EKOLOGIJA. 2005. Nr. 4. P. 1–7

Induction of micronuclei in Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) after treatment with bisphenol A, diallyl phthalate and tetrabromodiphenyl ether-47

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An *in vivo* study on the genotoxic effects of endocrine disruptors and flame retardant was carried out with marine fish Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*). The fish were exposed for 3 weeks to a sublethal concentration of bisphenol A, diallyl phthalate (50 ppb concentration for both) and to tetrabromodiphenyl ether BDE-47 (nominal concentration 5 ppb). Micronuclei (MN) were analyzed in blood (mature erythrocytes) and in immature erythrocytes of cephalic kidney. The mean MN frequencies in the cod ranged from 0.17 to 1.37 MN/1000 erythrocytes and in turbot from 0.60 to 1.18 MN/1000 erythrocytes. A significant increase of micronuclei was observed in turbot blood ($P < 0.0001$; Mann-Whitney U-test) and cod cephalic kidney after treatment with all three compounds (P values ranged from 0.0401 to 0.0027). In turbot blood, MN incidence increased 6.7-fold after exposure to bisphenol A, 9.4-fold after exposure to diallyl phthalate, and an 11.8-fold elevation was detected after treatment with tetrabromodiphenyl ether BDE-47. Treatment with either of the three compounds did not elevate MN formation in the blood of Atlantic cod and in the cephalic kidney of turbot. A species-specific response to bisphenol A and diallyl phthalate was observed in both types of erythrocytes, and tissue-specific differences in MN induction were found in both species after exposure to BDE-47.

Key words: micronuclei, cod, turbot, bisphenol A, diallyl phthalate, tetrabromodiphenyl ether BDE-47

INTRODUCTION

A growing interest in studies of environmental genotoxicity caused by different substances has led to the development of several tests for detecting genotoxicants in aquatic media. Cytogenetic methods, in particular the micronucleus test, have been widely applied for various groups of aquatic organisms. The micronucleus (MN) test has served as an index of cytogenetic damage for over 30 years. Micronuclei are produced from chromosomal fragments or whole chromosomes that lag at the cell division due to the lacking or damage of the centromere or a defect in cytokinesis. These small secondary structures of chromatin are surrounded by membranes located in the cytoplasm and have no detectable link to the cell nucleus (Heddle, 1973; Heddle et al., 1991; MacGregor, 1991; Seelbach et al., 1993; Zoll-Moreux, Ferrier, 1999).

Although originally the micronucleus test was developed for the application in mammals (Heddle, 1973), it was subsequently modified and used in fish (Hooftman, de Raat, 1982). Fish provide a relevant model for the evaluation of aquatic genotoxicity *in situ*, as well as the action of polluted effluents, sediments or toxic compounds (Hayashi et al., 1998).

In this paper, we report data on the frequency of micronuclei in blood mature erythrocytes and in immature erythrocytes from the cephalic kidney of turbot and Atlantic cod exposed in laboratory experiments to the endocrine disruptors bisphenol A and diallyl phtha-

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late, as well as to flame retardant tetrabromodiphenyl ether (BDE-47). Bisphenol A, a monomer of polycarbon plastics and epoxy resins, is contained in food-packing, can-coating chemicals and in dental sealants. This compound may be released into food, reach the circulating blood and cause genotoxic and cytotoxic effects. Disruption of the mitotic spindle, induction of metaphase arrest and micronuclei containing whole chromosomes / chromatids has been determined in human, mouse and Chinese hamster cells (Pfeiffer et al., 1997; Suarez et al., 2000; Lehmann, Metzler, 2004; Masuda et al., 2005). Polybrominated diphenyl ethers (PBDEs) belong to a novel group of environmental contaminants. These persistent compounds have been used extensively in the last decades and are widely distributed in wildlife samples from Europe, Australia, Azerbaijan, North America and the Arctic (Law et al., 2003). Since PBDEs are very hydrophobic and not easily dissolvable, they cannot be accumulated in tissues of organisms. Whilst, PBDE congeners have been identified in salmonids from Lake Michigan (Manchester-Neesvig et al., 2001), in the Baltic Sea perch, salmon (Olsson et al., 1999; Marsh et al., 2004), flounder and mussels (Barðienë et al., 2005), in different fish species from Greenland (Christensen et al., 2002). PBDE-47 has been determined in 89% of fish fillet samples and contributed 40–70% of the total PBDEs studied in fish from North American water basins (Hale et al., 2001). A preferential bioaccumulation of PBDEs appeared in fish liver (Kierkegaard et al., 1999; Voorspoels et al., 2003). Only fragmentary data exist on their mutagenic and clastogenic action (Evandri et al., 2003b). There have been no descriptions of the genotoxicity of diallyl phthalate.

The main aim of this work was to determine the genotoxic effects in fish blood and kidney caused by widely used endocrine disruptors and flame retardant. Mature erythrocytes from peripheral blood and immature erythrocytes from cephalic kidney of cod and turbot were used as "sentinel systems" for detecting micronuclei induction after chemical exposure considering differences of metabolism, induction of cytogenetic damage, DNA repair, as well as cell proliferation in different fish species, their tissues and organs.

MATERIALS AND METHODS

Atlantic cod and turbot were exposed at RF Akvamiljo (Norway) under controlled laboratory conditions (flow-through system with automatic regulation of chemical intake, oxygen content, water pH and temperature) to sublethal concentrations of bisphenol A, diallyl phthalate (for both, the nominal concentration was 50 ppb) and to tetrabromodiphenyl ether BDE-47 (nominal concentration 5 ppb). After 3 weeks of exposure, blood and cephalic kidney samples were collected from 95 fish specimens (Table 1).

A drop of blood from the caudal blood vessel of fish was directly smeared on slides and air-dried. After the sacrifice, a small piece of cephalic kidney was dissected, softly dragged along a clean slide and allowed to dry for one or two hours (Barðienë, 1980). Smears were subsequently fixed in methanol for 10 min and then stained with 5% Giemsa solution for 10–20 min. The frequency of micronuclei was evaluated (per 1000 cells) by scoring at a 1000×, or 1250× magnifications using either Olympus BX 51, Olympus CX 31 or PZO (Poland) bright-field microscopes. A total of 10,000 erythrocytes with intact cellular and nuclear membranes were examined for each fish specimen (5,000 mature erythrocytes from peripheral blood and 5,000 immature erythrocytes from cephalic kidney).

To minimize the technical variation, the blind scoring of micronuclei and other nuclear abnormalities was performed on coded slides without knowledge of the origin of samples. Only cells with an intact cellular and nuclear membrane were scored. Round or ovoid-shaped non-refractory particles with the color and structure similar to chromatin, with a diameter of 1/3–1/50 of the main nucleus and clearly detached from it were interpreted as micronuclei (Fig. 1). In general, the color intensity of MN should be the same or less than of the main nuclei.

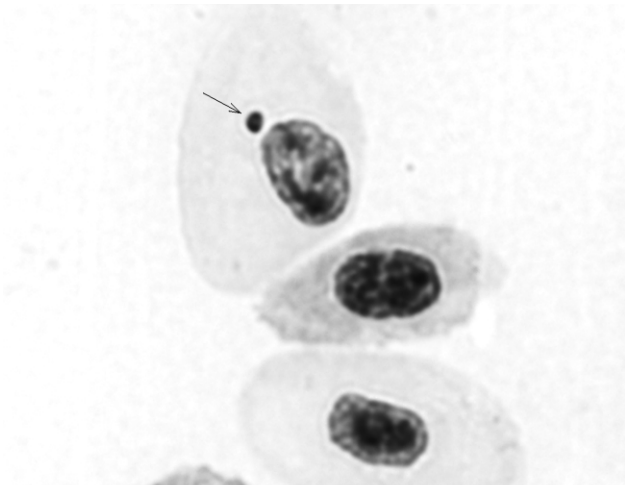

Fig. 1. Micronucleus in mature erythrocyte from turbot blood

Table 1. **Material for the micronuclei analysis in Atlantic cod and turbot**

Fish species/ exposure	Control	Bisphenol A	Diallyl phthalate	PBDE-47
Atlantic cod	12	13	15	15
Turbot	10	10	10	10

The statistical analysis was carried out using the PRISM statistical package. Means and standard errors were calculated for each experimental group. The non-parametric Mann–Whitney U-test was used to compare MN frequencies in the control and treatment groups, between different tissues and species.

RESULTS

Induction of MN in peripheral blood

The frequency of MN in the control group was equal to 0.22 in cod and 0.1 MN/1000 erythrocytes in turbot. In the treated fish, the levels of micronuclei frequencies were higher in turbot than in Atlantic cod. The highest value of MN (1.18 MN/1000 erythrocytes) was found in turbot exposed to BDE-47 (Fig. 2). In turbot blood, MN incidence increased 6.7-fold after exposure to bisphenol A, 9.4-fold after exposure to diallyl phthalate (DAP), and an 11.8-fold increase was detected after treatment with tetrabromodiphenyl ether. The Mann–Whitney U-test showed a significant increase ($P < 0.0001$) of micronuclei after treatment with any of the three compounds. However, in blood erythrocytes of Atlantic cod none of the three compounds induced a significant MN formation (P values varied in a range from 0.1005 to 0.7832).

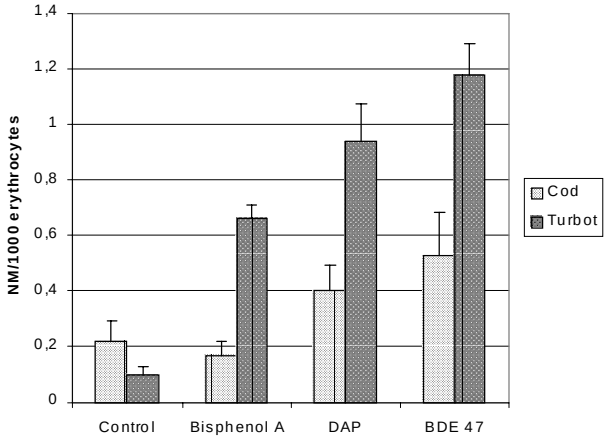

Fig. 2. The frequencies of micronuclei (mean ± SEM) in fish blood erythrocytes. Asterisks show statistically significant differences compared to control group (three asterisks – P at 0.0001 levels)

Induction of MN in cephalic kidney

The mean MN frequencies in treated cod ranged between 0.60 and 1.37 MN/1000 erythrocytes, in turbot – between 0.60 and 0.94 MN/1000 erythrocytes. A comparatively high level of MN was observed in the control group of turbot (0.64 MN/1000 erythrocytes) whereas in the cod control group, the level was half as high (Fig. 3). In cod, a significant increase of MN frequency was observed after treatment with bisphenol A ($P = 0.0027$), DAP ($P = 0.0080$) and BDE 47 ($P = 0.0401$). Micronuclei were not significantly induced in turbot after exposure to diallyl phthalate and PBDE 47, whereas a slight increase of MN frequency was found after exposure to bisphenol A (Fig. 3). However, a comparison with the control group showed that the induction was not statistically significant ($P = 0.1051$; Mann–Whitney U-test).

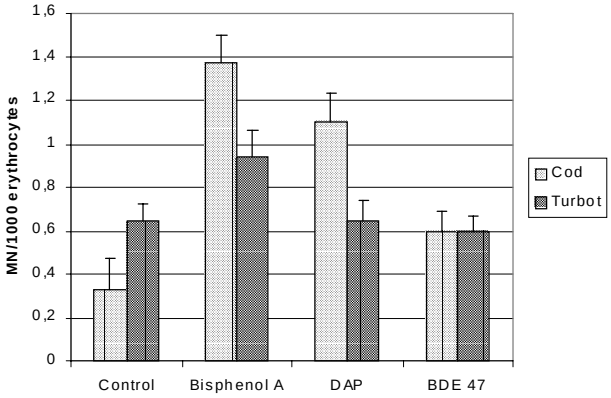

Fig. 3. The frequencies of micronuclei (mean ± SEM) in cod and turbot cephalic kidney erythrocytes. Asterisks show statistically significant differences compared to control group (one asterisk – P is at 0.01, two asterisks – P at 0.001 levels)

Species and tissue-specific induction of MN in fish

In control groups, significant inter-specific differences were observed between frequencies of micronuclei in immature erythrocytes from cephalic kidney but not found in mature erythrocytes. Bisphenol A and diallyl phthalate induced different levels of responses in both types of erythrocytes, whereas a species-specific pattern of BDE-47 genotoxicity was observed only in mature erythrocytes (Table 2). The same MN induction level (0.60 MN/1000 cells) was observed in cod and turbot kidney after exposure to BDE-47.

Tissue-specific differences were induced in both species after treatment with BDE-47. A statistically different response was found in cod mature and immature erythrocytes after exposure to diallyl phthalate and in the control group of turbot (Table 3).

Table 2. **Levels of statistical significance (P values; Mann-Whitney U-test) of MN frequencies in Atlantic cod and turbot**

Tissues / exposure	Control	Bisphenol A	Diallyl phthalate	BDE-47
Mature erythrocytes (blood)	0.3326	**0.0001**	**0.0017**	**0.0047**
Immature erythrocytes (cephalic kidney)	**0.0266**	**0.0312**	**0.0160**	0.8868

Table 3. **Levels of statistical significance (P values; Mann-Whitney U-test) of MN frequencies in fish mature and immature erythrocytes**

Nuclear abnormalities / exposure	Control	Bisphenol A	Diallyl phthalate	BDE-47
Atlantic cod	0.0146	0.7934	**0.0161**	**0.0177**
Turbot	**< 0.0001**	0.0630	0.0630	**0.0160**

DISCUSSION

The micronuclei test in fish has been applied for both laboratory treatments of *in vivo* and *in situ* exposure to environmental pollution. Induction of micronuclei by several well-known clastogenic / mutagenic agents such as cyclophosphamide, mitomycin-C, bleomycine, colchicine, ethyl methanesulphonate and vinblastin was assessed in freshwater and marine fish species (Hooftman, Raat, 1982; Williams, Metcalfe, 1992; Ueda et al., 1992; Bahari et al., 1994; Pacheco, Santos, 1996; Matsumoto, Colus, 2000; Grisolia, Cordeiro, 2000; Ayllon, Garcia-Vazquez, 2000, 2001; Gustavino et al., 2001; Palhares, Grisolia, 2002; Rodriguez-Cea et al., 2003). The efficacy of the MN test as an indicator of cytogenetic damage has already been proven, and the studies of MN formation have been successfully used as bioassays to measure the impacts after fish treatment with surface water disinfectants (Buschini et al., 2004), herbicides (Ateeq et al., 2002; Farah et al., 2003), insecticides (Nepomuceno, Spano, 1995; Cavas, Ergene-Gozukara, 2003), tributyltin (Ferraro et al., 2004), benzo[α]pyrene and other PAH compounds (Pacheco, Santos, 2002; Maria et al., 2002a, 2002b; Gravato, Santos, 2002, 2003).

Most of the studies have been carried out in circulating mature erythrocytes of fish. Blood smears provide thousands of scorable erythrocytes, and technically analysis of MN is easy to perform. However, the mature erythrocytes are non-dividing cells. Since MN can arise after cell division, a disadvantage of the MN test when performed on peripheral blood could be a comparatively low response to the action of genotoxic agents. Furthermore, the proportion of injured erythroblasts appearing in the peripheral blood is unknown (Hooftman, Raat, 1982), as is the extent of DNA repair and how long a complete red blood cell turnover in fish might take (Buschini et al., 2004). Therefore, the testing of genotoxicity of certain compounds should be performed in fish proliferate tissues. The MN test can be successfully employed for the assessment of cytogenetic damage in fish erythropoietic tissues (cephalic kidney, spleen), or in gill and liver cells. Cephalic kidney in our study was selected as target organ considering the high frequency of mitotic cells in fish (Barðienë, 1977; Pendas et al., 1993).

The highest level of genotoxicity was observed in cod cephalic kidney (1.37 MN/1000 erythrocytes) after exposure to bisphenol A and in turbot peripheral blood (1.18 MN/1000 erythrocytes) after exposure to BDE-47. There are literature data on the genotoxicity of bisphenol A. An increase in the frequency of micronucleated cells has been demonstrated in the bone marrow of mice (Gudi et al., 1992), in Chinese hamster cells (Pfeiffer et al., 1997), in human lymphocytes (Suarez et al., 2000), in human fibroblasts (Lehmann, Metzler, 2004) and in reticulocytes of mice (Masuda et al., 2005). The genotoxicity of bisphenol F has been shown in human peripheral blood lymphocytes (Sueiro et al., 2003).

There is increasing concern over the toxicity of polybrominated diphenyl ethers used as flame retardants, whereas until now only scarce literature data exist on the genotoxicity of such compounds. The mutagenic and clastogenic action of BDE-99, pentabrominated diphenyl ether was demonstrated using *Salmonella typhimurium*, *E. coli* and *Alium cepa* tests (Evandri et al., 2003b). As regards the other biological effects of PBDEs in aquatic organisms, the results are contradictory. A two-week treatment of juvenile salmon (*Salmo salar*) with penta-BDE and octa-BDE compounds did not reveal any significant changes in the expression of CYP1A, vitellogenin, and zona radiata proteins. A set of physiological and biochemical variables (condition factor, liver somatic index, spleen somatic index, hematocrit, level of leucocytes, haemaglobin, glutathione reductase, catalase and EROD) was measured after a 6-day and 22-day exposure of rainbow trout to PBDE. The study results showed that glutathione reductase activity, the levels of blood glucose and hematocrit were affected by penta-BDE exposure. Reduction of EROD activity was the only effect of the 22-day exposure to tetra-BDE in rainbow trout (Tjarnlung et al., 1998). An acute toxicity test in *Daphnia magna* showed a dose-dependent decrease in survival and diminished the reproduction of this aquatic organism in response to PBDE exposure (Evandri et al., 2003a).

The results of our study on the species and tissue-specific genotoxicity of bisphenol A, DAP and BDE-47 revealed a significant induction of micronuclei in cod cephalic kidney (P varied within 0.0027 and 0.0401) and in turbot peripheral blood ($P < 0.0001$). It should be stressed that neither of the test compounds induced significant elevation of micronuclei in the cephalic kidney of turbot and in the

blood of cod. Inter-tissue differences in cod could be the result of micronuclei formation in mitotic cells of the cephalic kidney and elimination of micronucleated erythrocytes from the circulating system of peripheral blood. It is known that chromosome breakage and the dysfunction of the mitotic apparatus are two basic phenomena leading to the development of micronuclei in mitotic cells (Norppa, Falck, 2003). In turbot, high response in blood and a weak elevation of micronuclei in immature erythrocytes of cephalic kidney could be due to intake of micronucleated erythrocytes from the other erythropoetic tissues of fish, such as spleen or liver.

Our study results revealed that MN analysis if only performed on fish blood can lead to the conclusion that the test compounds are non-genotoxic. Thus, our study confirmed the relevance of multitissue analysis to demonstrate genotoxic effects of endocrine disrupting compounds and flame retardant in fish. Furthermore, it indicates that species differences are obvious resulting in a lack of response to exposure of all compounds studied in turbot kidney or cod peripheral blood. Thus, analysis of micronuclei in different fish species and their tissues should be considered as a relevant approach in detection of potential genotoxic effects caused by harmful agents in aquatic media.

The appearance of the inter-specific or inter-tissue differences we observed in fish could be attributed to the specificity of DNA repair, cell turnover time, physiological peculiarities, contaminant uptake or biotransformation in the fish species studied. Since the ratio between MN elimination from cells and potential re-incorporation into the daughter nucleus remains unclear, tissue-specific differences could be suspected. On the other hand, age, sex, reproductive status, genetic constitution may affect MN frequency in fish (Al-Sabti, Metcalfe, 1995). However, turbot and cod in our study were selected from the same age groups. The exposure was performed in the Akvamiljo experimental unit (Norway) using a flow-through system, and the main hydrochemical parameters of the water were monitored. Therefore, the experimental conditions were controlled, and the inter-specific-tissue differences in MN incidences should not be attributed to the intrinsic problems of the experimental system used.

ACKNOWLEDGEMENTS

This work was supported by the European Commission (Research Directorate General, Environment Program – Marine Ecosystems) through the BEEP project “Biological Effects of Environmental Pollution in Marine Coastal Ecosystems” (contract EVK3-CT2000-00025).

Received 14 October 2005

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MIKROBRANDUOLIØ INDUKCIJA PAVEIKUS ATLANTINÆ MENKÆ (*GADUS MORHUA*) IR OTÀ (*SCOPHTHALMUS MAXIMUS*) BISFENOLIU A, DIALILFTALATU IR TETRABROMODIFENILETERIU-47

S a n t r a u k a

Endokrininës sistemos disruptoriø ir liepsnà slopinanèio junginio genotoksiðkumas tirtas jûriniø þuvø atlantinës menkës (*Gadus morhua*) ir oto (*Scophthalmus maximus*) audiniuose. Þuvys tris savaites buvo veikiamos subletalios koncentracijos 50 ppb bisfenoliu A ir dialilftalatu bei 5 ppb tetrabromodifenileteriu-47. Mikrobranduoliai (MB) analizuoti subrendusiuose kraujo ir nesubrendusiuose inkstø eritrocituose. Paveikus menkes, mikrobranduoliø daþnio vidurkis kito nuo 0,17 iki 1,37 MB/1000 eritrocitø, o otus – nuo 0,60 iki 1,18 MB/1000 eritrocitø. Statistiðkai patikimas MB daþnio padidëjimas buvo stebimas oto kraujo eritrocituose ($P < 0,0001$; Mann–Whitney U testas) ir menkiø inkstø eritrocituose, paveikus visais tirtais junginiais (P reikðmës kito nuo 0,0401 iki 0,0027). MB daþnis otø kraujo eritrocituose padidëjo 6,7 karto, paveikus bisfenoliu A, 9,4 karto – dialilftalatu, ir 11,8 karto – tetrabromfenileteriu eksperimente. Atlantiniø menkiø kraujo làstelëse ir otø inkstø làstelëse visi trys tirti junginiai statistiðkai patikimo mikrobranduoliø kiekio padidëjimo neindukavo. Rûðiai specifinis atsakas á bisfenolá ir dialilftalatà gautas tiek kraujo, tiek inkstø eritrocituose, tiriant MB testu. Abiejø rûðiø þuvyse po poveikio tetrabromodifenileteriu-47 MB daþnio skirtumai nustatyti, tiriant eritrocitus ið skirtingø (kraujo ir inkstø) audiniø.

Raktaþodþiai: mikrobranduoliai, menkë, otas, bisfenolis A, dialilftalatas, tetrabromodifenileteris-47