THE LEVEL OF DNA DAMAGE
AND THE FREQUENCY OF MICRONUCLEI
IN HAEMOLYMPH OF FRESHWATER MUSSELS
ANODONTA WOODIANA EXPOSED
TO BENZO[A]PYRENE

ABSTRACT

Introduction: Monitoring toxicant-induced changes in DNA organization through the detection of DNA breaks provides a powerful approach to toxicological screening.

Material and Methods: Large freshwater mussels Anodonta woodiana were exposed to two concentrations of benzo[a]pyrene per 4 and 7 days. The comet assay and the micronucleus test were used for genotoxicity analysis.

Results: A higher level of cell damage was observed after 4 days of exposure than after 7 days of exposure in both studied concentrations of benzo[a]pyrene.

Conclusions: The mussel A. woodiana-based assay to detect genotoxic potential of b[a]p and possibly other PAHs seems to have broad applications to aquatic toxicology and environmental monitoring.

Key words: B[a]P, freshwater mussels, chromatin damage

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INTRODUCTION

The evaluation of toxic effects is an important tool in the characterization of environmental samples. The degree of DNA damage may serve as a useful parameter for the evaluation of genotoxic potential of particular environmental pollution [1]. Genotoxic substances may induce the formation of the so called adducts in a single DNA thread or cause DNA breaks. Monitoring toxicant-induced changes in the sequence and organization of DNA through the detection of chromosome aberrations (e.g. micronuclei in interphase cells), provides a powerful approach to toxicological screening [2].

Freshwater mussels store toxicants in their tissues and pseudofaeces and may play a key role in maintaining water quality [3,4]. Freshwater mussels have been included in “mussel watch” programs with their marine counterparts [5]. The zebra mussel (Dreissena polymorpha) was used for genotoxicity testing of different chemicals by micronucleus test [6,7,8]. Recently, Pavlica et al. [9] have used comet assay on zebra mussel. The comet assay offers considerable advantages over other cytogenetic methods for DNA damage detection, because the cells studied do not need to be mitotically active [9].

The current investigation was designed to develop a genotoxic assay based on large freshwater mussel Anodonta woodiana. We report a system (micronucleus test and comet assay test) to study the genotoxicity of polycyclic aromatic hydrocarbons (PAHs) using the freshwater mussels’ haemolymph.

MATERIAL AND METHODS

Animals

Chinese mussels (Anodonta woodiana) of 10–15 cm in length were collected from Konin lakes system (Pątnowskie Lake and Wąsoski Channel) in Poland. For the experiment mussels were transferred into aerated glass tanks containing 6 dm³ of dechlorinated tap water. The temperature of the water ranged from 18 to 20°C. Six animals were used in each exposure study tank.

Treatment

Two concentrations of benzo[a]pyrene (Fluka, 2000285) were chosen: 75 ppb and 750 ppb, because the statistically significant increase of micronuclei number was described for benzo[a]pyrene concentrations as ranging from 50 ppb to 1000 ppb in marine mussel Mytilus galloprovincialis [10]. Two samples were used as controls (tap water and 0.005% DMSO — as a solvent control). The exposure period was 7 days. Haemolymph for comet assay was collected twice (after 4 days of the experiment and at the end of the experiment). Additional volume of haemolymph for micronucleus test was collected only at the end of the experiment, just before sacrificing the mussels. Hydrogen peroxide (500 mM) was used as positive control.

Comet assay

For the comet assay haemolymph of Chinese mussels (Anodonta woodiana) was collected in darkness from both posterior and anterior adductor muscles with a syringe. Comet assay was conducted as described by Singh et al. [11] with slight modifications. Frosted slides were precoated with 1% agarose (normal melting point; NMA) in PBS. After agarose was solidified, aliquots of 1 ml of haemolymph were centrifuged in Eppendorf tubes at 1000 rpm for 10 minutes. The supernatant was discarded and the pellet was mixed with 65 µl of 0.5% agarose (low melting point; LMA) and placed on the first agarose layer. A coverslip was added and agarose was allowed to solidify for 10 minutes on ice. After removing the coverslip, the third layer of 0.5% LMA agarose was added and left to solidify on ice. The coverslip was removed and the cells were lysed in freshly made lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris·HCl, 1% sodium sarcosinate, 1% Triton X-100 and 10% DMSO, pH = 10.0) for 1 h at 4°C. After lysis, the slides were placed on a horizontal gel box and covered with cold (about 4°C) alkaline buffer (10 N NaOH and 200 mM EDTA, pH = 12.3). The slides were left in the alkaline buffer for 20 minutes to allow the unwinding of DNA. Slides were electrophoresed at 25 V and 300 mA for 15 minutes. After electrophoresis, slides were neutralised three times for 5 minutes with cold neutralisation buffer (0.4 M Tris·HCl, pH = 7.5), stained with 20 µg/ml ethidium bromide and examined the same day using a Nikon Optiphot 2 epifluorescence microscope. For each animal 100 cells per slide (500 per sample) were visually scored at random and given an empirical score depending on the degree of damage they exhibited. The damage categories were allocated according to the approximate percentage of DNA in tail as described by Wilson et al [12]. As a positive control, a sample with 500 µM H₂O₂ was used.

Cell viability of control animals evaluated by 5–6 carboxyfluorescein diacetate (CFDA) with ethidium bromide ranged from 96% to 100%. This test was done in order to demonstrate that the procedures of cell isolation do not harm the cells [12,13].
Micronucleus test
For micronucleus test 1 ml of haemolymph was mixed with 0.3 ml of fixative (10% acetic acid in methanol) and centrifuged at 1000 rpm for 5 minutes. After fixation/centrifugation period the supernatant was discarded and the pellet was smeared onto a microscope slide and allowed to dry. After that the slides were fixed in methanol for 10 minutes, air dried and stained with 5% Giemsa in distilled water for 20 minutes. For each animal 250 cells were counted, which is the minimum required for satisfactory statistical evaluation of the results. In order to take into account possible heterogeneity of the smear, some fields in topographically separated areas of the slide were chosen.

Statistical analysis of the differences between samples was performed by Kruskal-Wallis test. Two levels were considered significant: \( p \leq 0.05 \) (*) and \( p \leq 0.01 \) (**).

RESULTS AND DISCUSSION
The current study was the performance of genotoxicity screening assay on freshwater mussel, *Anodonta woodiana*, to explore the capacity of this organism to detect the presence of genotoxic compounds.

Figure 1 shows the level of damaged cells \( (L_d) \) expressed as a total percentage of all (low, medium, high and extreme) damaged cells after 4 and 7 days of exposure *Anodonta woodiana* to two benzo[a]pyrene concentrations. The \( L_d \) values at the solvent (DMSO) were no different from those observed at tap water control after the first and the second haemolymph collection. The mussels exposed to 75 ppb and 750 ppb of benzo[a]pyrene showed more damaged cells. The \( L_d \) values were significantly different from either controls \( (p \leq 0.01) \) after 4 days of exposure to both b(a)p concentrations. On the last day of the experiment, however, the increase in the number of damaged cells was significant \( (p \leq 0.05) \) only in the higher (750 ppb) of the studied concentrations (Fig. 1). The positive control showed \( L_d = 100 \) (only highly and extremely damaged cells were observed).

The results of the other, micronucleus test obtained after 7 days of exposure to benzo[a]pyrene in Chinese mussel’s haemocytes (Tab. 1), were congruent with those obtained through comet assay. The presence of micronucleated cells was noticed mainly in the samples exposed to benzo[a]pyrene, but significant differences in the case of micronuclei were observed only between 750 ppb of b[a]p treated mussels and the control samples \( (p \leq 0.05) \).

Benzo[a]pyrene is a well-known mutagenic pollutant. The micronuclei formation by this compound was described by numerous authors in marine mussels [10,14] and freshwater fishes [15,16]. The response to benzo[a]pyrene treatment seems to be much lower in mussels than in fishes. Venier et al. [10] described the frequency of micronuclei in haemocytes of *Mytilus galloprovincialis* in 48h treatment of different benzo[a]pyrene concentrations. The frequency of micronuclei was rather low (from about 0.2% to about 1.6%) [10].

Interestingly, the comet assay test used in the current study on *Anodonta woodiana* showed that the mussels exposed to different but high concentrations of b[a]p for shorter duration (4-day period), expressed hemocyte cell

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of individuals examined</th>
<th>No. of cells examined</th>
<th>Total number of micronuclei (MN)</th>
<th>Frequency [%] of MN (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_0 )</td>
<td>5</td>
<td>5000</td>
<td>0</td>
<td>0.0±0.00</td>
</tr>
<tr>
<td>( K_{DMSO} )</td>
<td>5</td>
<td>5000</td>
<td>2</td>
<td>0.40±0.55</td>
</tr>
<tr>
<td>75 ppb b[a]p</td>
<td>5</td>
<td>5000</td>
<td>14</td>
<td>2.80±1.48</td>
</tr>
<tr>
<td>750 ppb b[a]p</td>
<td>5</td>
<td>5000</td>
<td>20</td>
<td>4.00±1.58*</td>
</tr>
</tbody>
</table>

* \( p \leq 0.05 \).
damage to a higher degree than those exposed for a longer time (7-day period) (Fig. 1). Similar results were obtained for the seawater mussel 

Mytilus edulis [17]. Whereas the authors suggest that mussels exposed to relatively high concentrations of different PAHs for the long time are able to react to the contaminated environment to prevent ongoing genotoxic damage, the mechanisms responsible for this adaptation are unknown at present.

The results of the present study point out the sufficiency of the mussel A. woodiana-based assay to detect genotoxic potential of benzo[a]pyrene and possibly other PAHs, with broad applications to aquatic toxicology and environmental monitoring. However, the period of treatment seems to be an important factor in adaptation to the toxicant [17].

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TEST KOMETOWY I MIKOJĄDROWY U MAŁŻY SŁODKOWODNYCH ANODONTA WOODIANA PODDANYCH DZIAŁANIU BENZO[A]PYRENU

Streszczenie


REFERENCES

16. Sanchez P, Llorente MT, Castano A. Flow cytometric detection of micronuclei and cell cycle alterations in fish-derived cells after exposure to three model genotoxic agents: mita-