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Determining Mutagenic Effect of Nonylphenol and Bisphenol A by Using Ames / *Salmonella* / Microsome Test

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Abstract

Environmental pollution has been shown to haveadverse effects on all aquatic and terrestrial organisms including human beings. Chemicals such as nonylphenol (NP) and bisphenol A (BPA) are widely known with their abundance in the environment since they are commonly used as non-ionic additives in pesticides, herbicides, detergents, cosmetics, and plasticwares. It has been reported that these compounds may cause many different health problems including cancer. It is well known that the mutation occurred in DNA is the first step towards to the development of cancer. Therefore, the aim of this study was to determine the mutagenicity and non-mutagenicity of NP and BPA by using Ames /*Salmonella*/ Microsome Test method. Different doses of these chemicals with S9 (metabolic activated) or without S9 (non-metabolic activated) have been investigated in the TA98 and TA100 strains of *Salmonella typhimurium*. Results showed that non-toxic 100 µg/plate, 10 µg/plate, 1 µg/plate, 0.1 µg/plate doses were not mutagenic in the experiments performed in both strains.

Keywords: Ames/*Salmonella*/Microsome Test, Bisphenol A,Nonylphenol, mutagenicity, *Salmonella typhimurium*

INTRODUCTION

House-hold, industrial and agricultural wastes have been shown to have adverse effectson vital activities of aquatic and terrestrial organisms such as reproduction and development.In general, these chemicals are phenolics, alcohols, aldehydes, sterols, pesticides, alkylphenols, hormones and anti-hormonal drugs. Degradation products of these chemicals (which are also called as Xenoestrogens) and themselves, can be also mutagenic, carcinogenic and toxic.The majority of these chemicals are stored by biomass in our environment [1-2].

All living organisms, including humans, are exposed to these chemicals, which are found in our environment, even more or less.For example, it is reported that detergents, herbicides, pesticides, food additives, and cosmetics contain these harmful chemicals which affectlive organisms adversely.It is thought that most of these chemicals have toxic and mutagenic effects [3].

Alkylphenol polyethoxylates (APEs), are considered as estrogenic environmental disrupters [4]. The world's annual productionof APEs is more than 500,000 metric tons, and it has been informed that more than 60% of this accumulated in water masses,including seas, rivers, streams,and lakes. The APEs in water undergo degradation process to give short-side-chain derivatives ofAPEs, such as octylphenol (OP), nonylphenol (NP) and butylphenol (BP) [5].

Measurement of alkylphenols in fish tissues indicate the presence of alkylphenol in rivers of Turkey. Both the literature and laboratory studies indicate that alkylphenols and particularly NP cause bioaccumulation of living tissues. Bioaccumulation is measured as bioconcentration factors. Alkylphenol in tissues are calculated according to the quantity of alkylphenol in water.Bioconcentration factor is reported to vary between 1 and 1000 in the literature.It has been found that NP causes pathological and biochemical abnormalities in fish liver, spermatozoa and gamet physiology. Alsoeffected on the hearing rats adversely [6, 7, 8, 9, 10].

Hung et al.,(2010) tested the hypothesis ofthe regulatory function of EDC's to dendritic cell's which are regulator of human immune system. As a result of the research, the estrogen receptors in mDC (myeloid dendritic cells) have been influenced by NP and OP by means of MKK 3/6-p38 MAPK signal ways, histon modificiations andcytosine responses in T-Cells.NP damages the sperm characteristics by adversely affecting mitochondrial membrane potential, motion kinetics and leading premature acrosome reaction in bovine [11, 12].

Ho et al. (2006), researched whether being exposed to active estrogens (particularly BPA) on low levels increased the prostate cancer risk. As a result of the research, it has been observed that rats exposed to low dosages (equivalent amount of environment) of BPA or estradiol's, have more pre-cancerous lesions and hormonal carcinogenesis which lead to prostate cancer [13].

BPA has been detected in 95%of urine samples and ovarian follicular fluid of adult woman. Few studies have shown that the affect of BPA on the antral follicles which are the main producers of sexual steroid hormones.Therefore, according to the hypothesis of this study and postnatal testis, BPA prevents antral follicle development and steroidogenesis. As a result, it was observed that BPA prevents the growth of the follicle and there is not continuation of cotreatment pregnenolone. In addition, BPA 44 ve 440mm also inhibits the production of progesterone, estradiol, testosterone and androstenedione [2].

Identification of mutagens is particularly important as it may cause cancer and damage in various cells that may cause adverse developments in the upcoming generations [14].

To appraise mutagenic activity, many techniques are used. The initial technique is the Ames test, also known as the *Salmonella*/microsome test. It is widely used in investigating the mutagenic effect of chemicals due to its reliability and time saving bacterial test system, which is cheap and provides results very rapidly [15, 16].The aim

of the present study was to investigate and determine any mutagenic and non- mutagenic effects of BPA and NP using the bacterial reverse mutation assay with *S. typhimurium* only TA98 and TA100 strains with or without S9 mix.

These standard test strains are auxotroph of histidine. However, recovery of each test strain has specific frequency and provides reproducing colonies in non-histidine agar. These colonies are called "spontaneous revertant".In this test,to admit a material as mutagenic it should have 2 times more TA98 and TA100 revertant colony than strain specific spontaneous revertant [17].In this case,test substance is inserted in the living body, metabolits formed as a result of metabolic reactions can be considered to increasethe interaction with DNA to some extent.

MATERIALS and METHODS

Chemicals: S9 from the liver of rats (Sprague Dawley), bacto agar, tetracycline, nutrient broth No.2 (Oxoid), ampicillin, β-nicotinamide-adenine dinucleotide phosphate(β-NADP),glucose-6-phosphate (G6P), mitomycin-C (MMC), and histidine were obtained from Sigma-Aldrich. sodium azide (SA), citric acid monohydrate, sodium hydroxide, potassium chloride, sodium chloride, and dimethyl sulfoxide (DMSO) were purchased from Riedel. 4-Nitro-*o*-phenylenediamine (NPD) and 2-aminofluorene (2AF) were purchased from Fluka. The other chemicals used were obtained from Merck.

Ames Mutagenicity Test: While the TA98 strains were used for determining the frame shift, TA100 was used to determine the base pair exchange. Preparation of the stock *S. typhimurium* TA98 and TA100 strains; the histidine requirement; the presence of rfa and *uvr*B mutations; and the R-factor genetics of these strains were all determined according to the method of Maron and Ames and kept at -80°C [18]. The cytotoxic effects of the different levels of NP and BPA were determined according to the method [19].

TA 98 and TA 100 strains of *S. typhimurium* ancestral strain which were developed through in vitro mutation were used. During the experiment, TA98 and TA100 strains which are LT2 parenteral strain of *S. typhimurium* obtained by *in vitro* mutations. These strains were provided by Department of Biology, Faculty of Science and Literature,Afyon Kocatepe University. TA98 strain used in the detection of mutations in codon shift and TA100 strain were used in the detection of base pair mutations in the transformation of type.

Preparation of experiment without S9: In the experiment that was carried out without metabolic activation, histidine biotin solution of 0.25ml was added to the test tubes containing 2 ml top agar and the tubes were heated to 45°C in waterbath.Later, 0.1 ml test substance and 0.1mlof fresh bacterial culture was added. The tubes were incubated after they were poured into MGA plates that were shaked and heated at 37°C. After incubation,colonies were counted.

In this study, three separate plates were prepared for each dose and two independenttests were carried out. In parallel with the experiments, in order to assess the results spontaneous control, solvent control (DMSO),0.1 µg/µl sodium azide (NaN_3) for TA 100 and 2 µg/µl 4-nitro-*o*-fenilendiamine (NPD) for TA 98 as a positive control were used.

In short,aim of the experiment that auxotrophic strains require for previously growth ofthe histidine amino acid,can be synthesized histidine repetition with used the test substance(prototrophic) is based on the conversion becomes [6].

The Ames Test was performed as a standard plate incorporation assay with *S. typhimurium* strains TA98 and TA100 with or without S9 mix [15].For each tester strain, a specific positive control was always used to test the experimental flaws, if any. Without S9 mix, NPD was used as a positive control for TA98, SA was used for TA100, 2AF were used as positive controls with metabolic activation. We added 500 µl of S9 mix (or 500 µl of phosphate buffer), 100 µl of the test solution for each concentration, and 100 µl of a cell suspension from an overnight culture ((1-2)×10^9 cells/ml) to 2 ml of top agar (kept at 45°C) and vortexed the mixture for 3 s. The entire mixture was spread on the minimal agar plate. The plates were incubated at 37°C for 72 h and then the revertant bacterial colonies on each plate were counted. Positive controls and negative controls (distilled water) were maintained concurrently. In the experiment with S9, with metabolic activation; the test compound, bacterial test strain and S9 mix were added to top agar and then they were poured into MGA plates. Samples were tested on triplicate plates in 2 independent parallel experiments. SPSS17.0 for Windows package program which One-Way ANOVA test was used that results of statistical analysis.

RESULTS

The Ames/*Salmonella*/microsome test is one of the primary test systemsused in investigating the mutagenic effect of chemicals. It is one of the most reliable short-term bacterial test systems, and is cheap and provides results very quickly [15, 16].In this study, NP and BPA were tested for their mutagenic effects using *S. typhimurium strains* (TA98 and TA100), in eitherthe with or with out of S9 mix.

In this study,when the cytotoxic dose was determined of NP, 0.01g NP was dissolved in 10 ml of DMSO. This percentage was detected as 10000 µg/plate dose quantity. While cytotoxic dose was determined to BPA, 0.1g BPA was dissolved in1000ml of DMSO. This rate was determined as 10000µg/plate dose.

At with S9 mix and without S9 mix assay *S.typhimurium*of TA98 and TA100 strains was used in a non-toxic doses and each doses were madeas 3 different plate.

Table 1. TA 98 and TA 100 strains of *S. typhimurium* NP tested (in the presence and absence of the S9 fraction) plate incorporation assay results

Material test	Experience dose (µg/plate)	Number ofRevertant Arithmetic Mean ± Standard Deviation			
		TA98		TA100	
		Without S9	With S9	Without S9	With S9
Control		22.0±2.6	31.00±2.6	78.00±3.6	108.66±4.9
DMSO		19.66±3.5	25.33±0.5	89.00±15.8	106.66±3.5
Sodium azide				1174.00±109.6*	1144.66±22.5*
2-aminofluorene			1426.66±178.8*		
4-nitro-*o*-fenilendiamine		1359.0±35.0*			
Nonylphenol	0.1 (10^{-5})	27.66±4.1	24.33±10.0	100.66±25.0	93.00±6.0
	1 (10^{-4})	30.00±4.0	29.66±4.0	79.00±8.7	109.3±8.3
	10 (10^{-3})	30.33±2.5	31.33±2.3	80.66±5.0	104.0±12.2
	100 (10^{-2})	26.00±1.0	31.00±7.2	58.00±13.2	77.66±2.0

*Represents significant results when compared to control p <0,05(One-Way ANOVA Test)

The results are given in the tables. The average revertant colony numbers were 22.0±2.6for TA98, 78.00±3.6 for TA100 in the without of S9 mix. In the presence of S9 mix, the numbers for the same strains were 31.00±2.6 and 108.66±4.9, respectively. Spontaneous revertants were within the normal values for the TA98 and TA100 strains examined.

The number of revertant colonies ranged between 22.00±2.6 and 48.6±3.2 for TA98, between 58.00±13.2 and 100.66±25.0 in TA100 without S9 mix. For tests with the S9 mix, the number of revertant colonies ranged between 24.33±10.0 and 54.33±1.5 in TA98, between 93.00±6.0 and 112.3±25.5 for TA100. The plates with the positive control mutagens (DMSO, Sodium azide, 2-aminofluorene, 4-nitro-*o*-fenilendiamine) showed significant increases relative to the spontaneous mutation rate in the TA98 and TA100 tested strains.

The highest value observed with S9 mix was with a BPA at a concentration of 10 µg/plate in the TA100 strain (112.3±25.5); the lowest value observed with S9 mix was with NP at a concentration of 0.1 µg/plate in the TA98 strain(24.33±10.0).

Without S9 mix, the highest value was again obtained in the TA100 strain using NP at a concentration of 0.1 µg/plate (100.66±25.0); the lowest values were noted in the TA98 with NPat a concentration of 100 µg/plate (26.00±1.0).

Most of the results varied when compared to the control group and applying the One-Way ANOVA Test to these fluctuations indicated that they were statistically significant at $p<0.05$ in the examined strains.

The result of the present study showed that all of the NP and BPA concentrations were not mutagenic to *S.typhimurium* TA98 and TA100 with S9 mix and without S9 mix.

Table.2. TA 98 and TA 100 strains of *S.typhimurium* BPA tested (in the presence and absence ofthe S9fraction) plate incorporation assay results

Material test	Experience dose (µg/plate)	Number ofRevertant Arithmetic Mean ± Standard Deviation			
		TA98		TA100	
		Without S9	With S9	Without S9	With S9
Control		22.0±2.6	31.00±2.6	78.00±3.6	108.66±4.9
DMSO		19.66±3.5	25.33±0.5	89.00±15.8	106.66±3.5
Sodium azide				1174.00±109.6*	1144.66±22.5*
2-aminofluorene			1426.66±178.8*		
4-nitro-*o*-fenilendiamine		1359.0±35.0*			
Bisphenol A	0.1 (10^{-5})	43.33±2.0	51.33±2.3	75.66±15.1	96.66±5.5
	1 (10^{-4})	48.6±3.2	54.33±1.5	81.00±7.2	105.6±17.0
	10 (10^{-3})	41.00±5.2	54.00±14.7	70.66±13.0	112.3±25.5
	100 (10^{-2})	29.00±5.2	32. 66±5.5	61.00±13.7	60.00±13.2

*Represents significant results when compared to control p<0.05(One-Way ANOVA Test)

DISCUSSION and CONCLUSIONS

Many negative effects especially have caused of environmental mutagens, in developed societies many scientific article have been reported in.Over the life time these influences of living organisms are exposed to genetic deterioration, and thus was believed to be precipitating the emergence of many kinds of cancer.

Since more than 10 years, risk amounts of BPA are discussed at the international level. In 2008 the U.S. National Toxicology Program (NTP) clarified its effects on the level of exposure to BPA on the development of toxicity.In this condition, the French Food Safety Agency (AFSSA)terms of toxicity of under 5 mg/kg/day doses, it was decided to be reviewed. Some questions come to mind related to the toxicity with BPA in low doses. At several studies have to understand the importance of the warning signals for human health BPA at low doses of endocrine disruptors, and more generally about the need to develop new methods for the evaluation concluded that the risks[20].

As a result of experiments made with NP the study of with S9, in tests with TA 98 and TA100 strains of *S. typhimurium,* the average number of revertant colonies obtained in the presence of S9 was higher than the number of revertant colonies in the absence of S9 was observed of all doses practised.

When NP and BPA are metabolized in the body, these metabolites can show increased amount of interaction with DNA.

The aim of our study is to investigate the mutagenic and non-mutagenic potential of NP and BPA with Ames/ Salmonella/Microsome Test. In this research, firstly non-toxic doses determined. Tested doses were 10000 µg/ plate; 1000 µg/plate; 100 µg/plate, 10 µg/plate; 1 µg/plate and 0.1 µg/ plate. 10000 µg/plate and 1000 µg/plate doses were determined to be toxic. Later were tested for their mutagenicity in TA98 and TA100 strains of *S. typhimurium* for both substances with S9 and without S9.

In this study, determination of mutagenic doses was aimed. But mutagenicity was not observed at the tested doses. However, since NP and BPA are used often in everyday life, it is also important to know what non-mutagenic doses are. Result of the present study revealed that 100 µg/plate, 10 µg/plate; 1 µg/plate and 0.1 µg/ plate doses of BPA and NP were not mutagenic with or without S9. These result can be helpful while deciding study doses in the future studies investigating the mutagenicity of BPA and NP.

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