Usefulness of immature golden hamster (Mesocricetus auratus) as a model for uterotrophic assay

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Abstract

The present study assessed the sensitivity of immature hamster uterotrophic assay to reference oestrogen agonists/antagonists in order to develop a sensitive model for evaluation of endocrine-active compounds in diets. After performing a baseline for control animals, the sensitivity of immature females (postnatal day 18) to reference compounds was evaluated in a three-day uterotrophic assay. The absolute and adjusted dry uterine weights, fold induction over control for absolute wet uterine weight, and wet uterine weight/body weight ratio (%) were used as endpoints. The significantly active doses for reference oestrogens were as follows: 0.6 µg/kg for 17α-ethinyloestradiol (s.c.); 1 µg/kg/day (s.c.) and 40 µg/kg (p.o.) for diethylstilboestrol; 40 mg/kg (s.c.) and 160 mg/kg (p.o.) for bisphenol A. Co-treatment with tamoxifen at a dose of 1 mg/kg significantly antagonised the uterotrophic effect induced by 1 µg/kg 17α-ethinyloestradiol, and showed the attenuated proliferative effect in histopathological examination. We found immature hamster uterotrophic assay as a sensitive model that could be a good alternative to the rat assay.

Keywords: immature golden hamster, uterotrophic assay, oestrogens, sensitivity.

Introduction

The rodent uterotrophic bioassay, long considered as the “gold standard” for determining oestrogenicity, is identified as a preferred in vivo screen. The assay is based on the principle that the growth phase of the uterus in the natural oestrous cycle is under control of oestrogens. When the natural source of oestrogens is not available, either because the animal is immature or because it has been ovariectomised, then the growth of the uterus becomes sensitive to external sources of oestrogens. The increase in its weight is due to the imbibition of fluid (primary response) and cell proliferation (secondary response). Chemicals that act as oestrogen agonists would be expected to cause a statistically significant increase in the uterine weight, while oestrogen antagonists, when co-administered with a potent reference oestrogen, would be expected to decrease the uterine growth. Up to now the preferable model is rat, the species commonly used in toxicological testing. The rat uterotrophic assay was validated and accepted as a regulatory screening assay for oestrogenicity by the Organization for Economic Co-operation and Development (OECD) (22) and US Environmental Protection Agency (EPA) (29). The OECD and US EPA differ in their preference for model and route when conducting the uterotrophic assay; the OECD recommends the immature rat due to animal welfare concerns regarding survival surgery (19). The immature model detects indirect- as well as direct-acting oestrogenic/anti-oestrogenic chemicals and mixtures (28). Moreover, it seems to be more sensitive than ovariectomised adult model (11).

Although much attention has been focused on identifying protocol variables and reproducibility among laboratories (12, 13, 14, 16, 23, 24), direct comparison of uterine responses among rodent species has been rarely...
addressed (26). However, it is expected that the risk to humans takes into account the results obtained in the most sensitive species (26, 30).

Our experiences in using golden hamster for embryotoxicity investigations showed that this species is more sensitive to teratogenic stimuli than rats (19). It seemed justified to provide an experimental proof of the usefulness of golden hamster in tests assessing the endocrine disrupting effect. Moreover, Hendry et al. (8) described some unique advantages of the hamster as a model for perinatal endocrine disruption. With respect to these findings, in the present study we evaluated whether the immature hamster may be used as a model for the uterotrophic assay. Therefore, the aim of our study was to perform baseline for the control animals and then to evaluate the sensitivity of immature female hamsters to the representative reference compounds.

Material and Methods

Chemicals and dosage formulations. The following chemicals: 17α-ethinyl-oestradiol (EE2, CAS no. 57-63-6, purity ≥98%), diethylstilboestrol (DES, CAS no. 56-53-1, purity 99%), bisphenol A (BPA, CAS no. 80-05-7, purity 99%), tamoxifen (TAM, CAS no. 10540-29-1, purity ≥99%) were purchased from Sigma. Stock solutions of test substances were prepared in a small amount of 96% ethanol (POCh Gliwice, Poland) and kept in brown glass containers at -4°C. To obtain the required dosing concentrations, formulations were prepared by dissolution of the test chemicals in corn oil (CAS no. 8001-30-7, Sigma). Formulations were prepared daily and used on the day of preparation.

Animals, housing, diet. Hamsters were housed in clear polypropylene cages (48 × 26.5 × 21 cm) and under controlled conditions; temperature (22 ± 2°C), lighting (light: dark cycle 14 h:10 h), and humidity (40%-60%). Tap water and certified hamster free diet (Altromin) were provided ad libitum. Timed pregnant females delivered their young on day 16 of gestation. Female pups were weaned on the postnatal day (PND) 18 and housed six per cage for uterotrophic assays.

Uterotrophic assays. On the day of weaning, the animals were weighed and the initial weight variations of the animals within a group were less than ±20% of the mean weight. The females, six per group, received three consecutive doses of chemicals (experimental groups) or corn oil (vehicle control groups). Approximately 24 h after administration of the last dose the animals were euthanized by an overdose of pentobarbital. The uterus and vagina were carefully dissected free from the adhering fat. Uteri were weighed immediately (wet weight) and after drying at 60°C for at least 24 h (dry weight). The experimental protocol was approved by the Local Ethics Committee at the University of Life Sciences in Lublin.

Historical control data for 3-day immature hamster uterotrophic assay. Initial (18 PND) and terminal (21 PND) weights were recorded in addition to the uterine weights: wet/dry. Wet uterine weight:body weight ratios were calculated for each animal by dividing wet uterine weight by the body weight and multiplying by 100.

Uterotrophic assays for oestrogen agonist and antagonist. Evaluation of the sensitivity of immature hamsters to the most frequently used reference oestrogen agonist, ethinylestradiol (EE2) and antagonist, tamoxifen (TAM) was performed. EE2 was administered s.c. at the doses of 0.1, 0.3, 0.6, 1, 3, 10, and 100 µg/kg b.w./day. The control animals received corn oil in a volume of 2 mL/kg s.c. The reference antagonist tamoxifen was co-administered s.c. with EE2 (1 µg/kg b.w./d s.c.). Four doses of TAM were applied, 0.1, 0.5, 1, and 2 mg/kg b.w./d. The control group consisted of females given EE2 s.c. at the dose of 1 µg/kg b.w./d. The representative uteri and vaginas from the EE2 and TAM groups were chosen for histopathological examination.

Uterotrophic assays for DES and BPA after either subcutaneous or oral exposure. DES was administered s.c. at the doses of 0.05, 0.1, 0.5, 1, 5, and 10 µg/kg b.w./day and p.o. at 5, 10, and 40 µg/kg b.w./day. BPA was applied at the doses of 8, 40, and 160 mg/kg b.w./d. (p.o. and s.c.).

Histological examination. The uterus and vagina were fixed in 10% neutral buffered formalin, and then routinely processed into paraffin blocks from which 5 µm sections were cut and stained with haematoxylin and eosin (H & E). The sections were evaluated for pathological changes using conventional light microscopy. Each stained section was semi-quantitatively evaluated for the changes observed in the uterus and vagina.

Statistical analysis. The results are presented as a mean ±SD. The statistical significance of the difference between the mean values was evaluated with analysis of variance followed by the Dunnett test.

Results

Historical cumulative data. Generally, the control values were consistent among the multiple uterotrophic assays that were performed over three years (2010-2012). Data (mean ±SD) for initial (18 PND) and terminal (21 PND) body weights ranged from 13.4 to 17.2 g and from 19.5 to 22.6 g respectively (Table 1). For uterine weights the values ranged from 13.8 to 17.0 mg. Wet uterine weight constituted 0.068% to 0.079% of body weight.

Sensitivity of immature hamsters to ethinylestradiol (EE2) and tamoxifen (TAM). The active dose of EE2, at which statistical significance was first achieved started at 0.6 µg/kg (Fig. 1). Similar
effects were observed after treatment with two higher doses used in this study, i.e. 1 and 3 µg/kg (Fig. 1).

Histological examination of the uterus and vagina showed changes in both organs (Fig. 3 C-D). In the uterus, there was moderate oedema of the endometrium, characterised by separation of the endometrial stromal cells, moderate endometrial epithelial and glandular hyperplasia, and hypertrophy of the smooth muscle cell layers (Fig. 3 C). Squamous hyperplasia and cornification of the vaginal epithelium was observed only focally in one case. In the vaginal epithelium mostly moderate hyperplasia and moderate mucification was observed (Fig. 3 D).

Significant antioestrogenic activity of TAM was recorded at the dose of 1 mg/kg co-treated with EE$_2$ (1 µg/kg) (Fig. 2). The uterus showed mild oedema of the endometrium, moderate endometrial epithelial and glandular hyperplasia, and mild myometrial hypertrophy (Fig. 3 E). In the vaginal epithelium mostly moderate hyperplasia and moderate mucification was observed (Fig. 3 F). Squamous hyperplasia and cornification of vaginal epithelium was not observed in any of the cases in this group.

**Sensitivity of immature hamsters to diethylstilboestrol (DES) and bisphenol A (BPA).** The doses at which DES induced a significant increase in the dry uterine weights, absolute and relative, was 1 µg/kg/day (s.c.) and 40 µg/kg (p.o.) (Table 2). The mean weights of absolute wet uterus in these groups were 1.37-fold and 2.05-fold over control, respectively. As suspected, BPA showed a weak uterotrophic activity. Significant uterotrophic effect (an increase in absolute and relative dry uterus weights) was recorded at the dose of 40 mg/kg (s.c) (Table 2). After oral dosing of 160 mg/kg of BPA absolute were significantly increased, as opposed to relative weights. The mean weights of absolute wet uterus in these groups were 1.64-fold and 1.76-fold over control respectively.

**Table 1.** Historical data values (mean ± SD) for five vehicle treated control groups of immature female hamsters performed in uterotrophic assays

<table>
<thead>
<tr>
<th>Date of experiment</th>
<th>Body weight, g</th>
<th>WUW/BW (%)</th>
<th>Uterine wet weight</th>
<th>Uterine dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Terminal</td>
<td>Absolute, mg</td>
<td>mg/100g</td>
</tr>
<tr>
<td></td>
<td>Terminal</td>
<td></td>
<td>mg/100g</td>
<td></td>
</tr>
<tr>
<td>22.11.2010 (n = 6)</td>
<td>17.2 ± 1.7</td>
<td>21.6 ± 2.3</td>
<td>0.073 ± 0.015</td>
<td>15.8 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>21.0 ± 2.7</td>
<td></td>
<td>0.071 ± 0.015</td>
<td>22.1 ± 2.9</td>
</tr>
<tr>
<td>08.01.2011 (n = 6)</td>
<td>13.4 ± 2.2</td>
<td>19.5 ± 4.6</td>
<td>0.071 ± 0.02</td>
<td>14.2 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>20.2 ± 5.1</td>
<td></td>
<td>0.071 ± 0.02</td>
<td>16.9 ± 5.0</td>
</tr>
<tr>
<td>15.05.2011 (n = 6)</td>
<td>14.9 ± 2.8</td>
<td>21.4 ± 3.7</td>
<td>0.079 ± 0.023</td>
<td>17.0 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>21.0 ± 3.6</td>
<td></td>
<td>0.079 ± 0.023</td>
<td>17.8 ± 3.2</td>
</tr>
<tr>
<td>09.07.2011 (n = 6)</td>
<td>15.2 ± 1.7</td>
<td>22.6 ± 2.2</td>
<td>0.067 ± 0.013</td>
<td>15.1 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>20.9 ± 4.5</td>
<td></td>
<td>0.067 ± 0.013</td>
<td>17.0 ± 2.7</td>
</tr>
<tr>
<td>08.03.2012 (n = 5)</td>
<td>13.7 ± 1.9</td>
<td>20.9 ± 5.7</td>
<td>0.068 ± 0.009</td>
<td>13.8 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>21.0 ± 3.0</td>
<td></td>
<td>0.068 ± 0.009</td>
<td>17.0 ± 2.6</td>
</tr>
</tbody>
</table>

Animals were weaned on postnatal day (PND) 18, dosed for three days with corn oil (2 mL/kg, s.c.) and necropsied approximately 24 h after the last injection. *WUW/BW (%) - data are presented as uterine weight/body weight ratio × 100

**Fig. 1.** The relative uterine weights (wet and dry) of immature female hamsters exposed subcutaneously to ethinyl oestadiol (EE$_2$) in a three-day (18-20 PND) uterotrophic assay. The data are expressed as the mean ± S.D. (n = 6). Significantly different from corn oil control group at *P < 0.05; ***P < 0.001
Fig. 2. Bar graphs represent the dose-response characteristics of antagonistic effect of tamoxifen (TAM) against co-administered ethinylöstradiol (EE₂), injected subcutaneously in the dose of 1 µg/kg b.w., for three consecutive days (18-20PND). The results are expressed as relative weight (mean±SD, n = 6), wet (A) and dry (B). * P < 0.05 - corn oil (control) vs EE₂; **P < 0.01; ***P < 0.001 - EE₂ vs EE₂ plus tamoxifen.

Fig. 3. Histopathological changes in the uterus and vagina of immature hamsters following subcutaneous (s.c.) treatment with 1 µg/kg/day ethinylöstradiol (EE₂) alone and EE₂ (1 µg/kg/day s.c.) + tamoxifen TAM (1 mg/kg/day s.c.) for three consecutive days (18-20PND). No oestrogenic changes were observed in the vehicle control uterus (A) and vagina (B). (C and D) changes caused by EE₂ treatment: uterus (C)-moderate oedema of the endometrial stroma (es) and endometrium (e), moderate endometrial epithelial (ee) and glandular (eg) hyperplasia, and mild myometrial (m) hypertrophy; vagina (D) - strong hyperplasia of the vaginal epithelium (ve), presence of moderate mucification and focally minimal squamous hyperplasia and cornification (asterix). (E and F) changes caused by EE₂ + TAM treatment: uterus (E)-mild oedema of the endometrial stroma (es) and endometrium (e), moderate endometrial epithelial (ee) and glandular (eg) hyperplasia, and mild myometrial (m) hypertrophy, vagina (F) - moderate hyperplasia of the vaginal epithelium (ve) and presence of moderate mucification. H & E 100x, original magnification.
Table 2. Uterine weights of immature hamsters treated for three consecutive days with diethylstilboestrol (DES) and bisphenol A (BPA) by either, subcutaneous injection (s.c.) or oral gavage (p.o.), control comprised only vehicle: corn oil

<table>
<thead>
<tr>
<th>Group</th>
<th>Doses</th>
<th>Body weight (g)</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Terminal</td>
</tr>
<tr>
<td>Control</td>
<td>Corn oil</td>
<td>15.5 ± 2.5</td>
<td>20.0 ± 4.2</td>
</tr>
<tr>
<td>0.05</td>
<td></td>
<td>16.8 ± 0.1</td>
<td>21.7 ± 1.0</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>18.4 ± 2.1</td>
<td>25.7 ± 3.1</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>20.6 ± 3.4</td>
<td>24.3 ± 3.7</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>18.2 ± 1.3</td>
<td>23.1 ± 1.7</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>20.8 ± 2.3</td>
<td>28.5 ± 2.9</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>19.1 ± 1.8</td>
<td>24.1 ± 1.8</td>
</tr>
<tr>
<td>DES µg/kg/d s.c.</td>
<td>5</td>
<td>17.6 ± 2.1</td>
<td>20.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>18.8 ± 1.2</td>
<td>21.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>18.3 ± 1.2</td>
<td>22.0 ± 2.0</td>
</tr>
<tr>
<td>DES µg/kg/d p.o.</td>
<td>8</td>
<td>11.8 ± 1.2</td>
<td>17.5 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>13.7 ± 2.7</td>
<td>21.5 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>14.8 ± 1.1</td>
<td>21.4 ± 4.3</td>
</tr>
<tr>
<td>BPA mg/kg/d s.c.</td>
<td>8</td>
<td>10.9 ± 1.6</td>
<td>15.8 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>14.8 ± 1.3</td>
<td>17.8 ± 4.1</td>
</tr>
<tr>
<td>BPA mg/kg/d p.o.</td>
<td>160</td>
<td>19.1 ± 1.8</td>
<td>27.1± 2.4</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.D. (n = 6). Significantly different from appropriate corn oil control group at *P < 0.05; **P < 0.01; ***P < 0.001

Discussion

Due to the fact that the endocrine system can be modulated by many experimental factors, the in vivo assay must be done in compliance with the rules and using appropriate indicators (2, 19).

The animal species and diets are of great importance (26, 30). Several studies demonstrated that some commonly used outbred rats and mice are less responsive to oestrogenic substances than certain inbred mouse and rat strains, at various oestrogen-sensitive endpoints. With regards to diet, many laboratory animal feeds exhibit oestrogenic activity (6, 15, 31). It was found that soy isofoavones present in feed at high amounts interfere with the value of animal models and with experimental results (10). An example of this was a decreased sensitivity of immature rats (5) and hamsters (20) in the uterotrophic bioassay.

The value of the uterotrophic assay depends also on the model used and the endpoints measured (19, 21). The adult ovariectomised model was reported to have increased specificity in comparison to the immature model (29). Conversely, the uterotrophic assay performed on the immature model was shown to be more sensitive to an increase in uterine weight evoked by oestrogenic compounds than the model of ovariectomised animals (17). In both versions the baselines for negative and positive controls are necessary for a rational interpretation of the results (19, 30).

In this study, the usefulness of immature female hamsters for uterotrophic assay was evaluated. The first set of experiments was designed to obtain a baseline for negative control regarding the most important endpoints of the assay, i.e. body weight and uterine weight. We demonstrated that the values for animal initial body weights were consistent across the studies and ranged from 13.4 to 17.2 g. The mean uterine wet weights ranged from 13.8 to 17 mg and the ratio of uterine wet weight to terminal body weight was low (ranged from 0.068% to 0.079% in different studies). These baseline uterine weights met the required criteria to yield sufficient assay sensitivity (<0.09%), which is very important for the detection of weak oestrogenic compounds (19).

In the second step of our study we evaluated the sensitivity of immature hamsters to the most frequently used reference oestrogen agonist, i.e. ethinylestradiol (EE2), and antagonist - tamoxifen (TAM). In the uterotrophic assay the uterine weight of hamsters treated with EE2 was higher than in hamsters that received the vehicle alone, and the uterine weight of hamsters treated with TAM plus EE2 was lower than that of animals treated with EE2 alone. Fixed significantly effective doses of EE2 were ≥0.6 µg/kg. Co-treatment with tamoxifen at the dose of 1 mg/kg significantly antagonised the uterotrophic effect induced by 1 µg/kg EE2. The mean uterine relative weight was approximately 60% of EE2 control value and histopathological examination confirmed attenuated...
proliferative effect. Our results are largely consistent with the literature data obtained using immature rats, in which case significant uterotrophic effects of EE2 were observed at doses from 0.3 to 3 µg/kg b.w./d (9, 12, 13, 14, 16, 24, 28) and antagonistic effects of tamoxifen at the dose of 1 mg/kg b.w./d (7).

The third stage of the present study consisted of four experiments in which the sensitivity of immature hamsters to known oestrogens, DES and BPA, was compared after either, subcutaneous (s.c.) or oral (p.o.) exposure. It was revealed that significant differences were noted at the doses which induced a wet uterine weight increase, at least 1.4-fold over control, with the uterine weight/body weight ratio higher than 0.1%. In our study immature hamsters were sensitive to the s.c. and p.o. doses of 1 and 40 µg/kg/d (DES) and 40 mg/kg/d and >160 mg/kg/d (BPA) respectively. The results obtained in the study are in line with the literature data regarding the overall uterotrophic potency i.e. strong (DES) and weak (BPA). When it comes to doses and administration routes, there is a large variation in evaluation. In previous studies on immature rats effective doses were the same after oral and subcutaneous exposure i.e. 40 µg/kg/d for DES and 400 mg/kg/d for BPA (4). In other studies, BPA is known to produce uterotrophy in immature rats at the doses of 8 mg/kg/d s.c and 160 mg/kg/d p.o. (32). Tinwell et al. (27) failed to define BPA as reproducibly active in immature mouse uterotrophic assay at the doses ranging from 0.02 to 300 µg/kg. In a more recent study, the sensitivity of immature mice and rats to s.c. injection of DES was the same as in our study on hamsters (1 µg/kg/d) (3).

A detailed analysis of a number of published studies suggests that the diversity in response depends on the endpoints measured (1, 18). Among many endpoints, adjusted uterine weight was reported to be the most appropriate to express data (25); however, the functioning opinion is that the more endpoints are introduced for assessment, the more sensitive the assay is (19, 21).

In conclusion, in our study we combined endpoints such as the absolute and adjusted dry uterine weights, fold induction over control for absolute wet uterine weight, wet uterine weight/body weight ratio (%), and optionally, histopathological changes. The results obtained indicate that under these criteria of evaluation the immature hamster uterotrophic assay is very sensitive to oestrogens and can be a good alternative to rat and mouse assays.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The experiment was approved by the 2nd Local Ethics Committee for Animal Experiments at University of Life Sciences in Lublin, Poland, consent no. 49/2009 of 9th September, 2009.

References

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