Effects of the aromatase inhibitor letrozole on in utero development in rats

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BACKGROUND: The aromatase inhibitor letrozole has recently been identified as a promising ovulation-inducing agent. As information regarding possible adverse effects on gestation outcome is limited, the present study was undertaken to evaluate the developmental toxicity potential of letrozole in the rat. METHODS: Pregnant Sprague-Dawley rats were exposed via drinking water to letrozole at 0 (control group), 0.01, 0.02, or 0.04 mg/kg during the period of organogenesis. Developmental endpoints, including intrauterine mortality, fetal growth and incidence of structural abnormalities, were evaluated near the end of gestation. RESULTS: Major treatment-related effects included: (i) a dose-dependent increase in post-implantation loss, which reached 47.2% following exposure to 0.04 mg/kg letrozole; (ii) minor vertebral anomalies affecting 32.2, 29.3 and 42.2% of fetuses exposed to 0.01, 0.02 and 0.04 mg/kg, respectively. CONCLUSION: Gestational exposure to doses of letrozole that are equal to or lower than the daily recommended human dose has toxic effects on prenatal development in rats.

Keywords: letrozole; rat; developmental toxicity

Introduction

Aromatase is a cytochrome P450 enzyme responsible for the last step in estrogen biosynthesis, catalyzing the aromatization of androstenedione and testosterone into estrone and estradiol (E2), respectively (Haynes et al., 2003). Aromatase (CYP19) is present in several tissue and organ systems, including ovary, fat, muscle, liver and breast (Haynes et al., 2003). Letrozole is a potent non-steroidal aromatase inhibitor that produces ~99% inhibition of estrogen biosynthesis at the dose of 2.5 mg/day. It was approved in 1997 as a first-line therapy for hormone receptor positive, metastatic breast cancer in post-menopausal women (Haynes et al., 2003).

Increased interest is currently being paid to the possible clinical application of aromatase inhibitors as ovulation-inducing agents (Casper and Mitwally, 2006; Holzer et al., 2006). Potential advantages offered by the use of aromatase inhibitors over clomiphene citrate in ovulation induction include a lack of peripheral anti-estrogenic effects, particularly on the endometrium and the cervical mucus, and an ability to limit the ovarian response to mono-ovulation (Casper, 2007). Treatment of endometriosis and uterine fibroids represent additional areas of application for aromatase inhibitors in pre-menopausal women (de Ziegler et al., 2005).

When a drug is used to treat conditions that affect women of reproductive age, any potential developmental hazard posed by the agent is a matter of particular concern. No information is currently available regarding the outcome of human pregnancies exposed to letrozole at any stage. When human clinical data are unavailable, animal data represent a source of information that is important to the process of human risk estimation, despite known species-specific differences in sensitivity to embryotoxicity. Data from the manufacturer (Femara® patient prescribing information) indicate that letrozole exerts potent embryotoxic effects in rats and rabbits, at doses lower than the daily recommended human dose. Exposure of pregnant rats during organogenesis to doses ≥0.003 mg/kg results in increased intrauterine mortality and congenital anomalies, including the absence and shortening of renal papilla, dilation of ureters, edema and incomplete ossification of the frontal skull and metatarsals. A 0.03 mg/kg dose causes fetal domed head and cervical/centrum vertebral fusion. In rabbits, letrozole is embryotoxic at doses ≥0.002 mg/kg, and fetotoxic when administered at 0.02 mg/kg. Fetal anomalies include incomplete ossification of the skull, sternebrae, forelegs and hind legs. Unfortunately, the aforementioned data have been reported in abbreviated form in the letrozole product information (Femara® patient prescribing information), rather than in peer-reviewed journals, with several reporting deficiencies limiting the utility and interpretation of the available risk assessment data. Thus, the present study was undertaken in...
order to further the understanding on the developmental toxicity potential of letrozole in rats.

Materials and Methods

The study was approved by the ethical committee of our institution, and was performed according to the Italian legislation for animal studies. Male and nulliparous female Sprague–Dawley rats were used. Animals were housed individually in standard plastic cages with stainless steel covers, had wood shavings as bedding and were kept in a bioclean room under controlled temperature (22 ± 1°C) and relative humidity (55 ± 5%). The photoperiod consisted of 12 h of artificial light and 12 h of darkness. Rodent laboratory chow (Altromin-MT®, Italy) and filtered tap water were provided ad libitum. To produce timed matings, individual males were placed into cages containing one female during the dark cycle. Detection of sperm in the vaginal smear (taken as evidence of mating) at the end of the dark cycle (8:00 am) was used to designate gestation day 0.

Letrozole (Femara®) is a product of Novartis (Basel, Switzerland). Tablets containing 2.5 mg of active drug were dissolved in 10% propylene glycol (Sigma, Milan, Italy) and added to the drinking water at various concentrations to achieve daily exposures of approximately 0.01, 0.02, or 0.04 mg/kg. Doses were identified on the basis of a preliminary study, which demonstrated that the 0.04 mg/kg dose resulted in intrauterine loss of about half of all litters. In order to have a sufficient number of term fetuses for evaluation of structural abnormalities. The maximum dose selected (0.04 mg/kg) corresponds to the recommended daily human dose (2.5 mg) in a person weighing 60 kg. The number of pregnant rats treated with letrozole at 0.01, 0.02 and 0.04 mg/kg were 10, 12 and 17, respectively. Control animals (14 rats) were exposed to propylene glycol alone. Animals were exposed during gestation days 6–16, a gestational phase which corresponds to the period of organogenesis in the rat. During the treatment period, animals were monitored daily for water consumption, general status and clinical signs of toxicity. Pregnancies were terminated near term, on gestational day 20, and the following parameters were recorded: maternal weight; maternal absolute weight (maternal body weight at term minus gravid uterine weight); number of early and late resorptions; number of live and dead fetuses; fetal sex; fetal weight; and number and type of external morphological abnormalities. The fetuses from each uterus were selected alternately either for skeletal examination using the double-staining methods of Inouye (1976) and Kimmel and Trammel (1981) as modified by Kuczuk and Scott (1984), or for assessment of visceral anomalies using the free-hand razor blade sectioning technique devised by Wilson (1965). All morphological evaluations were carried out under a stereo microscope. All abnormalities were named according to the standardized nomenclature of Wise et al. (1997).

For statistical analysis, continuous data were compared using Student’s t-test or ANOVA and post hoc Student–Newman–Keuls test for multiple comparisons. Binomial data were compared using the Chi-square test. Differences were considered statistically significant when P < 0.05.

Results

The presence of letrozole or vehicle in drinking water did not affect drinking behavior; animals consumed the expected daily amount of water (~60 ml). There were no cases of maternal death, and no detectable clinical signs of maternal toxicity resulted from letrozole exposure. Table I details maternal and litter parameters observed in control and treated animals. Letrozole did not alter the maternal endpoints considered, including maternal body weight at term and maternal absolute body weight. The prominent treatment-related effect noted was increased post-implantation loss, including early resorptions, late resorptions and dead fetuses. This response was dose-dependent, with exposure to 0.01, 0.02 or 0.04 mg/kg letrozole significantly increasing the level of post-implantation loss to 17.6, 37.1 and 47.2%, respectively, in comparison to 6.5% in control animals. Increased intrauterine mortality was attributable to early and late resorptions in groups treated with the lower and intermediate dose, but also to fetal mortality in the group exposed to the maximum dose. In some instances, hemorrhagic areas were seen within the gravid uteri of mothers treated with letrozole at 0.02 or 0.04 mg/kg (not shown).

Table I. Maternal and litter parameters in Sprague–Dawley rats administered letrozole.*

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>0 (vehicle)</th>
<th>0.01</th>
<th>0.02</th>
<th>0.04</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of pregnant animals treated</td>
<td>14</td>
<td>12</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Maternal body weight (g SEM) at term gestation</td>
<td>412.3 ± 8.9</td>
<td>386.9 ± 11.7</td>
<td>384.7 ± 7.0</td>
<td>396.1 ± 10.6</td>
</tr>
<tr>
<td>Maternal absolute weight (g SEM)**</td>
<td>323.5 ± 10.4</td>
<td>301.4 ± 9.4</td>
<td>302.2 ± 7.7</td>
<td>322.2 ± 7.8</td>
</tr>
<tr>
<td>No. of animals at term gestation with live fetuses</td>
<td>14</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Implantations</td>
<td>200</td>
<td>142</td>
<td>179</td>
<td></td>
</tr>
<tr>
<td>No. of implantations per litter (mean ± SEM)</td>
<td>14.3 ± 0.8</td>
<td>14.0 ± 0.6</td>
<td>14.9 ± 0.4</td>
<td>13.8 ± 1.1</td>
</tr>
<tr>
<td>Post-implantation loss§</td>
<td>13/200 (6.5%)</td>
<td>25/142 (17.6%)</td>
<td>68/179 (37.1%)</td>
<td>111/235 (47.2%)</td>
</tr>
<tr>
<td>Early resorptions§</td>
<td>13/200 (6.5%)</td>
<td>12/142 (8.4%)</td>
<td>24/179 (13.4%)</td>
<td>66/235 (28.1%)</td>
</tr>
<tr>
<td>Late resorptions§</td>
<td>0</td>
<td>13/142 (9.1%)</td>
<td>42/179 (23.5%)</td>
<td>26/235 (11.0%)</td>
</tr>
<tr>
<td>Dead fetuses§</td>
<td>0</td>
<td>0</td>
<td>2/179 (1.1%)</td>
<td>19/235 (8.1%)</td>
</tr>
<tr>
<td>No. of fetuses at necropsy</td>
<td>187</td>
<td>117</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>Sex ratio (Female/Male)</td>
<td>98/89</td>
<td>52/65</td>
<td>58/53</td>
<td></td>
</tr>
<tr>
<td>No. of viable fetuses per litter (mean ± SEM)</td>
<td>13.4 ± 1.0</td>
<td>11.5 ± 0.6</td>
<td>9.4 ± 1.7</td>
<td>7.3 ± 1.2</td>
</tr>
<tr>
<td>Mean fetal body weight per litter (g SEM)</td>
<td>3.6 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>3.6 ± 0.3</td>
<td>3.5 ± 0.1</td>
</tr>
</tbody>
</table>

* Rats received letrozole in their drinking water during gestation days 6–16. Controls were administered with vehicle on gestation days 6–16. ** Maternal body weight at term minus gravid uterine weight. † Including early resorptions, late resorptions and dead fetuses. ‡ Resorption bodies lacking of identifiable fetal/placental remnants. § Implantation sites with placenta and non-viable conceptus of reduced size or that were in macerated condition. ¶ Fetuses of normal size displaying no vital signs at the time of uterine dissection. ‡‡ Statistically significant (P < 0.05, χ² test) versus control group. ‡§ Statistically significant (P < 0.05, χ² test) versus control group. ‡‖ Statistically significant (P < 0.05, ANOVA and post hoc Student–Newman–Keuls test) versus control group.
The fetal sex ratio for letrozole-exposed groups did not differ from that of the control group. There were no treatment-related effects on fetal growth, as evidenced by the finding that the weights of treated and control fetuses were comparable. Regarding fetal phenotypes, vertebral anomalies were the only significant morphological effect induced by letrozole. These abnormalities were observed in 32.2, 29.3 and 42.2% of fetuses exposed to 0.01, 0.02 or 0.04 mg/kg, respectively (Table II). All frequencies of vertebral anomaly were significantly higher in fetuses exposed to letrozole than in the control group, where the incidence of vertebral anomalies per fetus was 6.1%. Anomalies consisted of bipartite centrum, bipartite ossification of the centrum, and especially dumbbell ossification of the centrum. Vertebral anomalies were found only in the thoracic and lumbar segments of the axial skeleton, ranging from the 16th to the 22nd vertebrae. Evaluation of treated fetuses fixed in Bouin’s fluid revealed distended bladder in only 2 (3.4%) fetuses treated with 0.01 mg/kg and in 4 (6.7%) fetuses exposed to 0.04 mg/kg letrozole. These frequencies were not statistically higher than those recorded in the control group (data not shown).

Discussion
The present study contributes to the characterization of the developmental toxic potential of letrozole in the rat. Notably, we found that the levels of exposure lower than or equal to the recommended human therapeutic dose resulted in a marked and dose-dependent increase in intrauterine lethality. Letrozole increased the frequency of early and late resorptions and, at the higher dosage, also increased the percentage of fetal mortality, suggesting that sensitivity to letrozole extends over different phases of development. Letrozole-mediated embryonic lethality did not appear to be influenced by the conceptus gender, since fetal sex ratios for treated and control fetuses were comparable. Treatment-related dysmorphic effects were limited to minor structural anomalies of the vertebral bodies. The fact that embryonic lethality prevailed over dysmorphogenesis may suggest that, while letrozole (under the selected experimental conditions) had the capacity to interfere with processes that are vital for the maintenance of pregnancy, it had a limited negative impact on organogenesis. Alternatively, it is feasible that letrozole may affect organogenesis, inducing severe malformations that are incompatible with the continuation of pregnancy. We failed to detect other previously reported (Femara® patient prescribing information) morphological changes, including renal and ureter anomalies, incomplete ossification of frontal skull and metatarsals and abnormal morphology of the head defined as ‘domed head’. Given the reporting deficiencies of the previously performed study, it is difficult to comment meaningfully on reasons for the lack of full concordance between the two studies. An important finding of our study is that letrozole was embryotoxic at doses that were not maternally toxic, suggesting that the adverse developmental outcomes recorded in the study were not dependent on perturbations of maternal health. It is noteworthy that drugs with the capacity to induce embryotoxicity at doses which are not associated to maternal toxicity (as in the case of letrozole) have been categorized as potentially harmful for the human conceptus (Webster and Freeman, 2001).

Anastrozole is a third generation non-steroidal aromatase inhibitor that is also under investigation for use in the induction of ovulation (Sipe et al., 2006). Thus, its developmental toxicological profile is germane to the scope of the present study. The potential developmental toxicity of anastrazole has been evaluated by the manufacturer (Admirex® prescribing information). Treatment of pregnant rats with anastrazole at doses ≥0.1 and 0.02 mg/kg/day (about 1 and 1/3, respectively, the recommended human dose in the treatment of breast cancer), caused a dose-dependent increase in pregnancy loss. Evidence of fetotoxicity, resulting in delayed fetal development (including incomplete ossification and depressed fetal body weight) was observed in rats that had been administered doses of 1.0 mg/kg/day; there was no evidence of teratogenicity in rats at the same dose levels. When the developmental toxicology profiles of letrozole and anastrazole are compared, it seems that the two agents share the capacity to initiate embryopathic effects at pharmacologically relevant doses, with increased embryonic and fetal mortality representing the most sensitive developmental endpoint. However, congenital anomalies resulted from exposure to letrozole, but not to anastrazole.

A central mechanistic issue to be addressed is whether the developmental injury induced by letrozole is causally related to estrogen deprivation, resulting from aromatase inhibition. The generation of aromatase-deficient mice has enabled the various physiological functions of aromatase to be explored

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>0</th>
<th>0.01</th>
<th>0.02</th>
<th>0.04</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuses with vertebral anomalies</td>
<td>6/98 (6.1%)</td>
<td>19/59 (32.2%)</td>
<td>17/58 (29.3%)</td>
<td>27/64 (42.2%)</td>
</tr>
<tr>
<td>Type of vertebral anomaly&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thoracic vertebrae</td>
<td>2/98 (2.0%)</td>
<td>1/59 (1.7%)</td>
<td>1/58 (1.7%)</td>
<td>4/64 (6.2%)</td>
</tr>
<tr>
<td>Bipartite centrum</td>
<td>1/98 (1.0%)</td>
<td>2/59 (3.4%)</td>
<td>5/58 (8.6%)</td>
<td>0/64 (0.0%)</td>
</tr>
<tr>
<td>Bipartite ossification of centrum</td>
<td>2/98 (2.0%)</td>
<td>15/59 (25.4%)</td>
<td>12/58 (20.7%)</td>
<td>25/64 (39.1%)</td>
</tr>
<tr>
<td>Dumbbell vertebrae</td>
<td>1/98 (1.0%)</td>
<td>1/59 (1.7%)</td>
<td>3/58 (5.2%)</td>
<td>2/64 (3.1%)</td>
</tr>
</tbody>
</table>

<sup>4</sup>Rats received letrozole (or vehicle) in drinking water during gestation days 6–16. <sup>4</sup>A single fetus may be represented more than once in listing individual morphologic abnormalities. <sup>1</sup>Statistically significant ($P < 0.05, \chi^2$ test) versus control group.
(Fisher et al., 1998; Honda et al., 1998). It was found that (i) male and female mice lacking aromatase are born phenotypically normal; (ii) the mutant allele is transmitted to the F2 generation via the expected Mendelian inheritance pattern, indicating that there is no appreciable survival disadvantage associated with the homologous or heterozygous condition. These observations seem to suggest that embryonic aromatase activity is not essential during organogenesis and fetal development. The possibility remains that maternal aromatase plays a vital role in maintaining pregnancy in the knockout model, considering that gonadal function is conserved in heterozygous pregnant mothers.

The idea that estrogen deprivation may have a mechanistic function in letrozole-induced developmental toxicity is supported by results obtained from a non-human primate model. Letrozole was used by Albrecht et al. (2000) to probe the impact of estrogen ablation on pregnancy maintenance in baboons. Pregnant animals received letrozole (0.1–2.0 mg/day) beginning on gestational days 30, 60 or 100. A subgroup of letrozole-treated baboons was also given E2 as replacement therapy. It was found that letrozole exposure periods ranging from 5 to 69 days caused a significant increase in pregnancies ending in spontaneous abortion, but that spontaneous abortion was completely prevented by E2 co-administration.

Within the context of possible embryopathic effects arising from interference with estrogen signaling, the developmental toxicology profile of clomiphene citrate is also of interest. This agent is a non-steroidal triphenylethylene derivative with both anti-estrogenic and estrogenic activities. It acts by blocking the negative effects of estrogen, thereby stimulating secretion of gonadotropins from the pituitary gland (Messinis, 2005). In experimental studies, clomiphene was found to be teratogenic in mice (Dziadek, 1993), rats (Eneroth et al., 1971), and guinea pigs (Motta and Hutchinson, 1991), but not in monkeys (Courtney and Valerio, 1968). Abnormal developmental outcomes included decreased implantation rates, fetal growth retardation and neural tube defects in mice after exposure to doses similar to those used in humans (Dziadek, 1993), hydramnios, cataracts (Eneroth et al., 1971), genital tract abnormalities in female offspring of rats exposed to doses similar to those used in humans (McCormack and Clark, 1979) and pregnancy loss in guinea pigs at clinically relevant doses (Motta and Hutchinson, 1991). The mechanisms and etiological factors involved in clomiphene citrate-induced abnormal development remain incompletely elucidated. Impairment of uterine function (Motta and Hutchinson, 1991; Dziadek, 1993), and direct or indirect luteolysis (Motta and Hutchinson, 1991) have been implicated in clomiphene citrate-associated pregnancy loss. The genetic tract abnormalities noted in rats were related to the ability of clomiphene citrate to cause long-term estrogenic stimulation (McCormack and Clark, 1979). With regard to human pregnancy, clomiphene citrate has not been associated with evidence-based proof of human embryopathy (Briggs et al., 2002). However, it is contraindicated in women who are or may become pregnant (Briggs et al., 2002). In addition, patients requiring clomiphene citrate should be cautioned that each new course of the drug should be started only after the possibility of pregnancy has been excluded (Briggs et al., 2002). Overall, the aforementioned data indicate that the role of estrogen deprivation in the occurrence of adverse pregnancy outcomes is poorly defined. We are planning to evaluate whether estrogen co-administration is able to alleviate the deleterious effects induced by letrozole in pregnant rats. Future analysis will also be aimed at testing the developmental toxicity of clomiphene citrate when administered under the same experimental conditions used in this study to characterize the developmental response to letrozole.

Animal models have an important function in the investigation of the impact of letrozole and other aromatase inhibitors on ovarian function. It is intriguing in this context to observe that treatment of female rats with letrozole (0.1 or 0.5 or 1 mg/kg/day for 21 days) resulted in histologic and biochemical findings consistent with human polycystic ovarian syndrome (Kafali et al., 2004). Similar findings were noted in mice with targeted disruption of the CYP19 gene (Fisher et al., 1998). Although it may not be possible to extrapolate directly from animal studies to human pathology (a concept also relevant to the present study), these findings raise the possibility that long-term exposure to letrozole can negatively affect ovarian function.

It is well known that, for the majority of teratogenic agents, organogenesis represents the most sensitive phase during which malformations are induced. This might explain why no increased teratogenic risk was found in a retrospective multicenter study (Tulandi et al., 2006), which analysed the type and prevalence of birth defects among 911 infants whose mothers received letrozole to induce ovulation (i.e. under exposure conditions predating pregnancy). It is noteworthy that pregnancy loss, the major adverse response induced by letrozole in our study, was not included within the pregnancy endpoints considered in the study. Although letrozole may not adversely affect morphogenesis when administered before fertilization, it should not necessarily be regarded as a safe agent for the induction of ovulation. Indeed, the possibility of inadvertent gestational exposure warrants careful consideration, as experience with clomiphene citrate demonstrates (Carlier et al., 1996; Bishai et al., 1999; Briggs et al., 2002).

This study increases our understanding of the developmental toxicity profile of the aromatase inhibitor letrozole in rats. The demonstration of elevated embryotoxicity raises concerns regarding its use as an ovulatory inducing agent in the human.

Funding
The study was supported by “Fondi di Ateneo per la ricerca scientifica ex art. 60%” from the University “G. d’Annunzio” Chieti-Pescara.

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Femara® patient prescribing information. Available at: www.pharma.us.novartis.com/product/pi/pdf/Femara.pdf


Submitted on November 2, 2007; resubmitted on February 25, 2008; accepted on March 4, 2008