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**Global DNA Hypomethylation and *Rassf1a* and *c-myc* Promoter
Hypermethylation in Rat Kidney Cells after Bisphenol A Exposure**

**Bisfenol A Maruziyeti Sonrası Sıçan Böbrek Hücrelerinde Global DNA
Hipometilasyonu ve *Rassf1a* ve *c-myc* Promotör Hipermetilasyonu**

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Short Title: Effects of bisphenol A on DNA methylation in kidney cells

Kısa Başlık: Böbrek hücrelerinde bisfenol A'nın DNA metilasyonu üzerine etkileri

ABSTRACT

Objectives: Bisphenol A (BPA), as a synthetic monomer used in the production of polycarbonate plastic and epoxy resins, has endocrine disruptor properties. The release of BPA from plastic carrier is concerned to exposed to high amounts of BPA which can cause high risk on human and environment health.

Materials and Methods: The study was aimed to investigate the effects of BPA on global DNA methylation, CpG promoter DNA methylation and gene expressions of *Rassf1a* and *c-myc* in rat kidney epithelial cells (NRK-52E).

Results: IC₅₀ value of BPA was determined as 105 μ M and 88 μ M in NRK-52E cells by MTT and NRU test, respectively. For DNA methylation analysis, the cells were treated with BPA at 1 nM, 10 nM, 100 nM, 1 μ M and 10 μ M concentrations for 24 h and at 100 nM concentration for 24, 48, 72, 96 h and 6 days. Decreased global DNA methylation at the concentration of 100 nM BPA was observed after 48, 72, 96 h and 6 days by using 5-methylcytosine elisa kit. Methylation changes were detected in promoter regions of *Rassf1a* and *c-myc* in BPA-treated NRK-52E cells by using methylation-specific polymerase chain reaction following bisulfite conversion. Expression levels of *Rassf1a* and *c-myc* altered in response to BPA at the high

concentrations after 24 h treatment, whereas 100 nM exposure of BPA was altered gene expressions for 48, 72 and 96 h.

Conclusion: Global and gene-specific methylation changes may play an important role in the mechanism of BPA toxicity in kidney cells.

Key Words: Bisphenol A, DNA methylation, *Rassf1a*, *c-myc*, NRK-52E cells

ÖZ

Amaç: Bisfenol A (BPA), polikarbonat plastik ve epoksi reçinelerinin üretiminde kullanılan sentetik bir monomer olup, endokrin bozucu özelliklere sahiptir. BPA'nın plastik taşıyıcılardan salınması yüksek oranda maruziyete sebep olabilir, bu durum insan ve çevre sağlığı üzerinde yüksek oranda riske sebep olabilir.

Yöntem ve Gereçler: Bu çalışmada, BPA'nın sıçan böbrek epitel hücrelerinde (NRK-52E) global DNA metilasyonu, *Rassf1a* and *c-myc* genlerinin CpG promotör bölge DNA metilasyonu ve gen ekspresyonu üzerindeki etkileri araştırıldı.

Bulgular: BPA'nın IC₅₀ değeri NRK-52E hücrelerinde MTT ve NRU testi ile sırasıyla 105 μ M ve 88 μ M olarak belirlendi. DNA metilasyonu analizleri için, hücreler 1 nM, 10 nM, 100 nM, 1 μ M ve 10 μ M konsantrasyonlarda BPA ile 24 saat boyunca ve 100 nM konsantrasyonda BPA ile 24, 48, 72, 96 saat ve 6 gün boyunca maruz bırakıldı. 48, 72, 96 saat ve 6 gün sonra 100 nM BPA konsantrasyonunda 5-metilsitozin elisa kiti ile global DNA metilasyonunda azalma gözlemlendi. BPA ile maruz bırakılmış NRK-52E hücrelerinde *Rassf1a* ve *c-myc* genlerinin promotör bölgelerinde metilasyonda değişiklikler bisülfid ile modifikasyonun ardından metilasyona özgü polimeraz zincir reaksiyonu analizi ile tespit edildi. *Rassf1a* ve *c-myc* genlerinin ekspresyon seviyeleri yüksek konsantrasyonlarda 24 saat BPA maruziyet ile ve 100 nM konsantrasyonda 48, 72 ve 96 saat BPA maruziyetleri ile değişiklik gösterdi.

Tartışma ve Sonuç: Global ve gene özgü DNA metilasyonundaki değişiklikler böbrek hücrelerinde BPA'nın toksisite mekanizmasında önemli bir rol oynayabilir.

Anahtar Kelimeler: Bisfenol A, DNA metilasyonu, *Rassf1a*, *c-myc*, NRK-52E hücreleri

INTRODUCTION

Bisphenol A (BPA; 4,4'-dihydroxy-2,2-diphenyl propane) is an organic compound with two phenol functional groups. BPA is found in the composition of many products such as medical supplies, inner coating of food and beverage packaging, coating of electrical materials, protective clothing.¹ It was determined that BPA can pass into food and beverages from many materials in very small amounts. Even in normal conditions, BPA is indicated to be released from plastic carriers. Various studies have shown that BPA affects enzyme activity and metabolism in various tissues, causes changes in the number of hormone receptors and in hormone receptor gene activity in target tissues, and deteriorates the endocrine system due to estrogenic and androgenic effects.² It was underlined that BPA may play a role in the etiology of many diseases such as diabetes, obesity, reproductive disorders, cardiovascular disorders, congenital anomalies, chronic respiratory and renal disorders and breast cancer.³

Many studies have been performed for the assessment of the toxicity of BPA, and studies performed on recent years emphasized that epigenetic mechanisms may play a role in the assessment of toxic effects on various systems.^{3,4} Therefore, epigenetic events such as DNA methylation may play an important role in the toxicity of BPA as an endocrine disrupting chemical.^{2,3,5,6} In this study, we aimed to examine the effects of BPA on global and gene specific DNA methylation to investigate the role of epigenetic mechanism in the BPA toxicity in the NRK-52E cells. Results obtained from this study are expected to contribute to provide a better understanding key molecular mechanisms for the toxicity of endocrine disrupting chemicals in the risk assessment process.

MATERIAL AND METHODS

Chemicals

BPA (99% purity) was obtained from Sigma-Aldrich (St Louis, Missouri, USA). A stock solution of BPA was prepared dissolving it in dimethyl sulfoxide (DMSO, Wisent Bioproducts, Saint-JeanBaptiste, QC, Canada) and kept at -20°C. Cell culture media and all other supplements were purchased from Wisent Bioproducts (Saint-

JeanBaptiste, QC, Canada) and sterile plastic materials were purchased from Greiner (Frickenhausen, Germany).

Cell culture and BPA treatment

The rat kidney proximal tubular epithelial cell line (NRK-52E) was obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM-F12), supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U-100 µg/mL) at 37°C in a humidified atmosphere with 5% CO₂. Subculturing was performed when the cells reached 70-80% confluence (every 2-3 days) using trypsinization. Cell counting was performed by Luna automated cell counter (Virginia, USA).

For cytotoxicity assays; cells (1×10^4 in 200 µL medium) were seeded in 96-well plates, then exposed to the BPA in the range of 31.25-1000 µM concentrations and DMSO (1%) as solvent control for 24 h.

For the DNA methylation analysis; cells (1.5×10^6 in 10 mL medium) were seeded in 25 cm² culture flasks, then exposed to BPA at the concentrations of 1 nM, 10 nM, 100 nM, 1 µM and 10 µM and DMSO (1%) as solvent control for 24 h, and BPA at the concentrations of 100 nM and DMSO (1%) as control for 48 h, 72h, 96h and 6 days. For all concentrations, it was tested in triplicates. Treated cells were performed for DNA isolation by 'High Pure PCR Template Preparation Kit' (Roche Applied Science, Mannheim, Germany), and for total RNA isolation by 'High Pure RNA Isolation Kit' (Roche Applied Science, Mannheim, Germany) according to the procedure provided by manufacturer.

Cytotoxicity

After 24 h of incubation with BPA, cytotoxicity tests were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) tests. Briefly, in MTT test, one of 96-well plate was removed from the incubator, and 20 µL of MTT solution was transferred, and mixed gently, then the plate incubated for 3 h at 37 °C, 5% CO₂. After the incubation, 100 µL of DMSO (100%) was added to each well and mixed gently. The resulting purple solution was spectrophotometrically measured at 560 nm with a reference wave length at 670 nm using a microplate spectrophotometer system (Biotek-Epoch, Winooski, USA). The other 96-well plate was removed from the incubator for NRU test, and washed with

150 μ L PBS solution. 100 μ L of neutral red vital dye was added to the cells then, the plate incubated for 3 h at 37 °C, 5% CO₂. The cells washed with PBS then, 100 μ L of neutral red vital dye dissolution was added to each well and mixed gently. Then, optical density of the plate was read at 540 nm using a microplate spectrophotometer system (Biotek-Epoch, Winooski, USA). The cytotoxicity results were calculated as a relative percentage to the control cells and expressed as 50% of inhibitory concentration (IC₅₀) of the compound that caused 50% inhibition of the enzyme activity in the cells. Based on our cytotoxicity results and also previous studies in different cell types⁷⁻¹², in present study we selected 1, 10, 100 nM, 1, 10 μ M concentrations of BPA for 24 h and 100 nM concentration for 24, 48, 72, 96 h and 6 days for the analysis of DNA methylation.

Global DNA methylation

Determination of global 5-methylcytosine (5-mC) levels in DNA was performed by using 5-mC DNA Elisa kit (Zymo Research, Irvine, CA, USA) according to the manual instructions. 100 ng of each DNA (in 2 μ L) was added to a PCR tube and make the final volume to 100 μ L with 5-mC Coating Buffer. DNA denaturated at 98°C for 5 minutes in a thermal cycler. Then transferred immediately to ice for 10 minutes. The denatured DNA was added to the wells, covered with foil, and incubated at 37 °C for 1 h. After the adding the buffers, samples were incubated with antibody mix consisting of anti-5-methylcytosine and secondary antibody. Then, after the washing steps, HRP developer was added to each well. Absorbance was measured at 405 nm using a microplate spectrophotometer system (Biotek-Epoch, Winooski, USA). Methylation ratio of DNA samples were acquired as % (percentage) by using calibration standard curves by preparing mixtures of the negative control (100 ng/ μ L) and positive control (100 ng/ μ L) standards.

Gene-specific DNA methylation

Determination of DNA methylation on CpG islands in promoter regions of *Rassf1a* and *c-myc* genes was performed methylation specific PCR (MSP). In our previous study we described the detailed study protocol.¹³ In MSP, genomic DNA is modified by treatment with sodium bisulfite, which converts all methylated cytosines to urasil, then to thymidine during the subsequent PCR step.^{14,15} Bisulfite DNA modification was performed by using EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manual instruction. Methylated (M) and unmethylated (U) primer pairs are used to amplify each region of interest. Primer sequences were

listed in Table 1. After PCR reaction, MSP products were analyzed by agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light (QuantumST4-Vilber Lourmat, Torcy, France).

Gene expression

The obtained RNA was used for the first-strand cDNA synthesis which was performed by using Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Mannheim, Germany) from 1000 ng of total RNA and the mixture of anchored-oligo (dT) and random hexamer primers according to the manual instructions. Expressions of *Rassf1a* and *c-myc* genes were performed by using real-time quantitative PCR employing Light Cycler 480 Probes Master with RealTime ready Custom Single Assays (Universal ProbeLibrary Probes, Roche Applied Science, Mannheim, Germany) which contain target specific primers for *Rassf1a* and *c-myc* according to our previous study.⁹ Ct of real-time quantitative PCR specific for *Rassf1a* and *c-myc* and the reference gene (β -actin) were determined. The relative expression was evaluated by the comparative Ct method.

Statistical analysis

Global methylation levels and cytotoxicity results were represented as mean $\pm$ standard deviation (SD). Statistical analysis was performed by ANOVA (Tukey's multiple comparison procedure) using "SPSS version 21.0 for Windows" statistical program (IBM Analytics, New York, USA). A p values <0.05 and <0.001 were considered statistically significant.

RESULTS AND DISCUSSION

BPA has been frequently exposed in daily life and has been shown to be harmful in living organisms due to endocrine disruptor properties.^{2,16,17} Various studies have revealed that BPA affects enzyme activity and metabolism in various tissues that causes change in the number of hormone receptors and hormone receptor gene activity in target tissues, and deteriorates the endocrine system.² Accordingly, BPA caused various health problems such as reproductive and developmental disorders, metabolic dysfunctions, behavioral changes, cardiovascular diseases, inflammation and cancer.^{3,18} It has been revealed that BPA might induce cancer development and various studies have explained its relations with several cancer types.¹⁹ BPA can induce epigenetic changes in early life in human and BPA may affect several cell signaling pathways via epigenetic mechanisms.²⁰ It has been appeared through

recent studies that epigenetic alterations could be useful biomarkers for the toxicity assessment of endocrine disrupting chemicals.^{6-9,21-23} Therefore, in present study, we investigated the effects of dose-dependent BPA exposure for 24 h and time-dependent BPA exposure at the 100 nM concentration of BPA on the alteration of global and gene specific DNA methylation, gene expression in NRK-52E cells. We chose the NRK-52E cells which have the characteristic properties of proximal tubule epithelial cells targeted by nephrotoxic agents to show the epigenetic effects of BPA in kidney cells.

Cell viability was determined by MTT and NRU assay in the BPA treated NRK-52E cells. It was shown in Figure 1 that the relationship between the exposure concentrations of BPA treated NRK-52E cells and % reduction in cell viability. IC₅₀ values of BPA were determined as 105 μ M and 88 μ M in NRK-52E cells by MTT and NRU test, respectively. Decreased global DNA methylation at the concentration of 100 nM BPA was observed in the range of 19.76%-25.30% after 48, 72, 96 h and 6 day (Figure 2). However, there was no change on the global DNA methylation at the concentrations of 1nM-10 μ M BPA for 24 h exposure (data not shown). Accordingly, in our previous studies, we showed decrease on the global levels of 5-mC% at 1 and 10 μ M after 96-h BPA treatment in HepG2 cells⁸ and 0.1 μ M and 1 μ M after 48 and 96 h BPA treatments in MCF-7 cells.⁷ Conversely, in another previous study, we observed no global methylation changes after 48 h exposure to BPA, whereas significant increase were shown on global level of 5-mC% (1.3 fold) after 10 μ M of BPA for 96 h exposure in neuroblastoma (SHSY-5Y) cells.⁹ According to results of this study, it has been shown that BPA caused global hypomethylation in rat liver²⁴, human fetal liver samples²⁵, and porcine oocyte culture at 250 μ M of concentration²⁶.

Rassf-1 is one of the tumor suppressor genes which has a significant role in cancer and it has been thought that its regulation was associated with CpG islands promoter DNA methylation. *c-myc*, proto-oncogene, codes a transcription factor and controls cell proliferation. It has been shown that increases the expression of *c-myc* gene were associated with hypomethylation which could be related with cell proliferation in liver and renal cancers.²⁷⁻²⁹ Increase in promoter methylation were detected in *Rassf-1* and *c-myc* genes at the BPA concentrations of 1 and 10 μ M in NRK-52E cells for 24 h while no methylation was detected in control samples by using MSP method

following bisulfide conversion. In addition, BPA caused increase in promoter methylation of *Rassf-1* and *c-myc* gene at the concentration of 100 nM at time-dependent manner (48 h, 72 h, 96 h, and 6 day) in NRK-52E cells. Representative profile of MSP for the *c-myc* gene using both methylated (M) and unmethylated (U) specific primers in NRK-52E cells treated with BPA has been shown in Figure 3. Expression of *Rassf1a* ve *c-myc* genes was measured by using RealTime 'ready custom single assays' primer probes master by RT-PCR. In response to BPA, expression of *Rassf1a* and *c-myc* was decreased at 1 μ M for 24 h (26.66% and 37.3%, respectively) and was decreased also at 10 μ M for 24 h (25.11% and 22.24%, respectively) (Figure 4). 100 nM exposure of BPA caused decrease on expression of *Rassf1a* and *c-myc* genes after 48, 72 and 96 h BPA treatment with regard to control samples, and it was observed non-significant increase for 6 day BPA treatment (Figure 5). According to the results, decrease on gene expression of *Rassf1a* ve *c-myc* was correlated with DNA methylation results which showed increase on CpG promoter methylations of the genes. In our previous study in HepG2 cells, no change were observed in promoter metylation and gene expression of *Rassf1a* gene after BPA exposure.⁸

CONCLUSION

In line with the findings of this study, BPA has been caused to alteration in expression and methylation on CpG islands in promoter region of the *Rassf1a* and *c-myc* genes associated with cancer related pathways in NRK-52E cells after 24 h – 96 h exposure periods. It has been suggested that epigenetic alterations in *Rassf1a* and *c-myc* genes might be key regulators for the evaluation of BPA toxicity. Further studies, evaluation on the expression of chromatin modifying enzymes and global and gene-specific histone modifications after BPA exposure in cell cultures might provide more detailed information for the assesment of epigenetic mechanisms in BPA induced toxicity.

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Table 1. Primer sets for MSP analysis

Primer set	Forward primer 5' → 3'	Reverse primer 3' → 5'	Annealing Temp. (°C)	Reference
<i>c-myc</i> – M	ttt gtt ttt tcg att tta gag aga c	tta tcc tac gta tat taa tca ccg c	55	13,30
<i>c-myc</i> – U	tgt ttt ttt gat ttt aga gag atg a	ctt atc cta cat ata tta atc acc aca	55	
<i>Rassf-1a</i> – M	ttt ttt tcg gtt ttt ttt cgt c	caa cta ata aat tcg taa cga acg taa	64	31
<i>Rassf-1a</i> – U	tgt ttt ttt tgg ttt ttt ttt gtt	caa cta ata aat tca taa caa aca	59	

Figure Legends

Figure 1. Effects of BPA (0–1000 μ M) on cytotoxicity by MTT and NR in NRK-52E cells after 24 h exposure. Data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed by ANOVA + Dunnett post hoc test. Statistically significant changes are indicated by (* $p < 0.05$; ** $p < 0.001$).

Figure 2. Effects of BPA (100 nM) in the levels of 5-mC% in NRK-52E cells after 24, 48, 72, 96 h and 6 days exposure. Data are presented as mean $\pm$ SD. Genomic DNA was extracted, then 5-mC% levels were detected using the Elisa kit. Statistical analysis was performed by ANOVA + Dunnett post hoc test. Statistically significant changes are indicated by (* $p < 0.05$).

Figure 3. Effects of BPA on methylation status of *c-myc* in NRK-52E cells. A representative sample of NRK-52E cells treated with BPA at the concentrations of 1 nM, 10 nM, 100 nM, 1 μ M and 10 μ M for 24 h and concentration of 100 nM for 24, 48, 72, 96 h and 6 days is shown. Methylation was determined by bisulfite modification of the genomic DNA and MSP using primers for the unmethylated (U) or methylated (M) promoter sequence. Contol 1 (C1) and Control 2 (C2) = DMSO (1%) as control instead of BPA treatment.

Figure 4. Effects of BPA (1 nM, 10 nM, 100 nM, 1 μ M and 10 μ M) on expression of *Rassf1a* and *c-myc* genes by Real-time PCR in NRK-52E cells after 24 h exposure. PCR reaction was done as described in Material and Methods section. The Real-time PCR results were standardized against β -actin, and the relative ratios were calculated (* $p < 0.05$).

Figure 5. Effects of BPA (100 nM) on expression of *Rassf1a* and *c-myc* genes by Real-time PCR in NRK-52E cells after 24, 48, 72, 96 h and 6 days exposure. PCR reaction was done as described in Material and Methods section. The Real-time PCR results were standardized against β -actin, and the relative ratios were calculated (*p < 0.05; **p < 0.001).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5