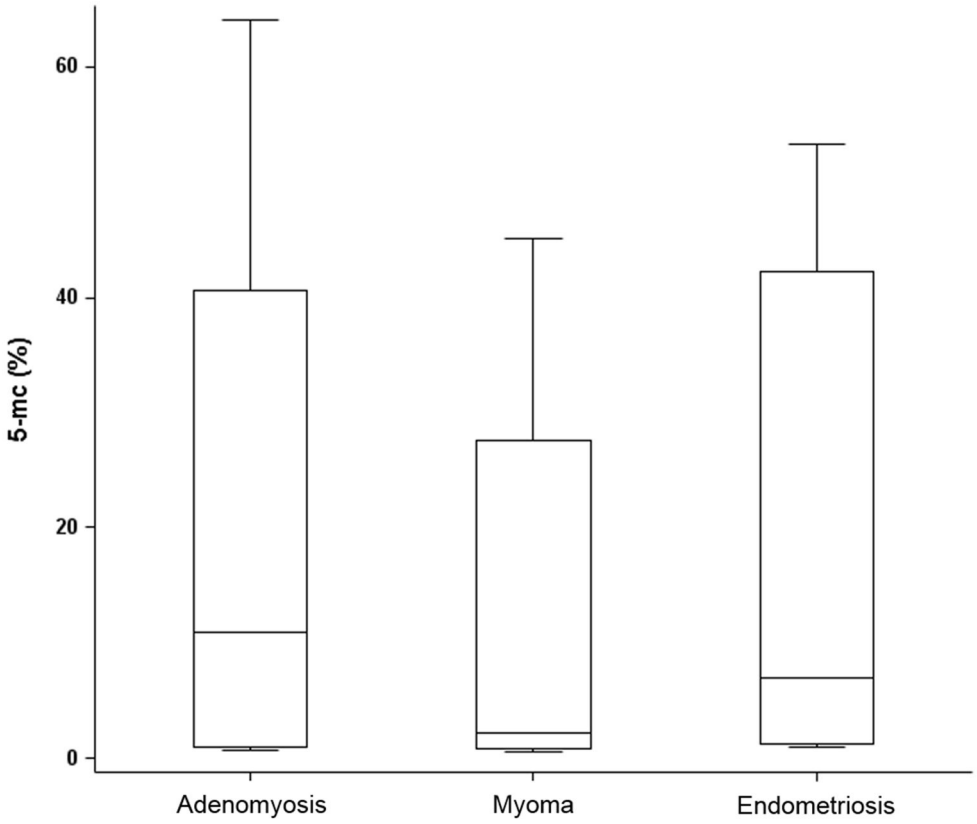


Figure 4.5 Comparison of global DNA methylation levels (5-mc (%)) between the groups. There were no significant differences among the groups. Global methylation level was not different in endometriosis or adenomyosis compared to in myoma.

3.3.2. Global DNA methylation level according to age, BMI, parity, smoking status, endometrial phase, and endometrial pathology

Table 4.7 showed about the effects on global DNA methylation by age, BMI, parity, smoking status, endometrial phase, and endometrial pathology. The 5-mc (%) level was significantly different by endometrium phase (p=0.03). The level of secretory phase was lower than that of proliferative phase (2.5 in secretory phase vs. 9.7 in proliferative phase). And endometrial polyps in myoma were hypomethylated than normal endometrium (0.7 in polyp of endometrial cells vs. 3.1 in normal endometrial cells).

Table 4.7 The effects of age, BMI, parity, smoking status, endometrial phase, and endometrial pathology on global DNA methylation

		Total subjects		Adenomyosis		Myoma		Endometriosis	
		N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)
Age (years)	<40	4	1.1 (0.4, 3.2)	-	-	-	-	4	1.1 (0.4, 3.2)
	40-49	25	4.7 (2.0, 11.1)	8	3.0 (0.5, 19.9)	11	4.6 (1.0, 21.4)	6	8.1 (0.8, 86.0)
	50<	15	7.3 (2.9, 18.6)	5	15.7 (2.0, 122.9)	5	2.0 (0.5, 7.9)	5	9.6 (1.2, 74.3)
	Kendall's τ	0.16		0.34		-0.11		0.31	
	p	0.24		0.20		0.64		0.20	
BMI (kg/m^2)	< 18.5	5	12.6 (0.7, 225.2)	-	-	-	-	5	12.6 (0.7, 225.2)
	18.5-23	20	4.6 (1.7, 12.3)	8	7.2 (1.0, 49.4)	6	4.0 (0.3, 54.7)	6	2.9 (0.4, 23.2)
	≥ 23	19	4.5 (1.9, 10.9)	5	5.2 (0.4, 73.1)	10	3.3 (0.9, 12.8)	4	8.1 (0.1, 763.2)
	Kendall's τ	-0.10		-0.05		0.04		-0.09	
	p	0.44		0.85		0.88		0.71	
Parity	0	8	1.7 (0.2, 15.8)	-	-	1	0.6	6	2.5 (0, 88.7)
	1	9	7.8 (2.0, 30.7)	2	1.0 (0.5, 1.8)	1	27.5	6	12.8 (2.3, 71.2)
	2	27	5.3 (2.5, 11.3)	9	9.7 (2.4, 38.7)	14	3.5 (1.1, 10.9)	3	3.7 (0, 659.4)
	Kendall's τ	0.02		0.32		0.17		-0.02	
	p	0.88		0.24		0.49		0.94	
Smoking status	No	40	4.7 (2.5, 8.7)	13	6.4 (1.8, 22.3)	14	3.4 (1.1, 10.5)	13	4.7 (1.4, 16.1)
	Yes	4	11.3 (0.5, 232.6)	-	-	2	4.9 (0-1436870000)	2	25.8 (0, 137560.2)

	Kendall's τ	0.09			-			0			0.33	
	p	0.51			-			1.00			0.20	
Endometrial phase	Proliferative	21	9.7 (4.2, 22.8)		5	13.7 (0.7, 285.5)		9	7.6 (1.7, 34.4)		7	10.7 (1.9, 60.3)
	Secretory	23	2.5 (1.2, 5.3)		8	4.1 (0.8, 22.4)		7	1.1 (0.5, 2.2)		8	2.9 (0.6, 14.7)
	Kendall's τ	-0.30			-0.20			-0.43			-0.27	
	p	0.03			0.45			0.08			0.29	
Endometrial pathology	Normal	35	7.0 (3.6, 13.4)		11	6.6 (1.7, 26.8)		13	3.1 (1.7, 19.4)		11	9.2 (2.3, 36.5)
	Polyp	9	2.2 (0.7, 6.9)		2	5.3 (0, 8756790)		3	0.7 (0.4, 1.3)		4	2.8 (0.2, 51.8)
	Kendall's τ	-0.26			-0.13			-0.50			-0.22	
	p	0.06			0.64			0.04			0.40	

Aberrations; GM, Geometric means; CI, Confidence interval
p, Tested by Kendall rank correlation coefficient (τ)

3.3.3. Correlation between global DNA methylation and urinary BPA concentration

Table 4.8 shows the GM value of global DNA methylation by the group of BPA levels. The median of BPA concentration (37.89 μg/g cr) was used as cut-point.

The relationships between BPA levels and global methylation level were not significant. But global DNA methylation level seems to slightly increase with BPA level, but not in adenomyosis. In each groups of BPA concentration, there were no significant differences among the groups. Also, the differences of global DNA methylation were not significant between myoma and endometriosis, or between myoma and adenomyosis.

Table 4.8 Geometric mean levels of the 5-mc (%) according to urinary BPA concentration in groups

	Total subjects		Adenomyosis		Myoma		Endometriosis		
BPA (μg/g cr)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
< 37.89	18	4.2 (1.8, 9.6)	5	11.7 (1.4, 97.8)	6	2.5 (0.4, 14.2)	7	3.1 (0.7, 13.1)	0.32
37.89 ≤	18	6.3 (2.5, 15.7)	6	3.8 (0.5, 30.7)	7	4.8 (0.8, 27.3)	5	16.4 (2.3, 118.3)	0.31
Kendall's τ	0.08		-0.15		0.07		0.31		
p	0.57		0.58		0.78		0.22		

[a], Kruskal-wallis test among the diseases

3.4. Effect of BPA on repeat element methylation

3.4.1. Levels of repeat element methylation in the groups

Repeat elements such as LINE-1 and Sat2 consist of interspersed repeats and tandem repeats comprise approximately 45 % of the human genome. Thus methylation status of LINE-1 and Sat2 can be used as a surrogate of global DNA methylation.

LINE-1 methylation levels were 63.12 ± 3.18, 64.04 ± 4.67, and 67.12 ± 2.03 in myoma, adenomyosis, and endometriosis, respectively (Figure 4.7). The differences were not significant among groups (p=0.67). Also LINE-1 methylation level was not significant difference between myoma and endometriosis, or between myoma and adenomyosis (p=0.37; p=0.68, Mann-whitney test). On the other hand, the level of Sat2 methylation were 57.04 ± 5.23, 76.56 ± 6.36, and 53.02 ± 6.28 in myoma, adenomyosis, and endometriosis, respectively (Figure 4.8). There were significant differences among the groups (p=0.01, Kruskal-wallis test). Sat2 methylation level was significantly different between myoma and adenomyosis (p=0.01, Mann-whitney test). However, the difference was not significant between myoma and endometriosis (p=0.68, Mann-whitney test).

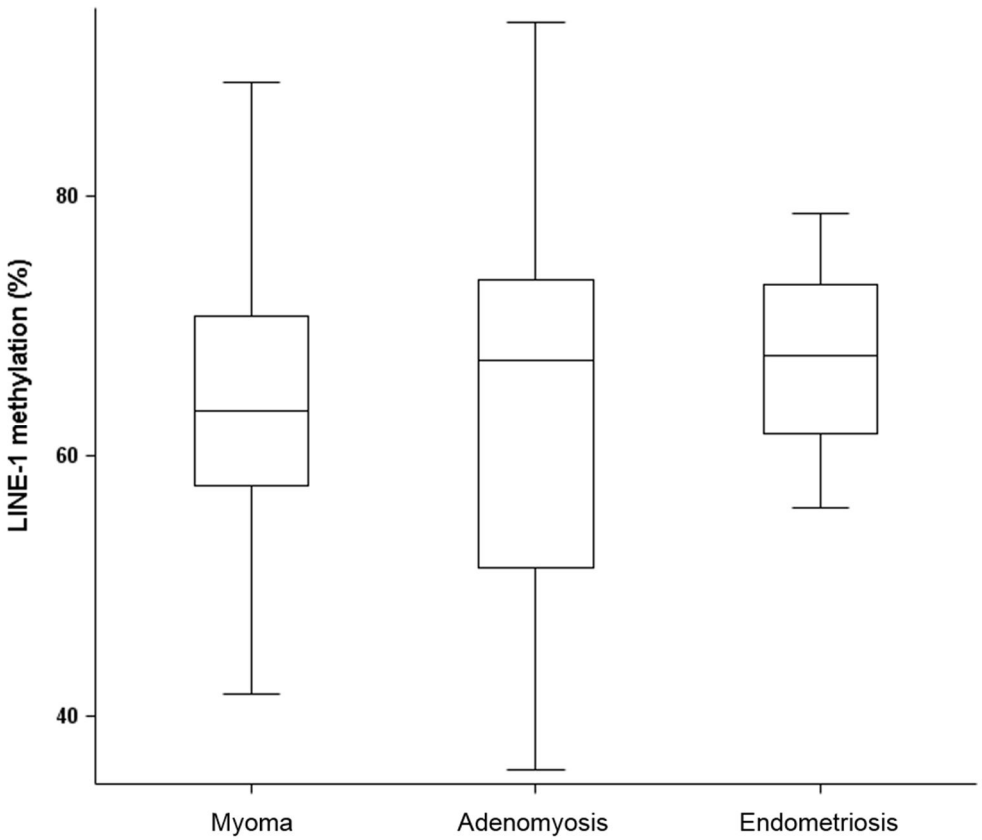


Figure 4.6 Comparison of LINE-1 methylation level among three groups. LINE-1 methylation levels were 63.12 ± 3.18, 64.04 ± 4.67, and 67.12 ± 2.03 in myoma, adenomyosis, and endometriosis, respectively. The difference was not significant (p=0.67, Kruskal-wallis test).

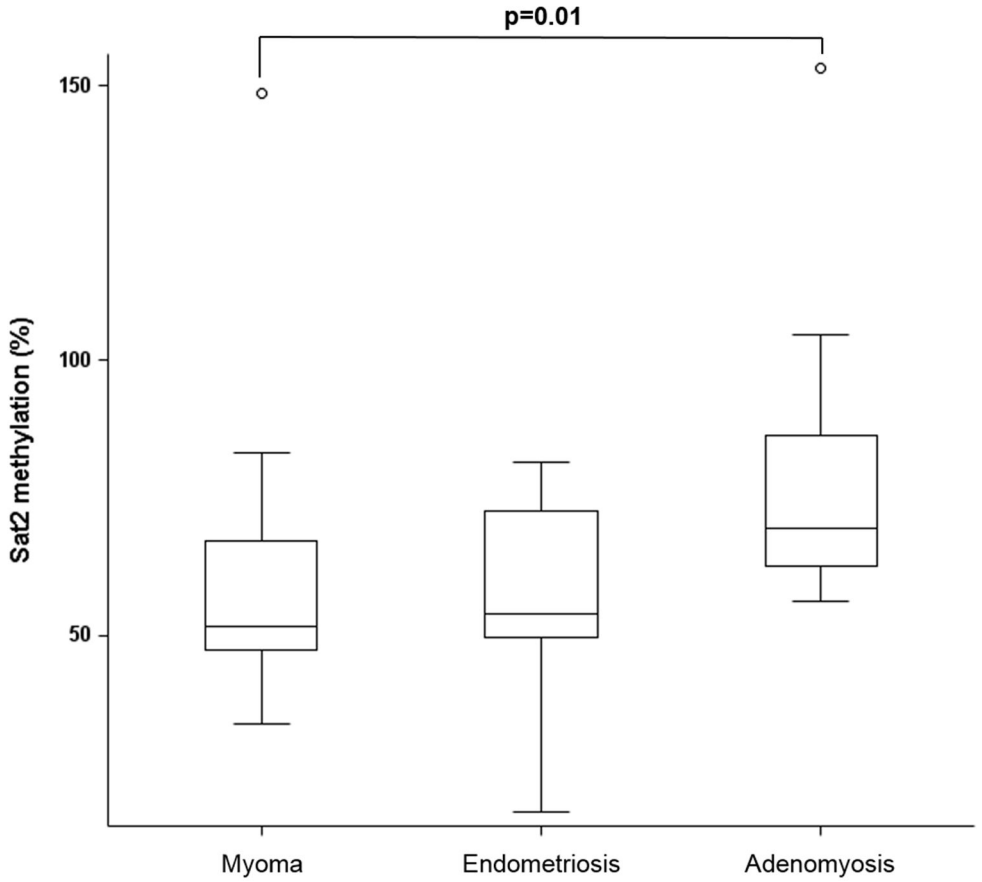


Figure 4. 7 Comparison of Sat2 methylation level among three groups. The levels of Sat2 methylation were 57.04 ± 5.23, 76.56 ± 6.36, and 53.02 ± 6.28 in myoma, adenomyosis, and endometriosis, respectively. There were significant differences among the groups (p=0.01, Kruskal-wallis test). And Sat2 methylation level was significantly different between myoma and adenomyosis (p=0.01, Mann-whitney test). Circles represent outliers.

3.4.2. Levels of repeat element methylation according to age, BMI, smoking status, parity, exercise, endometrial phase, and endometrial pathology

The effects of age, BMI, smoking status, parity, exercise, endometrial phase, and endometrial pathology on repeat element methylation in subjects were evaluated. The variables exhibited no association with LINE-1 methylation levels in the groups (Table 4.9). Sat2 methylation levels exhibited no association with any variables (Table 4.10).

Table 4. 9 The effects of age, BMI, number of children, smoking status, endometrial phase, and endometrial pathology on LINE-1 methylation

		Total subjects		Adenomyosis		Myoma		Endometriosis	
		N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE
Age (years)	<40	3	63.3 ± 3.0	-	-	-	-	3	63.3 ± 3.0
	40-49	21	67.1 ± 2.9	7	65.9 ± 6.7	11	66.4 ± 4.0	4	70.8 ± 1.5
	50<	15	61.5 ± 3.0	5	61.4 ± 6.8	5	56.6 ± 4.3	5	66.5 ± 4.3
	Kendall's τ	-0.09		-0.10		-0.11		0.16	
	p	0.48		0.68		0.64		0.51	
BMI (kg/m^2, %)	< 18.5	3	66.6 ± 3.6	-	-	-	-	3	66.6 ± 3.6
	18.5-23	20	65.0 ± 3.1	8	66.3 ± 5.7	6	64.1 ± 7.2	6	64.2 ± 2.9
	≥ 23	16	63.8 ± 2.8	4	59.6 ± 8.7	9	62.4 ± 2.9	3	73.4 ± 2.9
	Kendall's τ	-0.03		-0.13		-0.05		0.31	
	p	0.83		0.61		0.81		0.20	
Parity	0	6	67.2 ± 2.2	1	69.4	1	70.8	4	65.7 ± 3.2
	1	8	62.3 ± 4.8	2	56.7 ± 20.8	1	57.7	5	65.5 ± 3.6
	2	25	64.8 ± 2.6	9	65.1 ± 5.1	13	62.9 ± 3.6	3	71.8 ± 3.5
	Kendall's τ	-0.01		0.02		-0.02		0.27	
	p	0.91		0.93		0.93		0.27	
Smoking status	No	35	64.0 ± 2.1	12	64.0 ± 4.7	14	62.5 ± 3.5	10	65.9 ± 2.3
	Yes	4	70.2 ± 3.9	-	-	2	67.1 ± 8.5	2	73.3 ± 0.4

	Kendall's τ	0.17	-	0.11	0.33		
	p	0.19	-	0.61	0.20		
Endometrial phase	Proliferative	19	67.7 ± 2.6	4	72.8 ± 7.2	9	65.3 ± 4.2	6	67.8 ± 3.3
	Secretory	20	61.8 ± 2.8	8	59.7 ± 5.7	6	59.8 ± 5.0	6	66.5 ± 2.7
	Kendall's τ	-0.13		-0.22		-0.16		-0.12	
	p	0.33		0.40		0.48		0.63	
Endometrial pathology	Normal	31	64.9 ± 2.3	10	66.3 ± 5.2	12	62.4 ± 3.9	9	66.5 ± 2.6
	Polyp	8	63.7 ± 3.3	2	52.6 ± 8.0	3	65.9 ± 3.7	3	68.9 ± 2.9
	Kendall's τ	-0.04		-0.33		0.10		0.12	
	p	0.78		0.20		0.67		0.64	

Aberrations: SE, standard error
p, Tested by Kendall rank correlation coefficient (τ)

Table 4.10 The effects of age, BMI, parity, smoking status, and endometrial phase on Sat2 methylation

		Total subjects		Adenomyosis		Myoma		Endometriosis	
		N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)
Age (years)	<40	4	40.1 (6.7, 241.3)	-	-	-	-	4	40.1 (6.7, 241.3)
	40-49	25	65.7 (55.3,78.0)	7	85.1 (62.9, 115.1)	11	56.9 (42.9, 75.4)	6	59.8 (44.8, 79.8)
	50<	15	60.0 (52.0,69.1)	5	66.0 (55.1, 79.0)	5	57.4 (39.3, 83.8)	5	56.9 (38.5, 84.1)
	Kendall's τ	0.03		-0.40		0		0.20	
	p	0.84		0.12		1.00		0.42	
BMI (kg/m^2)	< 18.5	5	35.8 (8.1, 158.2)	-	-	-	-	5	35.8 (8.1, 158.2)
	18.5-23	20	68.8 (57.8, 81.9)	8	81.2 (61.9, 106.5)	6	62.8 (38.6, 102.4)	6	60.4 (44.2, 82.6)
	≥ 23	18	58.1 (50.9, 66.3)	4	68.1 (51.8, 89.4)	10	53.5 (43.4, 65.9)	4	60.4 (37.1, 98.5)
	Kendall's τ	-0.01		-0.30		-0.13		0.40	
	p	0.91		0.23		0.56		0.20	
Parity	0	8	49.8 (27.9, 88.8)	1	88.4	1	48.9	6	43.4 (16.4, 114.3)
	1	9	58.0 (47.1, 71.5)	2	59.5 (29.3, 120.7)	1	65.1	6	56.2 (37.8, 83.5)
	2	27	65.2 (56.2, 75.6)	9	79.7 (63.1, 100.7)	13	57.1 (45.4, 71.8)	3	63.0 (40.6, 97.8)
	Kendall's τ	0.14		0.16		-0.02		0.31	
	p	0.28		0.52		0.93		0.21	
Smoking status	No	38	61.5 (53.7, 70.5)	12	76.6 (63.8, 91.9)	13	56.3 (44.9, 70.5)	13	53.2 (38.5, 73.5)
	Yes	4	57.1 (42.1, 77.4)	-	-	2	62.5 (5.5, 715.0)	2	52.2 (29.2, 93.4)

	Kendall's τ	-0.06		-		0.19		-0.11	
	p	0.64		-		0.40		0.67	
Endometrial phase	Proliferative	20	58.1 (50.4, 67.1)	4	78.1 (54.3, 112.3)	9	51.1 (42.1, 62.0)	7	57.8 (42.5, 48.7)
	Secretory	22	64.0 (51.9, 78.8)	8	75.8 (57.6, 99.7)	6	67.2 (42.1, 107.2)	8	48.6 (28.3, 83.5)
	Kendall's τ	0.13		-0.17		0.27		-0.16	
	p	0.33		0.50		0.24		0.52	
Endometrial pathology	Normal	31	60.9 (54.3, 68.4)	10	72.7 (62.9, 84.1)	12	56.2 (43.9, 72.0)	9	55.8 (46.4, 67.0)
	Polyp	8	61.5 (37.3, 101.4)	2	99.0 (0.4, 2538.3)	3	60.4 (35.1, 104.1)	3	45.6 (6.1, 338.5)
	Kendall's τ	0.07		0.22		0.16		0.02	
	p	0.58		0.39		0.47		0.93	

Aberrations; GM, Geometric means; CI, Confidence interval
p, Tested by Kendall rank correlation coefficient (τ)

3.4.3. Levels of repeat element methylation according to BPA level by the disease groups

LINE-1 and Sat2 methylation was compared with diseases according to level of BPA group (Table 4.11 and Table 4.12). The log transformation of Sat2 methylation level was used to statistical analysis of comparison between disease groups. LINE-1 and Sat2 methylation were not significantly difference according to BPA level. LINE-1 methylation was not significantly difference among the disease groups, and Sat2 methylation level was significantly different in < 37.89 μg/g cr of BPA group. While there was no difference of Sat2 methylation level between myoma and endometriosis ($p=0.27$ in < 37.89 μg/g of BPA, $p=0.94$ in ≥ 37.89 μg/g cr of BPA, Mann-whitney test), Sat2 methylation level was significantly different between myoma and adenomyosis in < 37.89 μg/g of BPA ($p=0.01$, Mann-whitney test).

Table 4.11 The levels of LINE-1 methylation (%) according to the concentration of BPA

	Total subjects		Adenomyosis		Myoma		Endometriosis		
BPA (μg/g cr)	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	p-value [a]
< 37.89	19	64.2 ± 2.6	5	61.8 ± 6.8	6	64.5 ± 6.0	8	65.3 ± 2.4	0.93
37.89 ≤	20	65.1 ± 3.0	7	65.6 ± 6.7	9	62.2 ± 3.8	4	70.7 ± 3.6	0.36
Kendall's τ	0.05		0.02		-0.03		0.30		
p	0.71		0.94		0.91		0.23		

[a], Kruskal-wallis test among the disease groups

Table 4.12 Geometric mean levels of Sat2 methylation (%) according to the concentration of BPA

	Total subjects		Adenomyosis		Myoma		Endometriosis		
BPA (μg/g cr)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
< 37.89	19	56.6 (45.8, 70.1)	5	83.3 (52.2, 132.9)	6	46.9 (38.3, 57.4)	8	51.3 (33.9, 77.5)	0.02
37.89 ≤	20	65.6 (56.9, 75.6)	7	72.1 (59.0, 88.1)	9	65.0 (48.3, 87.5)	4	56.7 (41.5, 77.4)	0.18
Kendall's τ	0.15		-0.06		0.37		0		
p	0.26		0.81		0.10		1.00		

[a], Kruskal-wallis test among the disease groups

3.5. Effect of BPA on specific DNA methylation

To investigate the relation between the methylation of specific gene promoters and BPA concentration, the promoter methylation status assessed about six human genes: HOXA10, progesterone receptor, RASSF1A, p16, MLH1, estrogen receptor 1 (ER 1), estrogen receptor 2 (ER 2), and GSTP1. The methylation of p16, MLH1, ER 1, ER 2, and GSTP1 was rarely detected in subjects (data not shown).

HOXA10, PR, and RASSF1A methylation status was shown in Figure 4.9. Unexpectedly, there were no differences among the groups. Also endometriosis or adenomyosis showed no significant difference in promoter methylation levels compared to myoma.

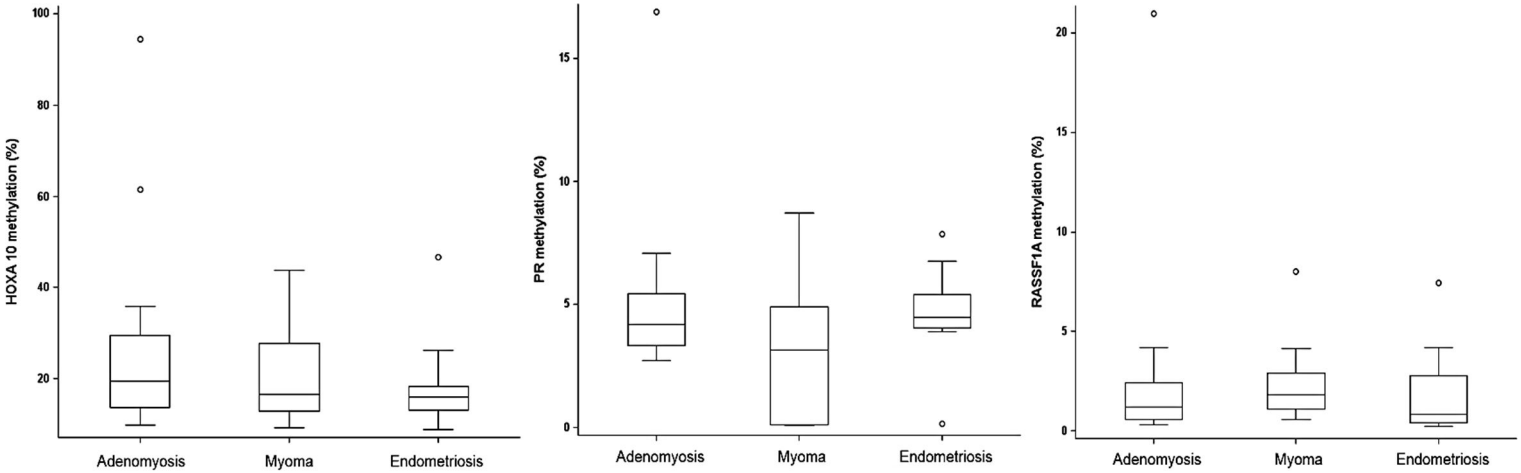


Figure 4.8 PMR values of HOXA 10, PR, and RASSF1A gene in the subjects. There were no significantly differences among these groups.

3.5.1. Association between BPA levels and HOXA 10 methylation

Table 4.13 showed about the effects on HOXA 10 methylation by age, BMI, parity, smoking status, endometrial phase, and endometrial pathology. In myoma group, the significant inverse relationship was found between age and HOXA 10 methylation level (Kendall's τ= -0.52, p= 0.02). And endometrial polyp was significant associated with HOXA 10 hypermethylation in adenomyosis (Kendall's τ= 0.55, p= 0.03).

Table 4.13 The effects of age, BMI, education, parity, smoking status, endometrial phase, and endometrial pathology on HOXA 10 methylation

		Total subjects		Adenomyosis		Myoma		Endometriosis	
		N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)
Age (years)	<40	3	15.3 (9.9, 23.7)	-	-	-	-	3	15.3 (9.9, 12.7)
	40-49	21	20.8 (16.3, 26.5)	7	23.2 (11.8, 45.5)	10	22.3 (16.7, 29.7)	4	14.5 (8.4, 25.1)
	50<	15	17.1 (12.6, 23.3)	5	20.9 (9.1, 47.8)	5	12.2 (8.1, 18.4)	5	19.8 (9.9, 39.7)
	Kendall's τ	-0.10		-0.10		-0.52		0.05	
	P	0.43		0.68		0.02		0.82	
BMI (kg/m^2, %)	< 18.5	3	15.5 (11.4, 21.2)	-	-	-	-	3	15.5 (11.4, 21.2)
	18.5-23	20	21.2 (15.6, 29.0)	8	27.8 (15.4, 50.0)	6	19.8 (9.9, 39.6)	6	15.9 (8.7, 28.8)
	≥ 23	16	16.9 (14.2, 20.0)	4	14.1 (8.3, 24.2)	9	17.2 (13.4, 22.2)	3	20.1 (10.7, 37.8)
	Kendall's τ	-0.04		-0.48		-0.05		0.17	
	P	0.74		0.06		0.81		0.50	
Parity	0	6	19.3 (12.3, 30.5)	1	21.0	1	43.8	4	15.4 (12.3, 19.5)
	1	8	18.3 (12.7, 26.2)	2	21.8 (9.5, 50.2)	1	13.8	5	18.0 (9.2, 35.2)
	2	25	18.9 (14.9, 24.1)	9	22.4 (12.2, 41.1)	13	17.4 (13.4, 22.6)	3	16.6 (4.1, 66.9)
	Kendall's τ	-0.02		-0.21		-0.17		0.05	
	P	0.86		0.41		0.45		0.82	
Smoking status	No	35	19.2 (16.0, 23.1)	13	22.2 (14.4, 34.1)	13	18.6 (14.1, 24.5)	12	16.9 (12.2, 23.5)
	Yes	4	16.0 (7.9, 32.6)	-	-	2	16.0 (0, 16907)	2	16.0 (13.7, 18.7)

	Kendall's τ	-0.09		-		-0.11		0	
	P	0.52		-		0.61		1.00	
Endometrial phase	Proliferative	19	17.8 (14.4, 22.0)	4	22.8 (7.8, 66.7)	9	17.6 (13.3, 23.2)	6	15.5 (10.5, 22.9)
	Secretory	20	19.9 (15.0, 26.4)	8	21.9 (11.9, 40.0)	6	19.3 (9.9, 37.5)	6	18.1 (11.0, 30.0)
	Kendall's τ	0.06		0		0.08		0.08	
	P	0.67		1.00		0.72		0.75	
Endometrial pathology	Normal	31	16.9 (14.8, 19.3)	10	17.3 (13.2, 22.7)	12	17.7 (13.6, 23.0)	11	15.4 (12.2, 19.4)
	Polyp	8	28.9 (14.5, 57.6)	2	76.3 (5.1, 1149.8)	3	20.4 (3.0, 140.3)	4	21.5 (3.9, 118.4)
	Kendall's τ	0.22		0.55		0.07		0.17	
	P	0.10		0.03		0.77		0.52	

Aberrations; GM, Geometric means; CI, Confidence interval
p, Tested by Kendall rank correlation coefficient (τ)

3.5.1.1. Correlation between HOXA 10 methylation and BPA concentration

Associations between the concentration of BPA and HOXA 10 methylation levels were evaluated (Table 4.14). And HOXA 10 methylation level was analyzed by disease. HOXA 10 methylation level was increased with BPA concentration, but it was not significant. Also, HOXA 10 methylation level was not different among groups, and there were no significant differences between myoma and endometriosis, or between myoma and adenomyosis, in each BPA concentration group.

Table 4.14 Geometric mean levels of HOXA10 methylation (%) according to the concentration of BPA

	Total subjects		Adenomyosis		Myoma		Endometriosis		
BPA (μg/g cr)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
< 37.89	19	18.2 (13.7, 24.1)	5	23.8 (7.7, 73.2)	6	17.1 (11.3, 25.9)	8	16.0 (10.7, 24.0)	0.69
37.89 ≤	20	19.6 (15.7, 24.4)	7	21.1 (12.9, 34.5)	9	19.0 (12.6, 28.7)	4	18.3 (11.7, 28.8)	0.88
Kendall's τ	0.15		0.06		0.13		0.26		
p	0.27		0.81		0.56		0.31		

[a], Kruskal-wallis test among the diseases

3.5.2. Association between BPA level and PR methylation

Table 4.15 showed about the effects on PR methylation by age, BMI, parity, smoking status, and endometrial phase. PR methylation level was increased according to age, but it was not significant. There were no significant changes in any variables.

Table 4.15 The effects of age, BMI, parity, smoking status, endometrial phase, and endometrial pathology on PR methylation

		Total subjects		Adenomyosis		Myoma		Endometriosis	
		N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)
Age (years)	<40	2	0.9 (0, 34058838)	-	-	-	-	2	0.9 (0, 340588383)
	40-49	19	2.2 (1.0, 4.9)	7	5.1 (2.8, 9.1)	10	0.9 (0.2, 3.9)	4	5.4 (3.0, 9.5)
	50<	14	4.1 (3.5, 4.9)	5	4.1 (3.3, 5.1)	4	3.6 (1.6, 8.0)	5	4.6 (4.0, 5.4)
	Kendall's τ	0.09		-0.10		0.23		0.23	
	P	0.49		0.68		0.32		0.32	
BMI (kg/m^2)	< 18.5	3	1.7 (0, 336.5)	-	-	-	-	3	1.7 (0, 336.5)
	18.5-23	19	3.1 (1.7, 5.7)	8	4.6 (2.9, 7.5)	6	1.1 (0.2, 8.3)	5	5.2 (4.2, 6.5)
	≥ 23	15	2.4 (1.0, 5.7)	4	4.6 (2.7, 8.0)	8	1.4 (0.3, 7.9)	3	4.1 (3.5, 4.9)
	Kendall's τ	-0.06		0.09		0.03		-0.24	
	P	0.67		0.73		0.90		0.35	
Parity	0	5	2.1 (0.3, 13.0)	1	3.3	1	4.0	3	1.5 (0, 184.1)
	1	8	5.0 (4.1, 6.0)	2	4.5 (0.4, 50.6)	1	4.7	5	5.2 (3.8, 7.2)
	2	24	2.3 (1.2, 4.5)	9	4.8 (3.2, 7.4)	12	1.1 (0.3, 3.8)	3	4.9 (2.4, 9.5)
	Kendall's τ	-0.07		0.07		-0.15		0.24	
	p	0.60		0.78		0.52		0.35	
Smoking status	No	34	2.8 (1.7, 4.6)	13	4.6 (3.4, 6.3)	12	1.5 (0.5, 5.0)	9	3.3 (1.3, 8.0)
	Yes	4	1.7 (0.1, 41.3)	-	-	2	0.5 (0, 241287387)	2	5.6 (0.1, 402.4)

	Kendall's τ	-0.08		-		-0.21		0.13	
	p	0.56		-		0.36		0.64	
Endometrial phase	Proliferative	18	2.2 (1.0, 4.8)	4	3.7 (2.9, 4.6)	8	0.8 (0.1, 4.8)	6	5.4 (4.1, 7.1)
	Secretory	19	3.2 (1.8, 5.8)	8	5.2 (3.2, 8.4)	6	2.4 (0.5, 12.3)	5	2.2 (0.3, 14.3)
	Kendall's τ	0.08		0.35		0.24		-0.49	
	p	0.54		0.17		0.30		0.07	
Endometrial pathology	Normal	29	2.5 (1.4, 4.3)	10	4.1 (3.3, 5.1)	11	0.9 (0.2, 3.5)	8	5.1 (4.1, 6.3)
	Polyp	8	3.5 (1.1, 11.1)	2	8.3 (0, 66773.4)	3	4.8 (1.2, 18.3)	3	1.5 (0, 188.6)
	Kendall's τ	0.07		0.28		0.27		-0.33	
	p	0.61		0.28		0.24		0.22	

Aberrations; GM, Geometric means; CI, Confidence interval
p, Tested by Kendall rank correlation coefficient (τ)

3.5.2.1. Correlation between PR methylation and BPA concentration

Associations between the concentration of BPA and PR methylation levels were evaluated (Table 4.16). And PR methylation was compared by disease. PR methylation level was decreased with BPA concentration, but it was not significant. The levels of PR methylation were not different among the diseases. And there were no differences of PR methylation level between myoma and endometriosis, or between myoma and adenomyosis, in each BPA concentration levels.

Table 4.16 Geometric mean levels of PR methylation (%) according to the concentration of BPA

	Total subjects		Adenomyosis		Myoma		Endometriosis		
BPA (μg/g cr)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
< 37.89	18	3.1 (1.6, 6.1)	5	5.2 (2.1, 12.6)	6	2.1 (0.3, 13.9)	7	3.0 (0.9, 10.3)	0.76
37.89 ≤	19	2.3 (1.1, 4.8)	7	4.3 (3.3, 5.6)	8	0.9 (0.2, 4.9)	4	4.9 (3.0, 8.2)	0.13
Kendall's τ	-0.13		-0.02		-0.21		0		
p	0.36		0.94		0.37		1.00		

[a], Kruskal-wallis test

3.5.3. Association between BPA levels and PR methylation

Table 4.17 shows the methylation level of RASSF1A by age, BMI, parity, smoking status, endometrial phase, and endometrial pathology. RASSF1A methylation level was not significantly different according those variables. Endometrial polyps showed slightly higher of RASSF1A methylation level than normal, but it was not significant.

Table 4.17 The effects of age, BMI, parity, smoking status, endometrial phase, and endometrial pathology on RASSF1A methylation

		Total subjects		Adenomyosis		Myoma		Endometriosis	
		N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)
Age (years)	<40	2	1.0 (0.1, 6.6)	-	-	-	-	2	1.0 (0.1, 6.6)
	40-49	20	1.6 (0.9, 2.8)	7	1.7 (0.5, 6.0)	9	2.2 (1.2, 4.2)	4	0.7 (0.1, 5.4)
	50<	15	1.2 (0.8, 2.0)	5	1.0 (0.3, 2.9)	5	1.3 (0.8, 2.0)	5	1.6 (0.4, 6.4)
	Kendall's τ	-0.06		-0.10		-0.33		0.13	
	p	0.63		0.68		0.16		0.61	
BMI (kg/m^2, %)	< 18.5	3	1.7 (0.2, 12.8)	-	-	-	-	3	1.7 (0.2, 12.8)
	18.5-23	18	1.3 (0.7, 2.3)	8	1.4 (0.4, 4.5)	5	1.6 (0.8, 3.5)	5	0.9 (0.2, 4.7)
	≥ 23	16	1.5 (1.0, 2.4)	4	1.3 (0.4, 4.2)	9	1.9 (1.0, 3.6)	3	0.9 (0.1, 10.5)
	Kendall's τ	0.05		0.17		0.08		-0.24	
	P	0.72		0.50		0.74		0.35	
Parity	0	5	1.0 (0.4, 2.1)	1	0.5	1	2.6	3	0.9 (0.5, 1.6)
	1	8	1.7 (0.7, 4.0)	2	1.3 (0, 37974.0)	1	2.8	5	1.7 (0.4, 7.5)
	2	24	1.5 (0.9, 2.3)	9	1.6 (0.6, 4.1)	12	1.7 (1.0, 2.8)	3	0.6 (0, 16.4)
	Kendall's τ	0.06		0.25		-0.15		-0.11	
	p	0.64		0.31		0.52		0.67	
Current smokers	No	33	1.4 (1.0, 2.0)	12	1.4 (0.6, 2.9)	12	1.9 (1.2, 3.1)	9	1.0 (0.4, 2.2)
	Yes	4	1.5 (0.5, 5.0)	-	-	2	1.4 (0.1, 17.3)	2	1.7 (0, 116970)

	Kendall's τ	0.03		-		-0.13		0.13	
	p	0.81		-		0.58		0.64	
Endometrial phase	Proliferative	19	1.2 (0.8, 1.9)	4	0.9 (0.2, 3.8)	9	1.8 (1.1, 3.0)	6	0.8 (0.2, 2.8)
	Secretory	18	1.7 (1.0, 2.8)	8	1.6 (0.5, 4.9)	5	1.9 (0.6, 6.1)	5	1.5 (0.5, 4.8)
	Kendall's τ	0.09		0.17		-0.08		0.30	
	p	0.52		0.50		0.74		0.27	
Endometrial pathology	Normal	29	1.21 (0.9, 1.6)	10	1.2 (0.7, 2.1)	11	1.6 (1.0, 2.4)	8	0.9 (0.4, 2.0)
	Polyp	8	2.5 (0.8, 8.0)	2	2.6 (0, 7282020)	3	3.2 (0.4, 24.3)	3	1.9 (0.1, 35.8)
	Kendall's τ	0.19		0		0.27		0.33	
	p	0.17		1.00		0.24		0.22	

Aberrations; GM, Geometric means; CI, Confidence interval
p, Tested by Kendall rank correlation coefficient (τ)

3.5.3.1. Correlation between RASSF1A methylation and BPA concentration

The relationships between BPA level and RASSF1A methylation were analyzed by disease (Table 4.18). The relationship between RASSF1A methylation and BPA concentration showed decrease patterns in disease groups, but endometriosis showed the opposite pattern. The relationship between RASSF1A methylation and BPA level in adenomyosis was significant ($p=0.04$). Also the RASSF1A methylation levels among the diseases were not significantly different, between myoma and endometriosis, and between myoma and adenomyosis.

Table 4.18 Geometric mean levels of RASSF1A methylation (%) according to the concentration of BPA

	Total subjects		Adenomyosis		Myoma		Endometriosis		
BPA (μg/g cr)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
< 36.33	18	1.7 (0.9, 3.0)	5	2.9 (0.6, 14.1)	6	2.3 (0.8, 6.2)	7	0.9 (0.3, 2.4)	0.12
36.33 ≤	19	1.2 (0.8, 1.8)	7	0.8 (0.4, 1.6)	8	1.5 (1.0, 2.4)	4	1.6 (0.3, 8.1)	0.21
Kendall's τ	-0.10		-0.52		-0.21		0.31		
p	0.45		0.04		0.37		0.26		

[a]; Kruskal-wallis test among the diseases

3.6. The level of DNA methylation by endometrium phase

3.6.1. Association between global DNA methylation and endometrium phase

By the endometrial phase, the geometric means of 5-mc (%) are represented in Table 4.19. While the 5-mc (%) level was increased according to BPA concentration in proliferative phase, the 5-mc (%) level was decreased in secretory phase. In the total subjects, 5-mc level (%) was significantly lower in secretory phase than in proliferative phase. Also, 5-mc level was significantly higher in proliferative phase than in secretory phase over 37.89 μg/g of BPA.

Table 4.19 Geometric means of the 5-mc (%) according to urinary BPA concentration by endometrium phase

	Proliferative		Secretory		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	19	9.7 (4.2, 22.8)	18	2.5 (1.2, 5.3)	0.03
BPA (μg/g cr)					
< 37.89	9	4.5 (1.2, 16.7)	8	4.7 (1.1, 20.2)	0.92
37.89 ≤	10	19.5 (6.3, 60.3)	10	1.6 (0.8, 2.9)	0.005
Kendall's τ	0.31		-0.20		
p	0.12		0.34		

[a], Mann-whitney test

3.6.2. Association between repeat elements methylation and endometrium phase

By the endometrial phase, the methylation levels of LINE-1 and Sat2 are represented in Table 4.20 and Table 4.21, respectively. Both LINE-1 and Sat2 methylation levels were not significantly different by endometrium phase.

Table 4.20 Means of the LINE-1 methylation (%) according to urinary BPA concentration by endometrium phase

	Proliferative		Secretory		
	N	Mean ± SE	N	Mean ± SE	p-value [a]
Total	19	67.7 ± 2.6	20	61.8 ± 2.8	0.33
BPA (μg/g cr)					
< 37.89	9	64.0 ± 4.1	9	64.2 ± 4.0	0.48
37.89 ≤	10	70.9 ± 3.3	12	61.8 ± 4.1	0.16
Kendall's τ	0.31		-0.17		
p	0.12		0.36		

[a], Mann-whitney test

Table 4.21 Geometric mean of the Sat2 methylation (%) according to urinary BPA concentration by endometrium phase

	Proliferative		Secretory		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	19	58.1 (50.4, 60.1)	20	64.0 (51.9, 78.8)	0.33
BPA (μg/g cr)					
< 37.89	9	52.0 (40.7, 66.4)	9	70.1 (54.2, 90.7)	0.08
37.89 ≤	10	64.3 (53.7, 76.9)	12	57.8 (41.8, 79.8)	0.58
Kendall's τ	0.32		0.03		
p	0.10		0.88		

[a], Mann-whitney test

3.6.3. Association between HOXA 10 methylation and endometrium phase

By the endometrial phase, the geometric means of HOXA 10 methylation are represented in Table 4.22. While the HOXA 10 methylation level was decreased according to BPA concentration in secretory phase, the HOXA 10 methylation level was significantly increased in proliferative phase. HOXA 10 methylation level was not different between proliferative and secretory phase.

Table 4.22 Geometric means of the HOXA 10 methylation according to urinary BPA concentration by endometrium phase

	Proliferative		Secretory		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	19	17.8 (14.4, 22.0)	18	19.9 (15.0, 26.4)	0.68
BPA (μg/g cr)					
< 37.89	9	14.8 (11.1, 19.6)	8	22.4 (12.8, 39.4)	0.25
37.89 ≤	10	21.1 (15.4, 29.0)	10	17.0 (12.3, 23.5)	0.34
Kendall's τ	0.40		-0.07		
p	0.04		0.71		

[a], Mann-whitney test

3.6.4. Association between PR methylation and endometrium phase

By the endometrial phase, the geometric means of PR methylation level were represented in Table 4.23. PR methylation was slightly higher in secretory phase, but it was not significant. PR methylation levels were decreased according to BPA level, but those were not significant.

Table 4.23 Geometric means of the PR methylation according to urinary BPA concentration by endometrium phase

	Proliferative		Secretory		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	19	2.2 (1.0, 4.8)	18	3.2 (1.8, 5.8)	0.55
BPA (μg/g cr)					
< 37.89	9	2.6 (0.9, 8.0)	8	5.5 (3.4, 8.8)	0.47
37.89 ≤	10	1.8 (0.4, 7.3)	10	2.3 (1.0, 5.7)	1.00
Kendall's τ	-0.13		-0.16		
p	0.51		0.41		

[a], Mann-whitney test

3.6.5. Association between RASSF1A methylation and endometrium phase

By the endometrial phase, the geometric means of RASSF1A methylation were represented in Table 4.24. RASSF1A methylation was not different between proliferative and secretory phase. RASSF1A methylation levels were not different according to BPA levels in each endometrial phase.

Table 4.24 Geometric means of the RASSF1A methylation according to urinary BPA concentration by endometrium phase

	Proliferative		Secretory		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	19	1.2 (0.8, 1.9)	18	1.7 (1.0, 2.8)	0.53
BPA (μg/g cr)					
< 37.89	9	1.1 (0.5, 2.3)	8	2.9 (1.0, 8.3)	0.11
37.89 ≤	10	1.4 (0.7, 2.5)	10	0.7 (0.3, 1.8)	0.31
Kendall's τ	0.10		-0.31		
p	0.62		0.12		

[a], Mann-whitney test

3.7. The level of DNA methylation by endometrial pathology

3.7.1. Association between global DNA methylation and endometrial polyps

Table 4.25 shows global DNA methylation levels according to BPA concentration by endometrial pathology. Pathology of endometrial carcinoma is classified into 4 grades; 1, polyp, 2, hyperplasia, 3, hyperplasia with atypia, 4, cancer. The endometrium of subjects had only polyps, the others were normal. The geometric mean of 5-mc level in polyp was significantly lower than that in normal.

Table 4.25 Geometric means of the 5-mc (%) according to urinary BPA concentration in normal of endometrial pathology

	Normal		Polyp		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	27	7.0 (3.6, 13.4)	9	2.1 (0.5, 7.8)	0.049
BPA (μg/g cr)					
< 37.89	13	7.3 (2.7, 19.8)	4	1.0 (0.6, 1.5)	0.06
37.89 ≤	14	6.6 (2.4, 18.2)	6	3.6 (0.5, 29.2)	0.43
Kendall's τ	-0.01		0.07		
p	0.96		0.81		

[a], Mann-whitney test

3.7.2. Association between repeat elements methylation and endometrial polyps

Table 4.26 and 4.27 shows LINE-1 and Sat2 methylation levels according to BPA concentration by endometrial pathology. There were no significant differences by endometrium pathology. The methylation level of LINE-1 and Sat2 were similar between endometrial pathology, normal and polyp.

Table 4.26 Means of the LINE-1 methylation (%) according to urinary BPA concentration by endometrium pathology

	Normal		Polyp		
	N	Mean ± SE	N	Mean ± SE	p-value [a]
Total	31	64.9 ± 2.3	9	63.7 ± 3.3	0.79
BPA (μg/g cr)					
< 37.89	14	64.3 ± 3.2	4	63.7 ± 6.5	0.79
37.89 ≤	17	65.4 ± 3.4	5	67.8 ± 4.6	0.94
Kendall's τ	0.07		-0.15		
p	0.63		0.65		

[a], Mann-whitney test

Table 4.27 Geometric mean of the Sat2 methylation (%) according to urinary BPA concentration by endometrium pathology

	Normal		Polyp		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	31	60.9 (54.3, 68.4)	9	61.5 (37.3, 101.4)	0.59
BPA (μg/g cr)					
< 37.89	14	55.1 (46.7, 64.9)	4	83.3 (43.0, 161.6)	0.08
37.89 ≤	17	66.3 (56.2, 78.1)	5	44.9 (22.4, 89.7)	0.14
Kendall's τ	0.24		-0.05		
p	0.11		0.88		

[a], Mann-whitney test

3.7.3. Association between HOXA 10 methylation and endometrial polyps

Table 4.28 shows HOXA 10 methylation levels according to BPA concentration by endometrial pathology. Each groups of BPA level, polyp showed slightly higher HOXA 10 methylation level than normal, but those were not significant. HOXA 10 methylation level was higher in polyp than in normal, and it showed significant difference ($p < 0.1$). For adjusting other factors, such as age, smoking status, endometrial phase, and the diseases, multiple linear regression was performed to assess the relation between endometrial pathology and HOXA 10 methylation. Endometrial pathology was significant associated with HOXA 10 methylation ($\beta=0.62$, $p < 0.01$, data not shown).

Table 4. 28 Geometric means of the HOXA 10 methylation according to urinary BPA concentration in normal of endometrial pathology

	Normal		Polyp		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	27	16.9 (14.8, 19.3)	9	28.9 (14.5, 57.6)	0.099
BPA (μg/g cr)					
< 37.89	13	15.4 (12.2, 19.4)	4	32.7 (7.9, 134.8)	0.12
37.89 ≤	14	18.2 (15.4, 21.5)	6	20.8 (7.0, 62.1)	0.94
Kendall's τ	0.26		-0.05		
p	0.09		0.88		

[a], Mann-whitney test

3.7.4. Association between PR methylation and endometrial polyps

Table 4.29 shows PR methylation levels according to BPA concentration by endometrial pathology. PR methylation level was slightly higher in polyp than in normal, but it was not significant. PR methylation was decreased according to BPA levels in each endometrial pathology, but it was not significant.

Table 4. 29 Geometric means of the PR methylation according to urinary BPA concentration in normal of endometrial pathology

	Normal		Polyp		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	27	2.5 (1.4, 4.3)	9	3.5 (1.1, 11.1)	0.62
BPA (μg/g cr)					
< 37.89	13	3.0 (1.4, 6.3)	4	7.4 (2.7, 20.3)	0.13
37.89 ≤	14	2.1 (0.9, 5.0)	6	2.0 (0.3, 12.2)	0.59
Kendall's τ	-0.03		-0.44		
p	0.83		0.18		

[a], Mann-whitney test

3.7.5. Association between RASSF1A methylation and endometrial polyps

Table 4.30 shows RASSF1A methylation levels according to BPA concentration by endometrial pathology. RASSF1A methylation levels were not different according to BPA level. Polyp showed slightly higher of RASSF1A methylation level than normal, especially under 37.89 μg/g of BPA level.

Table 4. 30 Geometric means of the RASSF1A methylation according to urinary BPA concentration in normal of endometrial pathology

	Normal		Polyp		
	N	GM (95% CI)	N	GM (95% CI)	p-value [a]
Total	27	1.2 (0.9, 1.6)	9	2.5 (0.8, 8.0)	0.18
BPA (μg/g cr)					
< 37.89	13	1.2 (0.7, 2.0)	4	6.1 (0.9, 42.5)	0.03
37.89 ≤	14	1.2 (0.8, 1.8)	6	0.5 (0, 5.1)	0.30
Kendall's τ	0.01		-0.34		
p	0.93		0.30		

[a], Mann-whitney test

4. Discussion

In this chapter, DNA methylation levels were assessed using endometrial tissue DNAs from women with endometrial disorders; adenomyosis, endometriosis, and myoma. There were no differences of urinary BPA concentrations among the disease groups. Global DNA methylation level was not different among the disease groups, and it was not associated with urinary BPA level. While LINE-1 methylation was not associated with endometrial disorders, Sat2 methylation level was significantly higher in adenomyosis than that in myoma. And Sat2 methylation level was significantly different between myoma and adenomyosis in < 37.89 μg/g of BPA. Promoter of HOXA 10, PR, and RASSF1A methylation were not associated with endometrial disorders or BPA levels. But global DNA methylation was associated with endometrium phase. Endometrial polyp showed significantly global DNA hypomethylation. In addition, endometrial polyp showed hypermethylation of HOXA 10 compared to normal, and RASSF1A methylation level was higher in endometrial polyp at < 37.89 μg/g of BPA.

BPA is one of the EDCs. BPA is extensively used as food-storage containers, baby bottles, bottle tops, water pipes, and medical equipment. The extensive use of BPA can increase its potential exposure to human. Several *in vivo* studies have demonstrated the prenatal BPA exposure had adverse endocrine disruptive effects on the developing female reproductive tract, as indicated by altered uterine morphology (Schonfelder et al. 2004), and the presence of endometriosis-like structure in the adipose tissue surrounding the genital tracts (Signorile et al. 2010). The risk of environmentally relevant doses of BPA on the human endocrine system is of increasing concern. There were lots of studies about monitoring of concentration of BPA in human. BPA is rapidly metabolized via glucuronidation and entirely excreted in urine. BPA is a non-persistent chemical

with a biological half-life < 6 h, with nearly complete urinary elimination in 24 h (Volkel et al. 2002). Therefore, urinary BPA level are reflective of recent BPA exposure, and most of studies were assessed BPA from single spot urine. In this study, the concentrations of BPA in single spot urine were investigated. However, changes in dietary patterns and exposure to other sources likely contribute to the high within-person variability of urinary BPA measures (Reeves et al. 2014). In this study, single spot urine samples were collected from participants at the time of the surgery. The subjects had to fast for 24 h before surgery, so the variability of urinary BPA caused by dietary could be ignored. Furthermore, urine samples were stored in polypropylene tubing at -80 °C until LC/MS-MS analysis. This should negate contamination during storage. There are several studies that monitoring of urinary BPA in Korean population was evaluated using various detection methods. Kim et al. (2014) investigated for 1852 Korean adults using GC-MS, and then the GM of BPA was 2.2 μg/g cr. Also the GM of urinary BPA was 1.79 μg/g cr in 1870 Korean adults using GC/MS (Kim et al. 2011). Yang et al. monitored urinary BPA in Koreans two times. They showed the GM of BPA was 8.91 μg/g cr in 73 Korean (Yang et al. 2003), and BPA was detected in urine samples from 172 Korean with a median BPA of 7.86 ng/ml (Yang et al. 2006). In this study, the GM of urinary BPA was 34.12 μg/g cr in total subjects. This GM value in women with gynecological disorder is higher than that in normal population assessed in other studies, and it is similar with BPA exposed workers' urinary BPA levels. Li et al. (2011) evaluated urinary BPA level in male workers with BPA exposure in the workplace, and the median value was 38.7 μg/g cr. However, Battal et al. (2014) showed the range of urinary BPA level from Turkish population, the range of urinary BPA was 0.24 - 615 μg/g cr. The range of urinary BPA in this subjects were 5.57 - 130.46 μg/g cr, the maximum value was lower than that of study of Battal et al. Although this study's subjects fasted for 24 h before collecting the urine, the reason why the GM of urinary BPA was as high as workers handled BPA is not exactly understood. Some reasons can be speculated about the high level of BPA in the subjects. First, all

subjects in this study had various diseases unavoidably, thus urinary BPA levels of the subjects may higher than the levels of previous studies. Kandaraki et al. (2011) evaluated serum BPA concentration in PCOS. BPA levels were significantly higher in the PCOS group compared with control group consisted of normal people. BPA concentration may higher in people with some diseases than normal people. In fact, urinary BPA levels from 4 normal women were assessed, and then the GM was 0.10 ng/ml (data not shown). Secondly, the urine samples might be exposed to medical equipment containing BPA. During the subjects had a hysterectomy, the urine samples were collected using catheters. BPA is used in medical equipment, so catheters may be composed of BPA. It should be assessed BPA level of urine passed the catheters. The migration levels of BPA from catheters were assessed by GC/MSD (Gas chromatography/mass selective detector). The catheters with 100 ml of urine placed into a waterbath at 37 ˚C for 4 h, and the urine was frozen at -80 ˚C. While the level of BPA in control urine was 0.02 ng/ml, the level of BPA in urine leached from the catheters was 14.27 ng/ml. This result indicated that BPA can be leached from the catheters about 14 ng/ml. If this value is subtracted from the each urinary BPA level of the subjects, the results will be minus values about 20 % of the urinary BPA concentration of total subjects, and the highest level of the urinary BPA will remain over than that of the other general population. Therefore, it is possible to conclude that catheters may not the reason of why the urinary BPA level in this study was higher than that of the other studies.

A previous study performed *in vivo* suggested that prenatal exposure to low-dose BPA (< 50 mg/kg bw/day) leads to obesity and elevated lipid levels (Carwile et al. 2011). Epidemiologic studies were also found that BPA is associated with abdominal obesity (Wang et al. 2011; Shankar et al. 2012). Although the precise mechanism about the relationship between obesity and BPA is unknown, there are several proposed pathways. Exposure of low-dose BPA in adipocytes and human adipose tissue explants trigger insulin resistance and tissue inflammation by

the inhibition of adiponectin release (Carwile and Michels, 2011). However, the current data showed that the relationship between BMI and BPA level was inversed after adjusting variables, such as age and smoking status (β= -0.14, p=0.09). Waist circumference also had negative association, but it was not significant (β= -0.01, p=0.79, data not shown). Shankar et al. (2012) assessed the association between BPA level and BMI in obese subjects (BMI ≥ 30 kg/m^2). In the present study, there are 10 subjects over 25 kg/m^2 of BMI; obese was defined as having a BMI of 25 kg/m^2 or higher according to Korean criteria. There was an inverse relationship between BMI and urinary BPA concentration, but it was not significant (β= -0.12, p=0.07). Meanwhile, abdominal obesity was defined as having a waist circumference of 85 cm or higher according to Korean criteria. Most of the subjects had less than 85 cm of waist circumference, except only 2 women in myoma. The two subjects had 86 and 91.4 cm, and the urinary BPA levels of the subjects were 25.46 and 23.59 μg/g cr, respectively. It is hard to find out the reason why the relationship between BPA concentration and BMI or waist circumference showed an inverse pattern differently from the previous studies. In fact, the subjects in this study have endometrium disorders and the size is too small, so I compared this data with that of large number of normal women. I investigated the relationship between BMI and urinary BPA levels in normal women using data from Korean National Environmental Health Survey (KNEHS) 1 (2009-2011). In the KNEHS 1 (n=3,350), the average age was 47.68 years with 23.77 kg/m^2 (BMI), and the urinary BPA level was 1.47 μg/L. The mean of age was similar between these two groups. However, BMI level was significantly lower in the subjects in this study compared with KHNEHS 1 (p=0.03). In addition, urinary BPA level was significantly higher in the subjects in this study compared with KHNEHS 1 (p<0.0001). For these reasons, it is hard to expect the similar result as a positive relationship between BMI and urinary BPA levels in normal people.

BPA has weak estrogenic activities and act as endocrine-disruptors since

they compete with endogenous estrogen binding to receptors, ER1 and ER2 (Singh and Li, 2012). The National Toxicology Program-Center for the Evaluation of Risks to Human Reproduction reviewed the impacts of BPA exposure on human health (Shelby, 2008). However, epidemiologic data are limited, especially with regard to endocrine-related diseases in women of reproductive age (Upson et al. 2014). Among the endocrine-related disorders, the possibility was come up for endometriosis affected by BPA exposure. Previous epidemiologic studies examined the relation between BPA exposure and endometriosis, but only few studies were performed. Louis et al. (2013) assessed urinary BPA level from 127 women. They indicated the association with BPA level and endometriosis, based on 14 women who were diagnosed with endometriosis within a screened population cohort of 127 women. In a second study, Cobellis et al. (2009) showed a positive association between serum BPA level and endometriosis. But Itoh et al. (2007) evaluated urinary BPA concentration from 140 women with endometriosis. There was no significant association of endometriosis with urinary BPA levels. Like these studies, the association between BPA level and endometriosis was not established and controversial issue. The current study could not find the differences about urinary BPA levels among the disease groups. The reasons can be speculated. First, urinary BPA levels from 44 women with endometriosis, adenomyosis, or myoma were evaluated in this study. Controls consisted of only 16 women with myoma, and cases were 28 women. And again, cases were divided into 2 groups; adenomyosis and endometriosis. Thus it was too hard to find out the relationships between urinary BPA level and endometrial disorders using limited size of subjects. Therefore, further studies will be needed to explore the relationship between endometrial disorders and urinary BPA level using bigger sample size. Secondly, the GM of urinary BPA level was 27.53 ng/ml in this study. This GM value can convert to 0.1 μM as a molar concentration using molecular weight of BPA (228.29 g/mol). Although the results in chapter 2 were clear and obvious, it was hard to find the relationships between urinary BPA and endometrial disorders, or DNA methylation

in chapter 4. Because the concentration of BPA from women was about 100-fold lower than the level used in chapter 2, the effects of BPA might weak on DNA methylation. Also there are many repair mechanisms about exposure to environmental contaminants in human. Thus the relations between BPA exposure and DNA methylation could not be found. Third, Exposed BPA in human body is rapidly and completely excreted in 24 h, urine is considered the most appropriate for assessment of BPA exposure (Upson et al. 2014; Iain et al. 2008). This quickly removed BPA reflects recent exposure than cumulative or long term exposure (Tsukioka et al. 2004; Volkel et al. 2002; Itoh et al. 2007). The chronic over recent exposure may explain the lack of association between BPA levels and endometrial disorders in this study (Itoh et al. 2007). Future study should be performed with the measurement of cumulative exposure to BPA.

Many studies found that BPA exposure can change on epigenetic status *in vitro* or *in vivo*. Endometriosis and adenomyosis are also referred as an epigenetic disease. Therefore it is plausible to speculate about the epigenetic modification of endometriosis or adenomyosis caused by BPA exposure.

Investigation of global DNA methylation can possible to understand mechanisms of early event of disease. Although the change of global DNA methylation level may not specific for BPA exposure, global DNA hypomethylation was associated with arsenic, cadmium, and polycyclic aromatic hydrocarbons (PAH). Global DNA hypomethylation results in chromosomal instability and increased mutation events (Guerrero-Preston et al. 2010). Therefore, it is needed to assess global DNA methylation level whether BPA affects global DNA methylation status. In this study, global DNA methylation level was measured by DNA methylation quantification kits, and the methylation levels were not significantly different among three disease groups. Also the associations with urinary BPA level and global DNA methylation were not observed. On the other hand, repeat elements, such as LINE-1 and Sat2, consist of interspersed repeats and tandem repeats, and

comprise approximately 45 % of the human genome and that they are normally highly methylated (Lander et al. 2001; Jordan et al. 2003). Thus repeat elements methylation have used as a surrogate marker for global DNA methylation levels to investigate the epigenetic changes induced by asbestos, cadmium, lead, pesticide, and benzene (Hou et al. 2011). Aberrant repeat element methylation is another common epigenetic change that evolves during carcinogenic process (Chalitchagorn et al. 2004; Estecio et al. 2007). It has been suggested that hypomethylation of the repeat elements may play multiple roles in the subsequent tumor progression (Kazazian and Goodier, 2002; Choi et al. 2009). DNA hypomethylation is known to contribute to carcinogenesis by inducing genomic instability and activating proto-oncogenes (Robertson, 2002; Weidman et al. 2007). DNA hypermethylation may be linked to structural changes in chromatin, decreased transposon movement and decreased gene expression (Robertson, 2005; Slotkin and Martienssen, 2007; Yauk et al. 2008). Especially, LINE-1 hypermethylation results DNA packaging in heterochromatin, appears to delay DNA strand break processing, leading to slow down repair (Rube et al. 2011). In this study, LINE-1 and Sat2 methylation was assessed by MethyLight assay. There were no significant differences about LINE-1 methylation among the disease groups. Sat2 methylation levels were lower in myoma than in adenomyosis or endometriosis. Meanwhile, in this study, LINE-1 methylation level was not significantly associated with urinary BPA level. Sat2 methylation level also showed non-significant association with urinary BPA level. This results showed that global DNA methylation is not adequate marker for explored BPA caused epigenetic modification in endometrial disorders. There were few studies about the relationship between BPA and global DNA methylation, but hypomethylation of LINE-1 according to BPA level was found in spermatozoa (Miao et al. 2013). The cases composed with male workers from epoxy resin manufacturers, the controls were recruited from a variety of industries without BPA exposure in the same area. The mean of urinary BPA level was significantly different between cases and controls (Case, 36.23 μg/g cr; Control, 1.38 μg/g cr).

Sperm LINE-1 methylation level was significantly lower in BPA exposed workers compared to controls. And there was a significant inverse association between sperm LINE-1 methylation and urine BPA levels.

Specific DNA methylation is one of the earliest detectable changes and precedes tumorigenesis. Also DNA methylation may provide prognostic information (Hesson et al. 2007). Promoter methylation status of the eight human genes [HOXA10, PR, RASSF1A, p16, MLH1, ER 1, ER 2, and GSTP1] was assessed by using MethyLight assay. The genes were chosen because these are involved in cellular responses to BPA or endometriosis and changes in their expression have been linked to cancer (Widschwendter et al. 2009; Wu et al. 2006; Liu et al. 2002; Fang et al. 2005; Gras et al. 2001; Ertunc et al. 2005; Xue et al. 2007). But p16, MLH1, ER1, ER2, and GSTP1 methylation changes were not observed, because the methylation levels of these genes were relatively low. Further studies are needed to analyze the effects of BPA on promoter methylation of these genes by using other assays.

As previously mentioned, BPA can act like estrogen through ER, so it is needed to investigate the effects of BPA at where lots of ER expressed tissues, especially endometrium. HOXA 10 encodes a transcriptional factor significant for successful implantation and expressed in endometrium throughout the menstrual cycle in human (Andersson et al. 2014). Defective HOXA 10 expression has been demonstrated in conditions associated with endometriosis and adenomyosis (Taylor et al. 1999; Fischer et al. 2011). HOXA 10 (-/-) mice are infertile due to the defect in uterine decidualization leading to failure of implantation (Bagot et al. 2000; Smith and Taylor, 2007). Decidualization defects may be the mediated by the altered HOXA 10 expression, either decreased or persistently elevated HOXA 10 expression can impair endometrial function (Smith and Taylor, 2007). Epigenetics defines meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself (Egger et al. 2004). DNA methylation is a

possible mechanism for altered gene expression (Andersson et al. 2014). Therefore, it is needed to assess the effects of BPA on HOXA 10 methylation in human endometrium. Wu et al. (2005) evaluated endometriosis patients had higher methylated promoter of HOXA 10 than normal people. HOXA 10 mRNA and protein expression were markedly lower in endometrium of endometriosis patients (Lu et al. 2013). And they found HOXA 10 mRNA and protein expression were significantly increased after treated with 5-azacytidine. These results mean that HOXA 10 was hypermethylated in endometrium of endometriosis. Lee et al. (2009) found hypermethylation of HOXA 10 in the endometriosis induced mouse. Unfortunately, in this study, HOXA 10 methylation was not significantly different among the disease groups. And urinary BPA level was not associated with HOXA 10 methylation. However, Bromer et al. (2010) found that BPA modified HOXA 10 methylation status in uterus of mouse.

Progesterone has important role for maintenance of pregnancy and treatment of endometriosis. Changes in PR expression caused an inadequate response to progesterone, so it can lead to infertility, pregnancy loss, or endometrial hyperplasia (Aldad et al. 2011). BPA exposure also was associated with pregnancy loss, or endometrial disorders. Also BPA can act as estrogen mimics, and estrogen induced PR expression. Thus it is possible to hypothesize that BPA can modify PR expression. Aldad et al. (2011) found the increased of PR expression treated with BPA in uteri of monkeys and ishikawa cell lines. Funabashi et al. (2004) also showed BPA increased the expression of PR mRNA *in vivo* study. In this study, the methylation level of PR was decreased with BPA level, but it was not significant. And endometriosis showed hypermethylation of PR compared with adenomyosis or myoma, but it was not significant. However, Wu et al. (2006) found hypermethylated PR in endometriosis compared with control, and the expression levels of PR-B are significantly lower in ectopic endometrium with endometriosis. They suggested that the progesterone resistance in endometriosis may result from

the hypermethylation of PR. Igarashi et al. (2005) also found BPA reduced expression of PR-B in the endometrium of women with endometriosis compared to normal women.

Ras-association domain family member 1 (RASSF1) gene is comprised of eight exons, and the major isoform is RASSF1A. RASSF1A can induce cell cycle arrest and inhibiting accumulation of cyclin D1, thus preventing G1/S-phase cell cycle progression (Shivakumar et al. 2002). Inactivation of RASSF1A by methylation is one of the most frequently hypermethylated genes in human cancer (Pijnenborg et al. 2007). There were no studies about assess the effects of BPA on RASSF1A methylation or expression. In this study, RASSF1A methylation level was not different according to BPA level, but there was a mild positive association between BPA level and RASSF1A methylation in endometriosis group (β=0.56, p=0.06). Pallarés et al. (2008) found RASSF1A was hypermethylated of promoter and reduced of gene expression in endometrial carcinoma.

Human endometrium undergoes cyclic morphological changes involving precise periods of growth, differentiation and regression, with each new menstrual cycle during reproductive ages (Curry and Osteen, 2003; Munro et al. 2010). The proliferative and the secretory phase of the endometrium are controlled by steroid hormones such as estrogen and progesterone (Curry and Osteen, 2003). During proliferative phase, serum estrogen levels are rising to stimulate endometrial cell growth. During secretory phase, serum progesterone levels are rising. Munro et al. (2010) proposed the endometrium may be subject to epigenetic regulation throughout the menstrual cycle. In this study, secretory phase showed significantly lower global DNA methylation than proliferative phase after adjusting with variables. The result in this study is similar with previous studies. Ghabreau et al. (2004) found a significant difference of the global DNA methylation level between proliferative and secretory phases of the endometrium. There was a strong decline of DNA methylation during the secretory phase. Yamagata et al. (2009) also found

DNMT mRNAs declined in the human endometrium during the secretory phase. Therefore epigenetic regulation is involved in the normal cyclic changes of the human endometrium.

On the other hand, endometrial tissues will grow too much, then creating an endometrial polyp. Polyps are attached to the wall of the uterus by a stalk and grow outward into the endometrial cavity. The presence of endometrial polyp has been described as a risk factor for future endometrial cancer (Ben-Arie et al. 2004; Torres et al. 2012). Endometrial polyp showed a significantly association with global DNA methylation. Also there was an association between HOXA 10 methylation and endometrial polyp in this study ($p < 0.1$). After adjusting variables, such as age, smoking status, endometrial phase, and the diseases, HOXA 10 methylation was significantly associated with endometrial polyp ($p < 0.01$, data not shown). Since the tissue DNA was extracted from eutopic endometrial tissue of the subjects in this study, global and HOXA 10 methylation may be more influenced by the pathology of endometrium. Previous study discovered the relationship between endometrial polyp and HOXA 10 expression (Rackow et al. 2011). Endometrial HOXA 10 mRNA expression was significantly decreased in uterine cavity with polyps, compared with controls. Global DNA methylation was significantly decreased in endometrial polyp compared to normal. However, the significant relationship was disappeared after adjusting other variables (data not shown). It is needed to further study using bigger sample size.

The present study has some limitations. First, the size of samples were very small. The subjects were composed to 44 women with various gynecological disorder in this study. There were some troubles to calculate the power of association, because of the limited number of subjects we evaluated. Second, this study classified the subjects into three groups; women with endometriosis, adenomyosis, or myoma. Adenomyosis occurs by endometrial cells, likely endometriosis. Myoma is a benign growth of smooth muscle in the wall of uterus.

Because these diseases are estrogen-dependent, thus these may be closely connected among each other and frequently occur at the same time. Therefore, there might be sampling bias, which may lead toward the null result of no association. Further study is necessary to clarify the effects of endometrial disorders. To do that, control will be composed women without any gynecologic disorders. And the third limitation is caused by the half-life of BPA. BPA is a non-persistent chemical with a urinary elimination half-life < 6 h. Because this subjects had been fasting at least 24 h, the urinary BPA level could not exactly reflect usual BPA exposures. Also, this study measured BPA level using a single spot urine sample collected after the onset of disease. Therefore, it is possible that urinary BPA levels didn't accurately represent participants' levels during the etiologically relevant time period for the development of gynecologic disorders. Fourth, human can exposure to not only BPA but multiple EDCs at the same time. Since EDCs can interaction among themselves in body, it can result in unexpected responses such as cross-talk or inhibition (Safe and Wormke, 2003, Yang et al. 2006). However, this study investigated the effects of only one kind of EDCs, BPA, so the relationships of BPA and health effects may be weak. Thus, effects of other EDC exposures should be studied further.

In conclusion, urinary BPA level was not associated with endometrial disorders or DNA methylation. Also, the relationships between DNA methylation and endometrial disorders were not found. However, global DNA methylation was significantly associated with endometrial phases. And HOXA 10 methylation level may be used as a potential biomarker for endometrial polyps.

Chapter 5.
Summary and conclusion

Human populations can simultaneously exposed to BPA (Calafat et al. 2005). The effects of BPA on human health have become of growing global concern and it became necessary to access for the toxicity of BPA. There were many studies about assess the relationship between BPA exposure and diseases. But there were few studies assessed the relation between BPA exposure and endometrial disorders, such as endometriosis, adenomyosis, or endometrial cancer.

In this thesis, three studies were performed to evaluate the effects of BPA on endometrium. First, the toxicity of BPA on bovine endometrial cells was assessed. BPA significantly reduced cell viability at 3 h. Apoptotic cells were significantly induced after 24 h treatment of 100 μM BPA. And BPA significantly induced intracellular ROS at 1 h and DNA damage at 3 h. On the other hand, global DNA methylation was decreased by BPA exposure for 3 or 24h. The methylation of HOXA 10 and RASSF1A levels were assessed. HOXA 10 methylation level was decreased according to BPA, RASSF1A methylation level was increased after 24 h treatment of 100 μM BPA. BPA can induced DNA methylation changes, along with cytotoxicity and DNA damage.

Secondly, to overcome the interspecies difference, the changes of DNA methylation induced by BPA exposure in human endometrial cells were analyzed. LINE-1 methylation level was increased, but Sat2 methylation level was slightly decreased by 100 μM BPA. HOXA 10 methylation level was decreased, and PR methylation level was different by treatment time. These experiments were performed using human endometrial cells of only two person, thus it is not possible to find significant differences about the effects of BPA. But the results of human endometrial cells were similar with bovine endometrial cells.

Third, urinary BPA levels from women with endometriosis, adenomyosis, or myoma were examined, and the relationship between urinary BPA concentration and endometrial disorders was analyzed. But there were no significant differences.

The global DNA methylation or specific DNA methylation level was assessed, but there were no differences among the disease groups. However, global DNA methylation level was significantly different by endometrial phase. HOXA 10 or global methylation level was significantly associated with endometrial polyp. Evaluation of DNA methylation was not adequate for BPA exposure, but HOXA 10 methylation may be a potential biomarker for endometrial polyp.

In previous studies, alteration of DNA methylation status induced by alteration of DNMTs, can change in gene expression levels. This study found that BPA can induce ROS, DNA damage, and apoptosis. Although the results in chapter 4 were not significant about the association between BPA levels and epigenetic changes, BPA changed DNA methylation status in chapter 2. The scheme of the effects of BPA on the pathogenesis of endometrium disorders is summarized in Figure 5.

In conclusion, bisphenol A can induce cytotoxicity and modify DNA methylation in bovine endometrial cells. Urinary bisphenol A concentration was not associated with endometrial disorders and DNA methylation level. However, this study found global DNA methylation was significantly different according to endometrial phase. Also, HOXA 10 methylation status may be used as a potential biomarker for endometrial polyp.

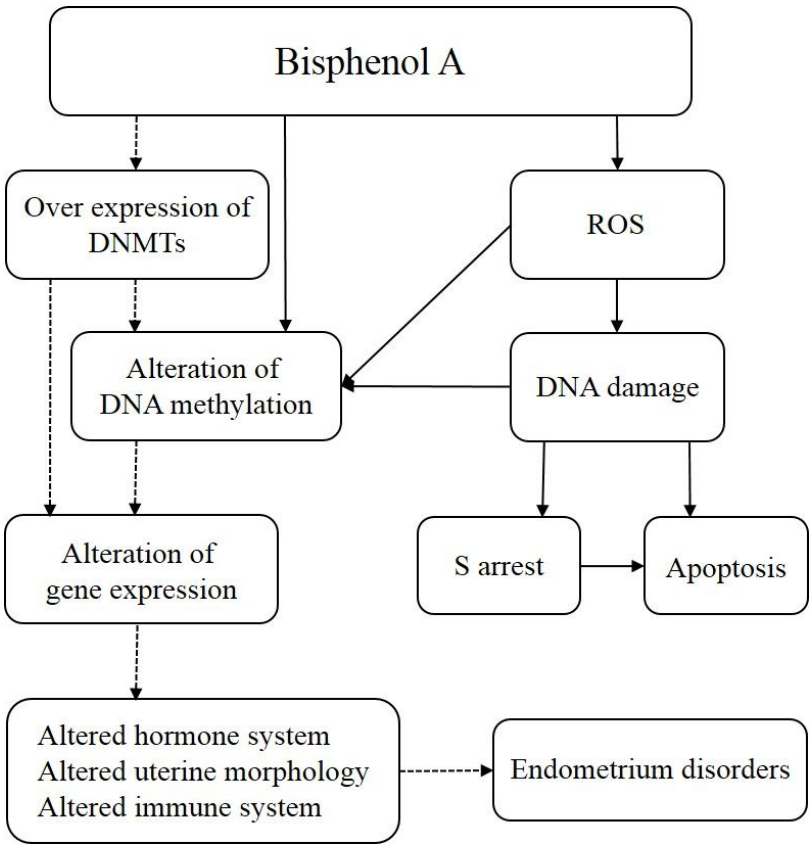


Figure 5 The interactions contributing to the pathogenesis of endometrium disorders. Solid arrows have been explored in this study, and dashed arrows represent established concepts (modified from Rosser, 2011).

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국문초록 (Abstract in Korean)

비스페놀 A와 자궁내막질환의 연관성에서 DNA 메틸화 변이의 역할

비스페놀 A는 내분비계 교란물질 중 하나로, 젖병, 플라스틱 식기, 캔 제품 내면의 부식을 방지하기 위해 사용되는 에폭시 수지의 원료 화학물질로 사용되고 있고, 치과 치료에 사용되는 등 여러 분야에서 대량으로 사용되고 있다. 비스페놀 A는 에스트로겐 수용체에 결합하여 에스트로겐과 비슷한 역할을 하는 것으로 알려져 있다. 또한 비스페놀 A는 후성유전학적 변화를 유발하는 물질로 보고되었다. 에스트로겐 수용체가 많이 분포하고 있는 자궁은 비스페놀 A의 표적 조직이 될 수 있으며, 많은 연구들이 자궁 관련 질환과 비스페놀 A의 관련성을 연구하였다. 하지만 비스페놀 A가 자궁내막에 미치는 세포독성 및 후성유전학적 변이를 평가한 연구는 드물고 연구 결과가 일관적이지 않다. 따라서 비스페놀 A와 자궁내막관련 질환과의 연관성을 파악해 볼 필요성이 있다.

본 연구에서는 소의 정상자궁내막세포를 이용하여 비스페놀 A가 자궁내막세포에 미치는 세포독성과 DNA 메틸화 변화를 평가하였다. 또한 자궁내막관련 질환 환자들의 소변 내 비스페놀 A 농도를 측정해서 자궁내막질환과 비스페놀 A의 연관성을 파악해보고, 자궁내막조직 DNA를 이용하여 후성유전학적 변화와 비스페놀 A의 연관성을 연구하였다.

비스페놀 A 100 μM 을 3시간 처리하였을 때, 세포생존율이 유의하게 감소하였다. 또한 100 μM 의 비스페놀 A를 24 시간 처리하였을 때 세포사멸이 유의하게 증가하였다. 비스페놀 A 를 3시간 처리하였을 때는 약한 S기 정지가 관찰되었고, 24시간 후에는 G2/M기 정지가 관찰되었다. 세포 내 활성산소종은 비스페놀 A 처리 1시간 후 유의하게 증가하였고, 처리 3시간 후에 DNA 손상 정도가 유의하게 증가하였다. 비스페놀 처리 3시간 후부터 글로벌 DNA 메틸화 정도가 감소하였고, HOXA 10 메틸화 역시 감소하였다. 한편, RASSF1A 유전자의 메틸화 수준은 100 μM 비스페놀 A 처리 24시간 후 증가하였다.

자궁내막증, 자궁근종, 자궁선근증 환자들의 자궁내막조직 DNA를 이용하여 소변 내 비스페놀 A 농도와 DNA 메틸화 수준, 질환과 DNA 메틸화의 관계를 파악하였다. 소변 내 비스페놀 A 농도는 질환 간에 유의한 차이가 없었고, DNA 메틸화 수준과 연관성도 찾지 못하였다. 하지만

자궁내막 증식기의 글로벌 DNA 메틸화 수준이 자궁내막 분비기의 글로벌 DNA 메틸화 보다 유의하게 높았고, HOXA 10 유전자는 자궁내막 용종에서 메틸화 정도가 유의하게 높았다.

이와 같이, 소의 정상자궁내막세포에서 비스페놀 A가 세포독성을 유발하고 DNA 메틸화 변화를 유도하는 것을 확인하였지만, 역학연구에서는 비스페놀 A와 자궁내막관련 질환, 또는 DNA 메틸화와의 관계를 찾을 수 없었다. 한편 자궁내막 주기에 따라 글로벌 DNA 메틸화가 유의하게 변화하는 것을 확인하였다. 또한 자궁내막 용종에서 HOXA 10 유전자의 과메틸화를 관찰함으로써, 자궁내막용종에 대한 생물학적 지표로서 유용하게 활용될 수 있을 것으로 기대된다.

표제어: 비스페놀 A, 자궁내막, 자궁내막증, 자궁근종, 자궁선근증, 자궁내막용종, DNA 메틸화, HOXA 10

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