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A. Fučić¹, R. Stojkovic², J. Katić¹, D. Markovic³, Z. Ferencic⁴, M. Koršić⁵, A.M. Jazbec⁶ and M. Gamulin⁵

¹Institute for Medical Research and Occupational Health, Zagreb, Croatia
²Division of Molecular Medicine, Rudjer Bošković Institute, Zagreb, Croatia
³GlaxoSmithKline Research Centre Zagreb Ltd., Zagreb, Croatia
⁴Children’s Hospital “Srebrnjak” and ⁵University Hospital Zagreb, Zagreb, Croatia
⁶Faculty of Forestry, University of Zagreb, Zagreb, Croatia

Abstract

Environmental xenoestrogens pose a significant health risk for all living organisms. There is growing evidence concerning the different susceptibility to xenoestrogens of developing and adult organisms, but little is known about their genotoxicity in pre-pubertal mammals. In the present study, we developed an animal model to test the sex- and age-specific genotoxicity of the synthetic estrogen diethylstilbestrol (DES) on the reticulocytes of 3-week-old pre-pubertal and 12-week-old adult BALB/CJ mice using the in vivo micronucleus (MN) assay. DES was administered intraperitoneally at doses of 0.05, 0.5, and 5 µg/kg for 3 days and animals were sampled 48, 72 and 96 h, and 2 weeks after exposure. Five animals were analyzed for each dose, sex, and age group. After the DES dose of 0.05 µg/kg, pre-pubertal mice showed a significant increase in MN frequency (P < 0.001), while adults continued to show reference values (5.3 vs 1.0 MN/1000 reticulocytes). At doses of 0.5 and 5 µg/kg, MN frequency significantly increased in both age groups. In pre-pubertal male animals, MN frequency remained above reference values for 2 weeks after exposure. Our animal model for pre-pubertal genotoxicity assessment using the in vivo MN assay proved to be sensitive enough to distinguish age and sex differences in genome damage caused by DES. This synthetic estrogen was found to be more genotoxic in pre-pubertal mice, males in particular. Our results are relevant for future investigations and the preparation of legislation for drugs and environmentally emitted agents, which should incorporate specific age and gender susceptibility.

Key words: Diethylstilbestrol; Estrogen; Puberty; In vivo micronucleus assay; Sex; Genotoxicity

Introduction

Estrogen is a paracrine and endocrine hormone, and probably also a neurotransmitter (1) with carcinogenic properties in animals and humans (2). Human exposure to xenoestrogens is alarming. Compounds, which express estrogen-like biological effects, are present in the environment in a variety of forms, such as metalloestrogens, pesticides, polychlorinated biphenyls, pharmacological estrogens, phytoestrogens, and heavy metals (3,4). Recent studies have shown that a number of radioactive, toxic, and/or genotoxic agents such as arsenic, cadmium and uranium also have estrogen-like activity. In transplacental exposure, these agents can cause cancer through mechanisms such as methylation (4,5). In the environment, they create complex environmental xenobiotic mixtures, and it is crucial to understand the effects of their interaction on organisms, especially during development.

As regulatory molecules, estrogens have been associated for decades with development, homeostasis, or pathology of the reproductive system. Growing evidence about the distribution of estrogen receptors in different mammalian tissues (6) suggests that estrogens could be central in orchestrating a number of pathways in developing and adult organisms (7).

New biomedical methods and data have shown that hormones such as estrogens may act as carcinogens at non-physiological doses (2,8). In addition, by decreasing glutathione-S-transferases, estrogens may increase oxidative DNA damage in estrogen-responsive tissues if the organism is simultaneously exposed to other genotoxicants, and launch carcinogenesis (9).
About 65 agents, mostly industrial products, are known today to express estrogen-like activity (10), although estimates of the number of such compounds vary (11). One such agent is the synthetic estrogen diethylstilbestrol (DES). DES has been used to reduce the risk of miscarriage, but was banned when it was demonstrated that this drug induced neoplasms and reproductive organ malformations, decreased fertility, and led to immune system disorders in transplacentally exposed mammals of both sexes, including humans (10,12).

The reactive genotoxic intermediate of DES is DES quinone (DES Q), which binds to DNA and forms chemically unstable adducts. DNA adducts caused by DES are 4-6 times more frequent in females than in males (13). DES also causes mitochondrial DNA damage specifically attacking cytochrome c oxidase (14).

In addition to chromosome rearrangements, the literature has reported the aneugenic potency of DES (8,15). Similar to colchicine, DES inhibits the intra-chain cross-linking of tubulin (16). Neonatal exposure to DES may cause disturbances of gene ontogeny by demethylation, with a transgenerational effect (12).

In mammals, DES also acts as an estrogen-like compound, and affects the kidney, prostate, vagina, and cervix, causing microsatellite instability and enhancing tyrosine phosphorylation, which may affect cellular integrity and cell cycle control (17).

Since the living environment is polluted with xenoestrogens, by-products of industrial or chemical processing that have estrogen-like effects, with unknown genome damage during development, we introduced and tested a pre-pubertal animal model for genotoxicity testing using DES as a xenoestrogen with a well-known pharmacodynamic and genotoxic mechanism. Pre-pubertal and adult animals were compared using the in vivo micronucleus (MN) assay (18), which can detect clastogen and aneugen damage (Figure 1). Additionally, the model is used to determine if genome damage caused by DES differs between the sexes.

**Material and Methods**

The study included 3-week-old young and 12-week-old adult BALB/CJ mice obtained from a breeding colony of the Ruder Bošković Institute (Zagreb, Croatia). During the experiment, animals were housed 4 to a cage. The bottom of the cage was covered with sawdust (Allspan®, Germany). Standard food for laboratory mice (4 RF 21 GLP Mucedola srl, Italy) was used. All animals had free access to food and water. Animals were kept under standard conditions with a 12-h light/dark cycle, temperature of 22°C, and 55% humidity. All experiments were performed according to the ILAR Guide for the Care and Use of Laboratory Animals, Council Directive (#86/609/EEC) and Croatian Animal Protection Act (#NN 135/06).

After the baseline MN frequency was established, each group of animals received 0.05, 0.5 or 5 µg/kg of DES (Sigma, USA) intraperitoneally (ip) for 3 consecutive days. Olive oil was used as a solvent. Treated animals were divided into groups of 5 each by age, sex, and treatment dose. In addition, one group of adult animals and one group of young animals of both sexes (5 animals each) served as positive control and received a single injection of 75 mg/kg cyclophosphamide (Sigma). One group of adult animals and one of young animals of both sexes (5 animals each) served as negative control and received olive oil injections for 3 consecutive days. The animals were sampled 48, 72 and 96 h, and 2 weeks after dosing.

Peripheral blood was collected from the tail vein (5 µL per sample) from all animals. Blood smears were prepared on acridine orange-coated slides, covered with a coverslip and analyzed according to Hayashi et al. (18). MN frequency was analyzed in 1000 reticulocytes per sample. Although

**Figure 1.** Reticulocyte stained with acridine orange (A) and reticulocyte-containing micronucleus (B).
usually there is only one micronucleus per reticulocyte, very rarely two micronuclei can be detected.

Analysis of variance (ANOVA) for repeated measurements was used to compare responses at each sampling time. Doses and groups were analyzed as fixed effects. The level of statistical significance was set at 5% (P < 0.05). Calculations were performed using the Statistica 6.0 software.

Results

Positive control animals exposed to cyclophosphamide showed a significant increase in MN frequency (61.4 ± 5.4 MN/1000 reticulocytes) 48 h after treatment. The frequencies at other sampling times were similar to the baseline frequency (0.77-1.33 MN/1000 reticulocytes). Negative control animals exposed to olive oil showed no differences from baseline values (0.25-1.25 MN/1000 reticulocytes).

Three-week-old male and female mice showed a significant increase in MN frequency (5.2/1000 and 5.6/1000 reticulocytes, respectively) 48 h after receiving 0.05 µg/kg DES. At 72 h, MN frequency had returned to baseline values in females, while in males it remained increased for as long as 2 weeks after exposure. In 12-week-old animals, MN frequency remained at baseline values (Figure 2).

At the dose of 0.5 µg/kg, MN frequency significantly increased regardless of age or sex (Figure 3). The highest frequency was detected 72 h after exposure in both age groups and sexes. In 3-week-old animals, MN frequency was not significantly higher than in 12-week-old animals. In adult animals, MN frequency dropped back to baseline values 96 h after exposure. Three-week-old females returned to baseline values 2 weeks after exposure, while in males MN frequency remained increased.

At the dose of 5 µg/kg, MN frequency increased in both age groups and sexes, peaking at 72 h in adults and at 48 h in 3-week-old animals (Figure 4). There was no significant difference in peak values between 3- and 12-week-old males. Three-week-old females had a significantly higher MN frequency than 12-week-old females.

Two weeks after exposure, MN frequency remained significantly increased only in 3-week-old males (Figure 4). There was a significant difference (P < 0.001) in MN elimination between age groups for all doses. Males had a significantly (P < 0.001) higher MN frequency than females (Table 1).

Some cells in the exposed animals, regardless of DES concentration, animal age, or sex, had several micronuclei in one reticulocyte.

Discussion

Estrogen is a hormone with genotoxic potential, which acts through its quinone metabolites. By inducing oxidants and aldehydes, they create mutagenic apurinic sites in DNA and cause lipid peroxidation (2).
Figure 3. Micronucleus (MN) frequency in A, 3- and B, 12-week-old male (blue full line) and female (red dashed line) mice exposed to 0.5 µg/kg diethylstilbestrol. For each dose, age and sex, 5 animals were analyzed. Micronucleus frequency is presented per 1000 reticulocytes. Vertical bars indicate 95% confidence intervals. ANOVA for repeated measurements was used to compare responses at each sampling time.

Figure 4. Micronucleus (MN) frequency in A, 3- and B, 12-week-old male (blue full line) and female (red dashed line) mice exposed to 5 µg/kg diethylstilbestrol. For each dose, age and sex, 5 animals were analyzed. Micronucleus frequency is presented per 1000 reticulocytes. Vertical bars indicate 95% confidence intervals. ANOVA for repeated measurements was used to compare responses at each sampling time.
Table 1. Descriptive statistics regarding micronucleus frequency in 3- and 12-week-old mice exposed to 0.05, 0.5, and 5 µg/kg diethylstilbestrol 48, 72 and 96 h and 2 weeks after exposure.

<table>
<thead>
<tr>
<th></th>
<th>0.05 µg/kg</th>
<th>0.5 µg/kg</th>
<th>5 µg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M 3 weeks</td>
<td>M 12 weeks</td>
<td>F 3 weeks</td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.31</td>
<td>1.00 ± 0.60</td>
<td>0.66 ± 0.33</td>
</tr>
<tr>
<td>48 h</td>
<td>5.20 ± 0.20</td>
<td>5.60 ± 0.83</td>
<td>4.25 ± 0.60</td>
</tr>
<tr>
<td>72 h</td>
<td>3.00 ± 0.22</td>
<td>1.60 ± 0.50</td>
<td>5.00 ± 0.60</td>
</tr>
<tr>
<td>96 h</td>
<td>3.20 ± 0.24</td>
<td>3.20 ± 0.58</td>
<td>2.75 ± 0.24</td>
</tr>
<tr>
<td>2 weeks</td>
<td>3.40 ± 0.37</td>
<td>1.80 ± 0.48</td>
<td>4.00 ± 0.48</td>
</tr>
</tbody>
</table>

Data are reported as means ± SD per 1000 reticulocytes. Five animals were analyzed for each dose, sex, and age group. M = male; F = female. *P < 0.005 for exposed animals compared to baseline frequencies (ANOVA for repeated measurements).

Estrogen-induced kidney tumors in animal models are characterized by highly aneuploid cells (92-94%) as a consequence of centrosome amplification related to the overexpression of Aurora A and B proteins (19), which probably involves caspase 3 (20). The ability of estrogen to interfere with tubulin polymerization is not related to its hormone activity (21).

DES is a well-known xenoestrogen that was used to prevent miscarriage between 1940 and 1971, when its application was banned because of the association established between in utero exposure and increased cancer incidence and non-neoplastic abnormalities of the reproductive system (22). Diethylstilbestrol seems to act through estrogen alpha receptor proteins (23). Like estrogens, this drug acts as a clastogen and aneugen through its quinone metabolites (21,22,24).

In the present study, we used a starting dose of 5 µg/kg, reported to be genotoxic (25), and reduced it to 0.5 and 0.05 µg/kg to find the threshold of its genotoxic activity on animals of pre-pubertal and adult age.

For the first time, we have shown a clear difference between age and sex in the level of genotoxic damage caused by DES. At 0.05 µg/kg, only pre-pubertal animals showed a significantly higher MN frequency, while this frequency did not differ from the negative control in adult animals. However, the higher dose of 0.5 µg/kg caused a significantly higher frequency of MN in both pre-pubertal and adult mice and could be suggested to be a threshold for adult animals. In adult animals, MN frequency reached control values 96 h after exposure, while in pre-pubertal animals it remained high for 2 weeks after exposure. Higher long-term genome damage detected in 3-week-old males may be the consequence of genome instability described after exposure to DES (26). One of the mechanisms responsible for this instability could be DNA hypomethylation (27). Estrogen-induced DNA methylation shows age-related differences in animals (28). Hypomethylation can also be related to aneuploidy since it impairs the function of the DNA-binding proteins in the satellite region of the centromere (29). This suggests that aneuploidy associated with exposure to endocrine disruptors (19) could also have its origin in their demethylating potency. Methylation is also age-dependent. Neonatal exposure to DES causes permanent disturbances in the level of DNA methylation (30). However, this phenomenon has not been investigated in puberty, which is the final stage of maturation directed by hormones.

In the present study, the mitotic arrest detected in adult animals probably reached the peak in MN frequency at 72 h after exposure, while in young animals the highest MN frequency was detected 48 h after exposure to 5 µg/kg (at the dose of 0.5 µg/kg the difference in MN frequency between 48 and 72 h post-dose was not significant). In an in vitro model, DES was reported to cause mitotic arrest (31). The age difference in the level of mitotic arrest may be explained by the higher cell turnover in young animals.

Since the genotoxic activity of DES is similar to that of estrogen, our results show that pre-pubertal animals are more susceptible to genotoxic estrogen actions and that males are more susceptible than females.

The reason for the age- and sex-related difference needs to be elucidated. Pre-pubertal girls have eight times higher estrogen levels than boys of the same age (32). Additionally, sexual dimorphism in metabolism has been reported for a number of xenobiotics (33,34). However, most studies still lack data about the age- and sex-specific
metabolism of xenobiotics, and this leaves no other option to researchers but to use models or approximations based on measurements in adults (34,35). Sex-specific DNA adduct production has been described in animal models. After exposure to estradiol, liver adduct formation was detected in male, but not in female, rats (36). Quinone reductase is known to reduce toxic quinones (26), which are the metabolites of estrogen and DES. The levels of this enzyme are age- and sex-specific, and as they are lower in males, this may contribute to a higher level of DES-induced free radicals in them than in females. In rats, higher levels of NADPH quinone reductase were detected in females than in males (37). This difference may be more pronounced in the pre-pubertal period. A sex-specific impact on metabolism has also been described for bisphenol A, an agent similar to DES (38).

The model proposed here may be incorporated into the Tier I test, the assay for pubertal female rodents designed to screen for xenoestrogen activity and recommended by the Endocrine Screening and Testing Advisory Committee (39,40).

Like transplacental genotoxicology, pubertal genotoxicology is a complex issue. Both developmental periods require application of tools for biology systems, including pattern recognition techniques, cluster analysis, component pathway analysis, and cognitive networks in order to obtain a reliable interpretation.

References