Effect of 4-octylphenol on germ cell number in cultured human fetal gonads

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This study evaluates whether a hormone disruptor found in environment, 4-octylphenol, affects the rate of proliferation of germ cells from human fetal gonads during a 3 week culture period. Five testis and five ovaries were obtained from fetuses of women undergoing legal abortions between the 6th and 9th week of fetal life, representing the period where early gonadal differentiation takes place. Each gonad was divided into equal sized test and control tissue. The test tissue was exposed to a continued presence of 10 \( \mu \text{mol/l} \) 4-octylphenol in the culture medium. The cultures were terminated by fixation of the tissues, which where then processed for histology and serially sectioned. The mitotic index of the germ cells (i.e. number of mitosis per 100 germ cells) and the number of germ cells per area was determined. Each of the five testes cultured in 4-octylphenol exhibited a significantly reduced mitotic index and number of pre-spermatogonia compared to the control, whereas none of the five ovaries exposed to 4-octylphenol revealed any difference compared to the control. It is concluded that 4-octylphenol exerts a sex-specific effect on male germ cells.

Key words: culture/fetal gonads/germ cells/human/octylphenol

Introduction

Chemicals in the environment exhibiting hormone-like activities have gained considerable attention during recent years, because of their ability to disrupt processes governed by hormones. The reproductive system seems to be especially vulnerable and, in connection with abnormally high exposures of such hormone disruptors, reproductive processes in animals, e.g. alligators, may be affected (Guillette et al., 1994). Moreover, hormone disruptors in the environment may also affect the reproductive system in humans (McLachlan et al., 1981; Colborn et al., 1993). The increased incidence of testicular cancer and malformation of genitals in humans, e.g. hypospadias and cryptorchidism, and the much debated presumed decline in sperm counts during the last decades, have been linked to exposure of substances in the environment which mimic oestriadiol – the so-called xenoestrogens (Carlsen et al., 1992; Sharpe and Skakkebaek, 1993; Toppari et al., 1996). These disorders may originate as a consequence of an inappropriate endocrine control during fetal life. The time around sex differentiation is especially important, because development of the secondary sex organs is governed by proper function of the gonads. Prenatal exposure of humans to the oestrogenic drug diethylstilboestrol (DES) induced genital tract alternations in adult life (Bibbo et al., 1977). However, it is not known whether the effect of DES or hormone disruptors is exerted on the duct systems, on the gonads or a combination of the two. Presently, there is only circumstantial evidence to suggest that hormone disruptors, found in the environment, interfere with the development and differentiation of human gonads.

Several chemicals, which mimic oestriadiol, are present in the environment. In this study 4-octylphenol (4-OPL), which is a degradation product of the widely used alkylphenol polyethoxylates (AP), was tested on human fetal gonadal tissue. 4-OPL has been studied in great detail, and has been shown negatively to affect reproductive processes of animals (Majdic et al., 1996; vom Saal et al., 1998; Raychoudhury et al., 1999). AP are used in laundry detergents and hard-surface cleaners, in paint and in connection with the use of herbicides and pesticides (Marcomini et al., 1988; White et al., 1994). These chemicals are used together with water and on fields, and have been detected in rivers, sewage treatment plants and drinking water (Stephanou and Giger, 1982; Ahel et al., 1987; Clark et al., 1992; Zoller, 1993). Consequently, humans are likely to be exposed to these chemicals. A hormone-like effect of 4-OPL has been documented by its ability to bind and activate the oestriadiol receptor (ER) in in-vitro systems (White et al., 1994; Soto et al., 1995; Arnold et al., 1996). Chronic administration of 4-OPL to adult rats resulted in a reduced size of the testis and disruption of spermatogenesis (Blake and Boockfor, 1997; Boockfor and Blake, 1997), and the
steroidogenic capacity of fetal Leydig cells obtained from fetuses where the mother was given 4-OPL in utero was reduced compared to controls, but similar to another group of animals receiving diethylstilboestrol (Majdic et al., 1996). Recently, it has been shown that 4-OPL exhibits toxic effects on cultured pre-spermatogonia and Sertoli cells from rats (Raychoudhury et al., 1999).

However, it is not known to what extent these chemicals have a negative effect on human reproduction. For obvious reasons, human fetal gonadal tissues are of limited availability. In this study, gonads of human fetuses, between the 6th and 9th week of fetal life, were obtained for in-vitro studies, after informed consent of women undergoing legal abortion. For the first time, a hormone disruptor was tested directly on human fetal gonads in vitro. The effect of 4-OPL, during a 3 week culture period, on proliferation and survival of germ cells in testes and ovaries was assessed.

Materials and methods

Human fetal gonads

Human fetuses were obtained from women referred to the Department of Gynaecology and Obstetrics, Odense University Hospital, Denmark, for legal abortion in the first trimester (6th to 12th gestational week, i.e. 4th to 10th fetal week). In all cases the women were informed orally and in writing about the aim and procedures of the project, before they gave their consent to participate by signature. The Medical Ethics Committee in the counties of Vejle and Fyn, Denmark, approved the project.

Operation technique

The operations were done according to the routine procedures for legal abortion in the department, and carried no additional risk for the participating women. In order to prevent severe damage to the fetus in the uterus, the procedure was slightly modified (Nauert and Freeman, 1994). All abortions were monitored with ultrasound equipment (Sonoline Prima; Siemens). The cervical canal was dilated, and a curette (Synevac7 Vacuum curette; Berkely Medevices Inc.) was inserted. A syringe (Becton Dickinson7 Plastipak7, 50 ml sterile syringe) was connected to the curette and a vacuum was applied manually. The recovered fetus was placed in a sterile cup with culture medium. All handling of the tissue was performed under sterile conditions. The fetuses were dissected under stereomicroscope a few minutes after they had been removed from the uterus. The gonads were identified and isolated in approximately 70% of the fetuses.

Determination of fetal age and sex

The age was determined by measuring the length of limbs and foot (Evtouchenko et al., 1996). The sex was determined by polymerase chain reaction (PCR) technique (Nakahori et al., 1991), and confirmed by the morphological appearance of the gonads prepared for histology, after culture.

Culture of the gonads

Five testes and five ovaries were obtained for the cultures. The gonadal-mesonephric-duct complex was removed from the fetus in toto and placed in sterile medium (Dulbecco’s modified Eagle’s medium, DMEM/F12; GibcoBRL) at 37°C. The mesonephric duct complex was carefully removed from the gonad. Because all gonads were too large for culture intact, they were divided into a cranial and a caudal part. One part was used as test tissue and the other part of the gonad as control tissue. It was a concern that regional differences could interfere with the results and, consequently, the decision whether to use the cranial part or the caudal part as test tissue was random. Also the decision of whether to use right or left gonad was random. The test tissue was incubated in basal culture medium (see below) containing 10 µmol/l 4-octylphenol (Bie&Berntsen a/s DK), i.e. 4-OPL medium. 4-OPL was dissolved in 96% ethanol, resulting in a final concentration of 0.96% ethanol in the medium. The control tissue was incubated in basal culture medium containing the same concentration of ethanol. Each tissue was placed on a 3 µm thick polycarbonate membrane in tissue culture inserts (Nunc™). The inserts were placed in wells of culture dishes (Nunc™). The basal culture medium was composed of DMEM enriched with 5 µg/ml transferrin (Sigma), 2 mmol/l glutamine (GibcoBRL), 20 µg/ml insulin (Sigma) and 50 IU/ml penicillin/50 µg/ml streptomycin (GibcoBRL). This culture medium was chosen after evaluation of the morphology of several human fetal gonadal tissues cultured for 1, 2 and 3 weeks. Since no or very little necrosis was present in the 3 week cultures, this culture period was studied closely in order to expose the tissue to 4-OPL for as long as possible. It was also hoped that it would be possible to cover the very early stages of meiosis in the ovarian tissue by this culture time. The culture dishes were incubated at 37°C in 100% humidified atmospheric air with 5% CO2. The medium was renewed twice a week. After incubation the tissue was fixed in Bouin’s fixative (Bie&Berntsen a/s) for 1 h, processed for paraffin embedding, cut into 5 µm thick serial sections and stained with haematoxylin and periodic acid Schiff (PAS).

Counting of germ cells

The preliminary studies showed that thick sections, i.e. more than 25 µm thick (necessary for the optical fractionator technique, a stereological method), of the small-sized cultures resulted in large variations in the thickness of the sections and, consequently, the coefficient of error (CE) of the individual number estimates (n), CE (n), would have been unacceptably large. It was therefore decided to count the cells in 5 µm thick sections, which only have insignificant variations in shrinkage, and we decided on determining the density (=concentration) of the cells:

\[
\text{density} = \frac{\text{(number of cell profiles/area)}}{\text{(mean size of profiles + mean thickness of sections)}}. 
\]

Because the nuclei are spherical and only have minor variations in diameter, and because the variation in the thickness of 5 µm thick sections is insignificant, the denominator was constant, and had the same value in test and control tissue. As it was the ratio between the concentration of cells in test and control tissue, and not the absolute number of cells, which was of interest in the present study, it was reasonable to ignore the denominator:

\[
\text{density of cells (=concentration)} = \frac{\text{number of cell-profiles}}{\text{area (here: total area covered with grids)}}, 
\]

The grids were positioned by a computer assisted stereological toolbox – grid system (CAST – grid system; Olympus®, Denmark) and, consequently, all regions of the gonadal tissue had equal opportunity of being sampled.

The size of the grids was dimensioned in a way that would result in the counting of around 1000 germ cells. However, this was not possible in the testicular explants because the total number of cells was small. In order to count as many germ cells as possible in the testicular tissue, the grids were made large (1336 µm² = 31 µm×43 µm), and were placed close to each other. However, in...
order to make sure that the grids did not overlap each other, the distance between the grids was 15–30 μm. Consequently, the step length was 60 μm in the x direction and 60 μm in the y direction. The concentration of germ cells in the female explants was larger than the concentration in the male explants. Consequently, the aim of counting around 1000 cells could be reached by using smaller grids, which were positioned further apart (the grids were 891 μm² (25 μm x 36 μm) and the step length was 100 μm in the x direction and 100 μm in the y direction).

The germ cells were identified by the morphology of the nucleus. Prespermatagonia have large spherical nuclei up to 9 μm in diameter, which are easy to distinguish from the elongated and often irregular nuclei of the Sertoli cells. Oogonia have large spherical nuclei up to 11 μm in diameter, which are easy to distinguish from the surrounding somatic cells with smaller and irregular nuclei. In order to make sure that no cells were counted twice, every fourth section (which equals a distance of approximately 15 μm in the serially cut tissue) was used for counting germ cells. The total number of germ cells, and the number of germ cells in mitosis (only cells in pro- or metaphase) were counted. The results from the counting made it possible to calculate the mitotic index (MI), defined as the number of cells in mitosis per 100 cells, for each tissue. Also the number of grids per tissue was counted, which made it possible to estimate the concentration of germ cells, by dividing the total number of germ cells with the total number of grids, which equals the average number of germ cells per grid. The average number of germ cells per grid reflects the concentration of germ cells in the tissues.

**Statistics**

**Mitotic index**

Odds ratios (no. of cells in mitosis in test tissue/no. of cells not in mitosis in test tissue)/(no. of cells in mitosis in control tissue/no. of cells not in mitosis in control tissue) were based on logistic regression. Common (e.g. all five tissues combined) odds ratios and corresponding confidence intervals and P values were adjusted for the different tissues.

**Concentration of germ cells**

Relative count ratios (no. of cells in test tissue/no. of grids in test tissue)/(no. of cells in control tissue/no. of grids in control tissue) were based on a Poisson regression. Common relative count ratios and corresponding P values and confidence intervals were based on robust variance estimates, taking into account the over-dispersion observable for this data.

**Significance**

The differences were considered significant if the P values < 0.05.

**Results**

**Appearance of the gonads before culture**

The sex of the gonads could not be determined with certainty when dissecting the fetus under stereomicroscope. The length of the gonads of both sexes ranged from 3–3.5 mm, and the diameter from 1–2 mm from the youngest to the oldest fetus.

**Morphology of the cultured gonads**

All tissues attached to the filter and a thin cell layer grew out in a circle from the explants, which also to some extent flattened out during the culture period. All tissues had a mean diameter >1 mm and most measured 2 mm in diameter at the time of harvesting. None of the tissues had coherent areas of necrosis but a few pyknotic nuclei were present. All tissues were sexually differentiated after culture (see below). None of the explants were covered with a distinct epithelium, but flattened cells lined some areas.

Comparing the morphology of the explants cultured in 4-OPL medium with the morphology of the explants cultured in control medium did not reveal any differences in either the testicular explants or in the ovarian explants.

**Testicular tissue**

Well-defined testicular cords were present in all testicular explants at all ages. In all explants, tests as well as controls, three zones were present (Figures 1 and 5). The centre was occupied with a network of closely packed, coiled cell cords with a diameter of around 20–45 μm, without pre-spermatogonia.
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Figure 9. Ovarian tissue from fetus number 10 (8 weeks) cultured for 3 weeks in control medium. The tissue is homogeneous with oogonia and somatic cells scattered all over. ×100.

Figure 10. Higher magnification of Figure 9 showing oogonia (arrows). ×630.

Figure 11. Higher magnification of Figure 9 showing oogonia. One of them is in mitosis (m). ×1000.

At the periphery of the explants, clusters of tightly packed, rounded cells with spherical nuclei and a large cytoplasm with a distinctive PAS-positive area were present (Figures 4 and 8). These cells resemble and will be termed Leydig-like cells. Leydig cells were also seen between the testicular cords.

Ovarian tissue
All explants were compact, homogeneous in appearance with oogonia scattered all over between somatic cells (Figures 9 and 12). The oogonia tended to be organized in small groups. The nuclei of the oogonia were spherical with a diameter up to 11 µm and possessed one large, dense, often spherical nucleolus and smaller ones. Most of the somatic cell nuclei were elongated and often smaller than nuclei of the oogonia (Figures 10 and 13). Some of the oogonia were in mitosis, and a few in the early stages of meiosis (Figures 11, 13 and 14).

Counting of germ cells
Testicular tissue
The number of pre-spermatogonia counted per test tissue was 21–298 and 131–598 per control tissue.

The MI of pre-spermatogonia in test tissue was significantly reduced compared to the MI in the corresponding control tissue (Table I). The common odds (CO) ratio was 0.46. The corresponding confidence interval (CI) range was between 0.24 and 0.87 and P value = 0.016.

The concentration of pre-spermatogonia in test tissue was also significantly reduced compared to the corresponding...
control tissue (Table II). The common relative count ratio was 0.46. The corresponding CI range was between 0.40 and 0.53 and \( P \) value < 0.0005.

**Ovarian tissue**

The number of oogonia counted per test tissue was 724–1288 and 397–1437 per control tissue.

The MI of oogonia in test tissue was not significantly reduced compared to the MI in the corresponding control tissue (Table III). The CO was 0.87. The corresponding CI range was between 0.63 and 1.20.

Similarly, the concentration of oogonia in test tissue was insignificantly reduced compared to the corresponding control tissue (Table IV). The common relative count ratio was 1.05. The corresponding CI range was between 0.97 and 1.14.

**Discussion**

This study demonstrates that 4-OPL has a profound negative effect on proliferation and concentration of germ cells in human fetal testis during a 3 week culture period, contrasting observations in cultured fetal ovaries, where no decline in proliferation and concentration of germ cells could be detected. This excludes a general toxicity to germ cells and indicates that 4-OPL exhibits a specific negative action, directly or indirectly, on fetal male germ cells in vitro.

It was decided to keep the tissues in vitro for this extended time because the possible influence of 4-OPL on a living fetus would be extended. After having evaluated the morphology of several human fetal gonadal tissues cultured in vitro in our laboratory (the cultures had not been exposed to 4-OPL), it was concluded that the tissues did not show any signs of necrosis or gross changes in morphology, compared to in-vivo gonads, after this extended culture period. The concentration of 4-OPL (10 µmol/l) was chosen because this concentration has resulted in significant effects in other experiments: e.g. it has been shown that this concentration had mitogenic effects on cultured MCF-7 cells (White et al., 1994).

The mode of action of 4-OPL is not yet clarified. 4-OPL may interact with steroid receptors in the testis and perhaps, in fact, oestradiol receptors (ER) (Brand-emberger et al., 1997) as well as androgen receptors (Wilson and McPhaul, 1996) have been detected in the human fetal testis. In the fetal rat testis ER are expressed in Sertoli cells as well as in gonocytes (Saunders et al., 1998), which suggests

<table>
<thead>
<tr>
<th>Fetal age at the start of incubation (weeks + days)</th>
<th>Fetal identification number</th>
<th>Right gonad</th>
<th>Left gonad</th>
<th>Number of pre-spermatogonia</th>
<th>Number of pre-spermatogonia in mitosis</th>
<th>Mitotic index (MI)</th>
<th>Odds ratio (OR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 + 5</td>
<td>1 – 4-OPL</td>
<td>Caudal end</td>
<td></td>
<td>68</td>
<td>2</td>
<td>2.9</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>1 – control</td>
<td></td>
<td></td>
<td>194</td>
<td>13</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>7 + 0</td>
<td>2 – 4-OPL</td>
<td>Cranial end</td>
<td></td>
<td>21</td>
<td>1</td>
<td>4.8</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>2 – control</td>
<td></td>
<td></td>
<td>145</td>
<td>17</td>
<td>11.7</td>
<td></td>
</tr>
<tr>
<td>7 + 0</td>
<td>3 – 4-OPL</td>
<td>Cranial end</td>
<td></td>
<td>44</td>
<td>2</td>
<td>4.5</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>3 – control</td>
<td></td>
<td></td>
<td>131</td>
<td>11</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>8 + 0</td>
<td>4 – 4-OPL</td>
<td>Cranial end</td>
<td></td>
<td>298</td>
<td>3</td>
<td>1.0</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>4 – control</td>
<td></td>
<td></td>
<td>405</td>
<td>13</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>9 + 1</td>
<td>5 – 4-OPL</td>
<td>Caudal end</td>
<td></td>
<td>242</td>
<td>4</td>
<td>1.7</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>5 – control</td>
<td></td>
<td></td>
<td>598</td>
<td>13</td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>

Right gonad, left gonad, cranial end, caudal end: the decision of whether to use right or left gonad was random, also, the decision of whether to use the cranial end or the caudal end for test tissue was random.

Mitotic index: \((2/68) \times 100 = 2.9\) etc.

Odds ratio: see ‘Statistics’ section.

4-OPL = 4-octylphenol.
Effect of 4-octylphenol on cultured human fetal gonads

Table II. Concentration of pre-spermatogonia from 4-OPL treated and control tissues

<table>
<thead>
<tr>
<th>Fetal age at the start of incubation (weeks + days)</th>
<th>Fetal identification number</th>
<th>Right gonad</th>
<th>Left gonad</th>
<th>Number of pre-spermatogonia</th>
<th>Number of grids</th>
<th>Relative count ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 + 5</td>
<td>1 – 4-OPL</td>
<td>Caudal end</td>
<td>68</td>
<td>4.509</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 – control</td>
<td></td>
<td>194</td>
<td>5.748</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>7 + 0</td>
<td>2 – 4-OPL</td>
<td>Cranial end</td>
<td>21</td>
<td>2.146</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 – control</td>
<td></td>
<td>145</td>
<td>6.197</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 + 0</td>
<td>3 – 4-OPL</td>
<td>Cranial end</td>
<td>44</td>
<td>4.651</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 – control</td>
<td></td>
<td>131</td>
<td>6.275</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 + 0</td>
<td>4 – 4-OPL</td>
<td>Cranial end</td>
<td>298</td>
<td>5.847</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 – control</td>
<td></td>
<td>405</td>
<td>4.425</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 + 1</td>
<td>5 – 4-OPL</td>
<td>Caudal end</td>
<td>242</td>
<td>6.364</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 – control</td>
<td></td>
<td>598</td>
<td>6.270</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Right gonad, left gonad, cranial end, caudal end: the decision of whether to use right or left gonad was random, also, the decision of whether to use the cranial end or the caudal end for test tissue was random. Number of grids: the total number of grids, used for counting germ cells, in the explants. Relative count ratio: see ‘Statistics’ section.

Table III. Mitotic index of oogonia from 4-OPL treated and control tissue

<table>
<thead>
<tr>
<th>Fetal age at the start of incubation (weeks + days)</th>
<th>Fetal identification number</th>
<th>Right gonad</th>
<th>Left gonad</th>
<th>Number of oogonia in mitosis</th>
<th>Mitotic index (MI)</th>
<th>Odds ratio (OR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 + 5</td>
<td>6 – 4-OPL</td>
<td>Caudal end</td>
<td>724</td>
<td>12</td>
<td>1.7</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>6 – control</td>
<td></td>
<td>397</td>
<td>8</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>6 + 6</td>
<td>7 – 4-OPL</td>
<td>Caudal end</td>
<td>1288</td>
<td>25</td>
<td>1.9</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>7 – control</td>
<td></td>
<td>1039</td>
<td>23</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>7 + 4</td>
<td>8 – 4-OPL</td>
<td>Cranial end</td>
<td>1114</td>
<td>12</td>
<td>1.1</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>8 – control</td>
<td></td>
<td>698</td>
<td>10</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>8 + 1</td>
<td>9 – 4-OPL</td>
<td>Cranial end</td>
<td>756</td>
<td>11</td>
<td>1.5</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>9 – control</td>
<td></td>
<td>1437</td>
<td>24</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>8 + 4</td>
<td>10 – 4-OPL</td>
<td>Caudal end</td>
<td>1159</td>
<td>17</td>
<td>1.5</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>10 – control</td>
<td></td>
<td>1062</td>
<td>16</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

Right gonad, left gonad, cranial end, caudal end: the decision of whether to use right or left gonad was random, also, the decision of whether to use the cranial end or the caudal end for test tissue was random. Mitotic index: \((12/724) \times 100 = 1.7\) etc. Odds ratio: see ‘Statistics’ section.

Table IV. Concentration of oogonia from 4-OPL treated and control tissue

<table>
<thead>
<tr>
<th>Fetal age at the start of incubation (weeks + days)</th>
<th>Fetal identification number</th>
<th>Right gonad</th>
<th>Left gonad</th>
<th>Number of oogonia</th>
<th>Number of grids</th>
<th>Relative count ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 + 5</td>
<td>6 – 4-OPL</td>
<td>Caudal end</td>
<td>724</td>
<td>1.258</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 – control</td>
<td></td>
<td>397</td>
<td>801</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 + 6</td>
<td>7 – 4-OPL</td>
<td>Caudal end</td>
<td>1288</td>
<td>1.368</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 – control</td>
<td></td>
<td>1039</td>
<td>1.150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 + 4</td>
<td>8 – 4-OPL</td>
<td>Cranial end</td>
<td>1114</td>
<td>1.231</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 – control</td>
<td></td>
<td>698</td>
<td>911</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 + 1</td>
<td>9 – 4-OPL</td>
<td>Cranial end</td>
<td>756</td>
<td>1.181</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 – control</td>
<td></td>
<td>1437</td>
<td>1.950</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 + 4</td>
<td>10 – 4-OPL</td>
<td>Caudal end</td>
<td>1159</td>
<td>1.630</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 – control</td>
<td></td>
<td>1062</td>
<td>1.669</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Right gonad, left gonad, cranial end, caudal end: the decision of whether to use right or left gonad was random, also, the decision of whether to use the cranial end or the caudal end for test tissue was random. Number of grids: the total number of grids, used for counting germ cells, in the explants. Relative count ratio: see ‘Statistics’ section.
that oestradiol may affect germ cells directly during testicular development and spermatogenesis (van Pelt et al., 1999). This is supported by the observation that oestrogen (Kalla, 1987) and 4-OPL (Sharpe et al., 1995) have a negative effect on spermatogenesis in rats. Another study shows that 4-OPL given to pregnant ewes resulted in a reduction in the Sertoli cell number in the male offspring (Sweeney et al., 2000).

Differentiation of the human fetal testis starts around postcoital week 7 (Wagenen and Simpson, 1965; Jirasek, 1968; Motta et al., 1997), and encompasses the enclosure of male germ cells and Sertoli cells in testicular cords and differentiation of Leydig cells outside the cords (Byskov and Hoyer, 1994). Thus, the present experiments were performed at a developmental stage, where testicular cords and Leydig cells differentiate, and where testosterone (Winter et al., 1977) and anti-Müllerian hormone (Josso et al., 1993; Josso et al., 1998) are produced by the human fetal testis. The observed harmful effect of 4-OPL on pre-spermatogonia may be the result of interference with the hormone production by the developing testes.

Male gametogenesis involves two successive proliferation waves: the pre-spermatogonic wave, which ceases around the 22nd week of pregnancy, and the wave which introduces spermatogenesis (Hilscher and Hilscher, 1976; Hilscher, 1991; Hilscher and Engemann, 1992). One study shows that a certain concentration of intratubular testosterone is required for quantitative maintenance of spermatogenesis in the rat (Sharpe et al., 1988), suggesting that also the proliferation of germ cells in the pre-spermatogonic wave is dependent on a certain concentration of testosterone. 4-OPL might interfere with the production of testosterone, and thereby indirectly reduce the proliferation rate of pre-spermatogonia. That 4-OPL may interfere with fetal Leydig cell steroidogenesis is supported by the observation that maternal exposure to 4-OPL results in reduced expression of messenger RNA and cytochrome p450c17 in rat Leydig cells of the fetuses (Majdic et al., 1996).

New findings reveal that the mode of action of 4-OPL and other endocrine disruptors is complex and not solely confined to its binding to ER, as shown in mouse Leydig tumour cells cultured with bisphenol A, an anti-androgen, 4-OPL or oestradiol. Bisphenol A and 4-OPL inhibited human chorionic gonadotrophin, stimulated cAMP and progesterone production, whereas oestradiol had no effect on cAMP production, revealing a different mode of action (Nikula et al., 1999). Studies of Leydig cells from neonatal rats also indicated that 4-OPL utilizes a different signal transduction pathway in addition to the classic ER pathway (Murono et al., 1999). Actually, one study suggests that 4-OPL is directly toxic to spermatogonia and Sertoli cells in culture, exerting its effects through a calcium-independent apoptotic pathway (Raychoudhury et al., 1999). The effect of 4-OPL on proliferation and number of pre-spermatogonia in the present study might be explained by similar mechanisms, but further investigation is needed to elucidate this.

Recently, it was shown that oogonia of human fetal ovaries around the 12th week of gestation are immunoreactive for ER (Gould et al., 2000). Since 4-OPL had no effect on proliferation and number of oogonia in the present study, it is suggested that 4-OPL may not exert its effects by activation of ER. On the other hand, it cannot be excluded that male and female germ cells react differently to activation of ER.

In the present study the concentration of germ cells was determined following a 3 week culture period. It was not possible to evaluate whether the total number of germ cells in the explants cultured in control media equals the number present in in-vivo testes of similar ages, because the number of pre-spermatogonia in human fetal testes, to our knowledge, is unknown. Therefore, it cannot be determined whether the culture procedure itself affects the number of germ cells. Nevertheless, a significant and harmful effect of 4-OPL on pre-spermatogonia compared to the controls was clear. Since the morphology of the cultured gonads within each sex were similar between tests and controls, it was concluded that 4-OPL reduces mitotic activity and concentration of germ cells in cultured human fetal testes, but leaves oogonia in cultured human fetal ovaries of similar age unaffected.

The model used here for evaluation of harmful effects of 4-OPL on fetal germ cells is obviously not directly applicable to an in-vivo situation. A human fetus would probably never be exposed to such concentrations of 4-OPL. However, 4-OPL may exert harmful effects in the human fetal testis, which later on in life may result in reduced fertility. This emphasizes the need for studies on human tissue in order to obtain reliable results in connection with human exposure.

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References


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