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Araştırma Makalesi
Research Article

Cytotoxic and Cytogenetic Effects of Bisphenol-A in *Mytilus galloprovincialis* (Lamarck 1819)

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Abstract

Bisphenol-A (BPA), is one of the most important industrial chemicals synthesized for diverse applications. The environmental concentrations of BPA are at high risk level due to use widely in many fields of industry according to the latest studies. However, less knowledge has been founded to the toxicity of BPA. No studies have been founded genotoxicity of BPA to the *Mytilus galloprovincialis* (Lamarck 1819). The Micronucleus test is a biomarker of mutagenicity/genotoxicty used for determining changes in DNA fragments exposure to pollution and chemicals. For this purpose, frequency of MN and other nuclear abnormalities (binucleated, nuclear buds, fragmented/apoptotic cells) was analyzed. Increasing concentrations of BPA (12.5-100 µg-BPA/L) were tested for determination of MN under semi-static conditions in the laboratory. According to the results of the study, it is revealed that all concentration of BPA increases the genotoxic damage, and elevates the micronucleus frequency from ‰ 11, 33 to ‰ 37, 35 Thus, it was tried to determine whether BPA affected the cells of *M. galloprovincialis* (Lamarck 1819) at the level of DNA through to the MN assay.

***Keywords*:** Micronucleus Assay, Bisphenol-a, *Mytilus galloprovincialis,* genotoxicty.

Öz

Bisfenol-A'nın M*ytilus galloprovincialis* (Lamarck 1819) Üzerine Sitotoksik ve Genotoksik Etkileri

Bisfenol-A (BPA), çeşitli uygulamalar için sentezlenen önemli endüstriyel kimyasallardan biridir. En son çalışmalara göre sanayinin birçok alanında yaygın kullanımı nedeniyle BPA'nın çevresel konsantrasyonları yüksek risk düzeyinde bulunmaktadır. BPA'nın *Mytilus galloprovincialis* (Lamarck 1819) üzerine genotoksisitesinin belirlendiği çalışma bulunmamaktadır. Mikronukleus denemeleri, interfaz hücrelerin sitoplazmasındaki DNA parçacıklarının değişimine neden olan kimyasalların ve kirliliğin belirlenmesinde kullanılan mutajenite test sistemidir. Bu amaçla, micronukleus (MN) ve diğer çekirdek anormallikleri (nuklear bud, binukleus, apoptatcik çekirdek) incelenmiştir. Midyeler BPA'nın(12.5-100 µg-BPA/L) artan konsantrasyonları semi-statik labaratuvar şartlarında maruz bırakılmışlardır. Çalışmanın sonuçlarına göre; BPA'nın tüm konsantrasyonları genotoxic hasarı arttırmakta ve ‰ 11, 33 to ‰ 37, 35 MN oluşumun aneden olduğu gözlenmiştir. Bu nedenle, MN testiile BPA'nın DNA düzeyinde, *M. galloprovincialis*' in hücreleri etkileyip etkilemediğini belirlenmeye çalışılmıştır.

***AnahtarKelimeler*:** Micronukleus Testi, Bisphenol-a, *Mytilus galloprovincialis,* Genotoksisite.

Introduction

BisphenolA is an organicsynthetic compound which is used for make certain plastics / polycarbonate and epoxy resins. BPA widely used such as; water bottles, sports eguipment,

baby bottles, medical equipments, cds, and dvds and also used to coatings on the inside of many food and beverage cans, thermal paper and line water pipes (Anonymous, 2003). This compound persistent toxic chemicals for humans and wildlife (Arslan and Parlak, 2008).

It is possible in the environment because of the potential released during manufacturing and discharges of treated industrial waste water (West et al., 2001) intomarine environments (Arslan and Parlak, 2008). The aquatic environment is an important zone for the study of BPA which is enter into the aquatic environment through effluent from wastewater treatment plants and landfill sites and present from the migration of BPA-based products into rivers and marine waters (Lyons, 2000).

Several publications are reported measured levels of BPA in streams and rivers in Japan, Europe and the United States. Cousins et al. (2002) reported that the water concentrations of BPA from 21 European and 13 United States studies as 0.016 and 0.5 µg/L respectively. Recently, a Japanese study reported detectable levels of BPA in water samples selected downstream rivers, was 0.01 µg/L (Staples *et al.,* 1998). Previous studies are reported that BPA levels in surface waters was 6.44-15.6 ng/L from the coastline of China and 60 to 1900 ng/L from Tokyo Bay (Yeru *et al.*, 2001; Hando *et al.,* 2003). Report of IUPAC (2003) indicate that the median measured concentrations of BPA on several industrial effluent as 41 µg/L (metal/wood), 17 µg/L (paper), 18 µg/L (chemical industry) and 25-146 µg/L have been found in compost water and waste dump (IUPAC, 2003). BPA was moderate to slightly toxic to aquatic organisms including freshwater and saltwater algae, invertebrates and fish. LC50 values range from 1000 to 20,000 µg/L (Alexander *et al.,* 1988; Colborn *et al.*, 1996; Arslan and Parlak, 2008).

Numerous studies showed that some of chemicals polluted to the environment have carcinogenic/mutagenic effects (Arslan *et al.,* 2010-2015). Genotoxic pollutants caused damages on the DNA, which is the first event in the aquatic organisms. DNA damage at the level of chromosome confirm a necessary part of genetic toxicology because mutation plays the big significant role in cancer formation (Fenech, 2000). There are lots of dangerous chemicals/compounds in the water and sediment which are caused cellular and DNA damage (Harvey *et al.,* 1999; Bolognesi *et al.,*1999-2004; Bolognesi and Fenech 2012).

The micronucleus (MN) test, most frequently used in environmental genotoxicity studies which is served as an index of cytogenetic damage (Fenech, 2000). MN assays is reasonable to determining the broken chrosomes due to its more objective results than other tests in detecting chromosomal damages (Arslan *et al.,* 2015; OECD, 2004). The being of nuclear buds, binucleated cells, fragmented-apoptotic cells have also been usefull endpoints for determination of genotoxic and cytotoxic effects of contaminants in molluscs (Baršienė *et al.*, 2006).

In our investigations, considering the knowledge above, we have attempted to determine the genotoxic effects of BPA using *M.galloprovincialis* (Lamarck 1819). As it is well known the genotoxicty of chemicals were ranged according to species. Although the toxic effects of BPA on some Mollusca species examined, there are lack of information about the genotoxic effect and micronucleus formation on cells of *M.galloprovincialis* (Lamarck 1819).

Materials and Methods

Mussels were collected from a clean area which is not exposed to any domestic and industrial waste water, in the Izmir-Çeşme, (Aegean coast of Turkey). Before the experiment, all mussel were taken from a stock tank (260x80x70). They were acclimatized in the laboratory in tanks of artificial sea water aerated continuously at 17.5 ± 1°C. In the experiment, 57x39x28 cm glass aquariums were used. All treatments were tested as two replicates in glass aquariums which contain 10 mussel. Mussels were fed daily with phytoplankton *Chlorella sp.*

Bisphenol A [$(CH_3)_2C$ (C_6H_4OH)] purchased from Aldrich (Cat.No:85-05-7, Mol weight: 228.229g), was dissolved in Dimethyl sulphoxide (DMSO) (Sigma:67-68-5). Maximum DMSO concentration were tested as a solvent control had no observed micronucleus induction. According to the European directive 67/548/EEC, DMSO is not classified as dangerous (Anonymous, 2016). Test concentrations were prepared by adding the chemicals from stock solution directly to the test medium. Semi-static test conditions were prepared for chronic the toxicity experiment. BPA concentration levels used in the experiment were calculated as 12.5, 25, 50, 75, 100 µg-BPA/L. During the study, a total of 10 mussel were investigated, which the mean total length was 6.4 ± 1.65 cm. For the MN assay in gill enzymatic digestion was performed as pre-viously reported by Arslan *et al.* (2010). For this purpose, gills of the mussels were kept for low enzymatic activity and then, centrifuging at x1000 rpm.

This process was repeated twice and the pellet was fixed with ethanol: acetic acid (3:1) (Arslan *et al.,* 2010). The fixed pellet was smeared on slide. Then, dried samples were stained with Giemsa (5%, Sigma, Aldrich) and following kept in ethanol for 10 minutes. For analyzis of hemolymph which was taken and mixed with a fixative (3:1 methanol: acetic acid) and centrifuged at x1000 rpm. The pellet is smeared on the slide then fixed with methanol for 10 minutes. It is allowed to dry, then stained with 5% Giemsa followed by the rinsed with distilled water and closed with a cover slide. The preparation is covered by entelan avoid to expose to the air (Wóznicki,2004). MN/BN frequency was calculated as ‰ number of MN and BN (Figure 1).

The cells with MN and BN were counted at x100 magnification (immersion oil) by light microscopy. Five hundred gill and hemolymph cells from each preparation and 1500 from each individual were inspected (9000 cells per concentrations).

Results

In this study, genotoxic effects of BPA, on *M. galloprovincialis* (Lamarck 1819), an economically important species, were studied. The study was performed by an in vivo method on hemolymph and gill cells of *M.galloprovincialis* (Lamarck 1819) using sampling times (30 days). The present study performed to identification the genotoxic potential of BPA using MN test. Frequency of MN was determined and also other nuclear abnormalities were observed such as nuclear bats. Micronuclei and their frequency found in cells of 10 individuals of mussels from each concentration are shown in Table 1.

The mean frequency of MN in the gills of the control group was 6,17‰ while the BPA exposure resulted in MN value range 17,22-55,13 ‰ (Table 1).

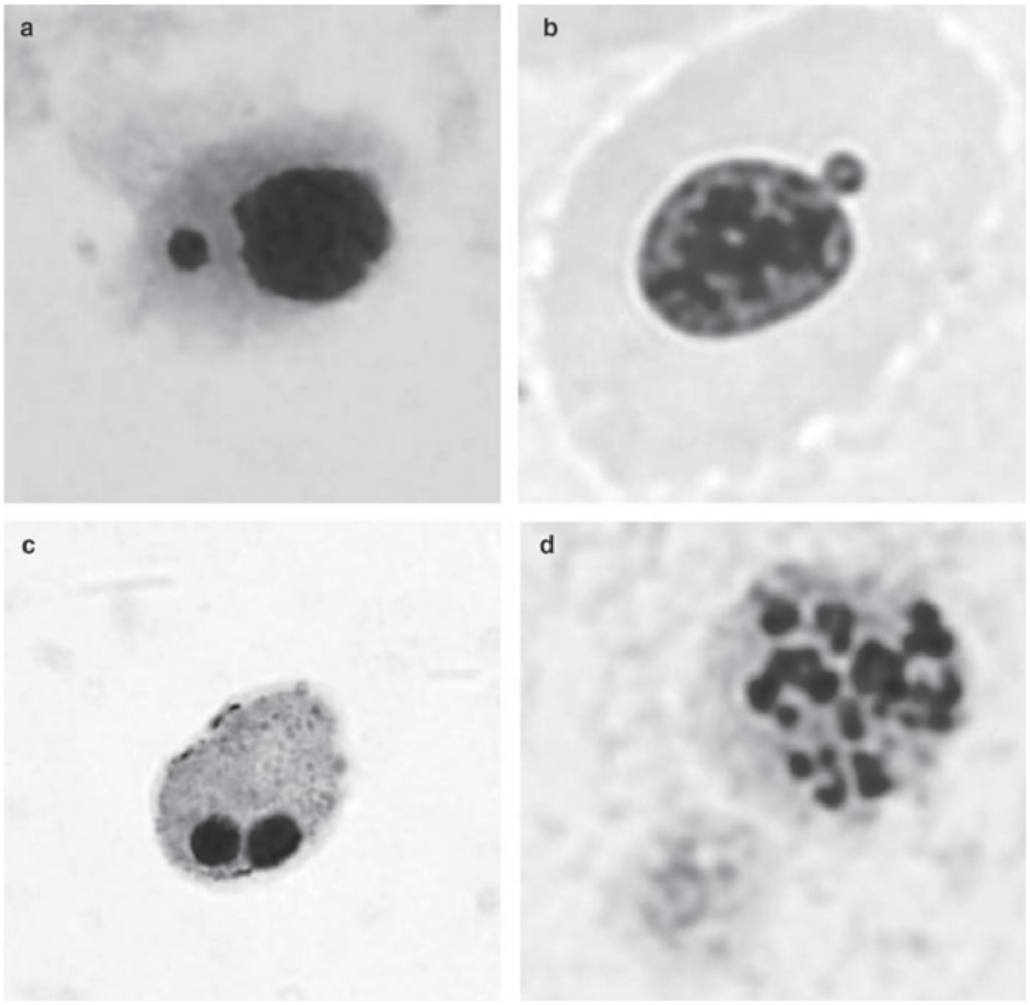

Figure 1. Nuclear abnormalities in the gills of mussels: micronuclei (a), nuclear buds (b), bi-nucleated cells (c) and fragmented-apoptotic cells (d).

The exposure to BPA induced the MN between 11,33 to 41,38 ‰ cells in hemolymph cells while this value was observing 3,33 ‰ in the control group (Table 1). MN inductions were observed at concentrations ranging from 12.5 to 100 µg-BPA/L. Table 1 gives the averages and standard errors of nuclear abnormalities (Figure 1) in cells of mussels from 5 exposure concentrations ($p<0.005$).

In the control group of mussel, the frequency of nuclear buds, bi-nucleated cells, fragmented-apoptotic cells was not observed in hemolymph and gill cells. BPA action was most significant in induction of bi-nucleated and fragmented-apoptotic cells as well as nuclear buds (Table 1). Experimental results demonstrated that the exposure to BPA increased the level of nuclear buds, bi-nucleated and fragmented-apoptotic cells.

As can be seen in Table 1, higher MN and nuclear abnormality frequencies were found in mussel gills taken from 100µg-BPA/L medium. As mentioned previously, considering that micronuclei and nuclear abnormalities vary depending on increasing concentrations and micronuclei and nuclear buds, bi-nucleated cells, fragmented-apoptotic cells frequencies frequencies showed statistically significant differences when were compared with control group ($p<0.005$).

Table 1. Nuclear abnormalities observed in gill and haemolymph cells of *Mytilus galloprovincialis* (Mean ± standart error)

Concentrations (µg/L)	Nucleus	Micronucleus	Binucleus	Apoptic	Nuclear Bud	Nucleoplasmic Bridge
Haemolymph Cells						
Control	996,7±0,33	3,33±0,33	0±0	0±0	0±0	0±0
12,5	982,2±1,06	11,33±0,53*	0±0	3,56±0,80*	0±0	2,89±0,42*
25	975,8±0,91	14,67±0,50*	0,5±0,22*	5,83±0,83*	0±0	3,17±0,75*
50	958,5±1,28	20,67±0,33*	2,33±0,42*	12,67±0,88*	0±0	5,83±0,79*
75	917,1±3,61	37,35±1,85*	3,35±0,30*	26,95±1,35*	9,45±0,91*	5,9±0,56*
100	885,6±5,62	41,38±5,05*	3,53±0,46*	47,30±2,82*	13,61±2,19*	8,53±1,65*
DMSO	995,5±0,25	4,5±0,25	0±0	0±0	0±0	0±0
Gill Cells						
Control	993,8±0,40	6,17±0,40	0±0	0±0	0±0	0±0
12,5	945,4±1,65	17,22±0,49*	0,44±0,24*	17,89±0,56*	15,44±0,71*	3,56±0,67*
25	924±1,77	22,17±0,70*	4,5±0,34*	22,33±1,12*	20,33±0,76*	6,67±0,61*
50	910,7±2,60	27±1,15*	5±0,63*	24,67±0,80*	24,67±1,08*	8±1,01*
75	875,6±3,75	38,57±1,14*	6,14±0,31*	37,38±1,75*	34,28±1,34*	8,04±0,21*
100	808,1±3,87	55,13±1,01*	10,53±0,59*	59,93±1,08*	56,93±1,55*	9,4±0,53*
DMSO	992,9±0,45	7,1±0,45	0±0	0±0	0±0	0±0

***p<0.005**

Discussion

The MN tests were used as a biomarker because it gave dependable results and alsoits easy applicability. When the results obtained from this study examined samples with different concentrations to determine genotoxic effects were found to be one of the pollutants that cause genotoxic damage in the light of BPA.

Bivalves feed by filtering (Jĝrgensen, 1990). Mussels have potential for both low and high bioaccumulation and biotransformation to the inorganic and organic contaminants (Cunha *et al.,* 2005). So that they are suitable for pollution monitoring activities. Because of the gills, independently of the elaboration of mussels seems most appropriate tissue used in biomonitoring study.

Induction of MN is indirect marker of numeric and structural chromosomal damages cause in the cells by many agents. The organisms are highly exposing to negative effects of toxic, carcinogenic, and mutagenic agents because of increasing pollution. Thus, the genetic damage in the organisms at locations where extensive pollution exists has been determined using MN test (Arslan *et al.,* 2015; Dolcetti and Venier, 2002).

Numerous studies are investigated for micronucleus formation both on organisms collected from their natural environment and

laboratory conditions. Genotoxicity assessment performed by Siu et al. (2004) MN induction were observed in gill cells of *Pernaviridis.* For this purpose, mussels were exposed to the same net amount of a genotoxicant mixture of four polycyclic aromatic hydrocarbons (PAHs): anthracene, fluoranthene, pyrene, and benzo[*a*]pyrene) and four organochlorine pesticides (OCs); α-hexachlorocyclohexane (HCH), Aldrin, dieldrin, and p, p′-DDT) for MN test. In this study the highest MN frequencies were recorded as 22.3 ± 1.12% for constant exposure, 18.9 ± 2.41% for increasing concentrations, 19 ± 1.72% decreasing concentrations. They are reported that, MN frequency depended on treatment and correlated with both nominal contaminant levels. And they are suggested that MN responses can be a sensitive indicator of exposure to genotoxicants and that MN response in mussel gill cells can be a stable biomarker of genotoxicity. Dolcetti and Venier (2002) were examined MN frequencies in Mediterranean mussel *Mytilusgalloprovincialis* to determine genetic ofbenzopyrene in laboratory setting. In that study, MN frequency found in the gills (about 8.5‰) was higher than that found in the hemolymph (about 48‰). And also it was found that MN frequency increased parallel to the increasing concentrations of benzopyrene.

MN test was carried out in gill cells of *Dreissena polymorpha* (Bolognesi *et al.,* 2004) in order to determine effects of surface waters which are present sodium hypochlorite and chlorine dioxide and peracetic acid disinfectants (Lake Trasimeno, Italy). After the collection of mussel, they were exposed the contaminated surface water during 10 and 20 days for MN test. Results of that study MN frequency were reported as in gill cells (mean frequency 2.79 and 3.15 per 1000 cells) (Bolognesi *et al.,* 2004). These results near to our present study which the frequency value, depending on the toxicant, began to increase (begin with 5.73 ‰ to 31.26 ‰ in gill cells). *Limnoperna fortunei* mussel species are left exposed in different concentration copper sulfate (CuSO4) and Pentachlorophenol (PCP). After 24 h exposure to Copper sulfate 3.75 and 7.50 mg/ml concentrations, MN frequency was increasing significantly reported. PCP for 24 hours and 48 hours after exposure at all concentrations (10, 80, 100, 150 mg /ml) a significant increase compared to the control group MN frequency has been reported to be observed (Villela *et al.,* 2006). In another study with mussels 80, 100 and 150 mg/ L Pentachlorophenol (PCP) concentrations have applied to *Dreissena polymorpha* mussel for 7 days. The significant damage occurring in hemolymph cells was determined by the MN test (Pavlica *et al.,* 2000).

Bolognesi *et al.,* (1999) have used *Mytilus galloprovincialis* hemolymph and gill cells in their work for effects of heavy metal. After 5 days of exposure $CuCl_2$ (5, 10, 20, 40, 80 mg/L), detected MN frequencies was reported for the cells in the gills ranged from 2.90±0.2 and 2.26±1.0 ‰. MN frequencies found for hemolymph cells are between 2.3 ± 0.26 and 3.7±0.86 ‰. In the same study $CdCl_2$ (1.84, 18.4, 184μg / L) for the detected cells in the gills MN frequency varies between 1.1 ‰ ± 4.77 and 4.03 ± 0.23 ‰. While they have identified MN frequencies ranging between 4.0±1.3 and 4.2 ± 1.1 ‰ for hemolymph. $HgCl_2$ (32.0 mg/L) found for MN frequency hemolymph cells was 3.8 ± 0.1 ‰ gill cells was 3.4±0.9 ‰. The study found that MN frequencies increases in the form of three heavy metals Cd> Cu> Hg.

Barsiene *et al.* (2006) were reported that micronuclei, nuclear buds, bi-polynucleated

and fragmented-apoptotic cells in gills of blue mussels exposed for to sublethal concentrations of bisphenol A, diallyl phthalate (50 ppb) and to tetrabromodiphenyl ether-47 (5 ppb). There are found that a significant increase in micronuclei frequency after the treatment with bisphenol A, diallyl phthalate and tetrabromodiphenyl ether-47. Although they were found the BPA was caused only in induction of micronuclei but not any other kind of nuclear injuries, in our results we found cytotoxic effects of BPA by scoring of nuclear abnormalities.

Negreiros et al.(2011) reported that sub lethal concentrations of crude oil (18ml/L) were caused significant increase in MN frequency erythrocyte of *H. reiki,* after 8 hours exposure. In a study Barón et al. (2016) *Mytilus galloprovincialis* are exposed to the DPI (dechlor) and B-209 (decabromodiphenyl ether). Micronucleus formation in the study on the highest concentration of each chemical (100 mg/L DP and 200 mg/L B-209) has been reported to control over 18%. DP of low exposure concentrations (5.6, 56 mg/L) B-209 (56,100 μg/L) caused a similar effect has been reported. In the investigations by Liu et al. (2014) mussels *Pernaviridis* were exposed to increasing concentrations of (0.1, 1, 10, 100, 1000 mg/L) the perfluorinated compounds from perfluorooctane sulfonate (PFOS), perflorooctanoic acid (PFOA), perfluorono-nanoic acid (PFNA) is perfluorodecanoic acid (PFDA). The confidence interval for the EC50 value of the compound PFOS, PFOA, PFNA, PFDA of respectively, with 33 (29-37), 594 (341-1036), 195 (144-265), 78 (73-84) ‰ mg/L as reported. The maximum frequency of MN PFOS has been seen in the study results. Then the PFDA> PFNA> PFOA has been continued. PFOS in this study, reported to constant DNA damage that may lead to results in thick exposed to high concentrations. In the comparison between the tests compounds PFOS other perfluorinated compounds has been reported that more than 30% may cause apoptosis. For measuring comet tail length of the primary DNA fragmentation. The results of the trial were similar to the results of Comet MN test.

PFOS compounds are reported and PFDA may also cause DNA fragmentation at low exposure levels (Liu *et al.,* 2014). This study confirmed that the present study and indicated that PFOS were mutagenic / carcinogenic compound.

In conclusion, this study has shown BPA to be genotoxic and cytotoxic to the mussel species *M. galloprovincialis* (Lamarck 1819). In addition there was a correlation between the sublethal concentration of BPA and micronuclei formation. Results conclusively indicated that Bisphenol A is genotoxic for *Mytilus galloprovincialis*.

In sum of our study showed that increasing concentrations of BPA have mutagenic and genotoxic properties in cells of *M. galloprovincialis* (Lamarck 1819), and also indicates that the MN test in mussels give sensitive results in monitoring genotoxic/mutagenic effects of chemicals might be used as a standard method in the regular determination of genotoxic effects of BPA compounds.

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