Impact of estrogen replacement on letrozole-induced embryopathic effects

G.M. Tiboni¹³, F. Marotta¹, A.P. Castigliego¹, and C. Rossi²

¹Sezione di Ostetricia e Ginecologia, Dipartimento di Medicina e Scienze dell’Invecchiamento, Facoltà di Medicina e Chirurgia, Università ‘G. d’Annunzio’, Chieti-Pescara Ospedale ‘SS. Annunziata’, Via dei Vestini, 66013 Chieti, Italy ²Centro Studi per l’Invecchiamento (Ce.S.I.), Università degli Studi ‘G. d’Annunzio’, Chieti-Pescara, Chieti, Italy
³Correspondence address. Tel: +39-0871-540034; Fax: +39-0871-540037; E-mail: tiboni@unich.it

BACKGROUND: The aromatase inhibitor, letrozole, exerts embryo toxic effects in rats, causing increased embryo lethality and anomalies of the axial skeleton at pharmacologically relevant doses. Letrozole acts by inhibiting estrogen biosynthesis. It may thus be feasible that estrogen deprivation is a crucial determinant of the elicited developmental toxic effects. In order to gain insight on this hypothesis, the present study tested the capacity of estrogen replacement in preventing letrozole-mediated embryopathy.

METHODS: Pregnant Sprague Dawley rats were exposed to letrozole alone (0.04 mg/kg), or in combination with estradiol cyclopentylpropionate (ECP) at 0.5, 1 or 2 µg/rat. A control group receiving only the vehicles was also included. Animals were exposed during gestation Days 6–16 (corresponding approximately to 3–10 weeks of gestation in the human). Developmental end-points, including intrauterine mortality, fetal growth, placental weight and incidence of structural abnormalities, were evaluated near term gestation.

RESULTS: Exposure to letrozole alone was lethal for 41% of conceptuses, and caused minor axial skeletal anomalies in 51% of live fetuses. ECP co-administration effectively prevented letrozole-induced embryolethality, but failed to reduce the incidence of axial skeletal alterations.

CONCLUSION: The obtained results support the concept that inhibition of estrogen biosynthesis represents a critical determinant of letrozole-induced embryonic mortality. A mechanism other than estrogen deprivation appears to underlie the initiation of skeletal anomalies.

Key words: letrozole / estrogen replacement / estradiol cyclopentylpropionate / rat / developmental toxicity

Introduction

Letrozole is a third generation aromatase inhibitor registered for the treatment of breast cancer in post-menopausal women (Haynes et al., 2003). It produces approximately 99% inhibition of estrogen biosynthesis at the daily dose of 2.5 mg. Letrozole has been also found to be effective as an ovulating inducing agent (Casper and Miewally, 2006), and an increasing number of studies on this subject have been published in recent years (Requena et al., 2008). Extension of the therapeutic application to women of reproductive potential has raised concerns regarding the developmental hazard potential of letrozole and other aromatase inhibitors (Tiboni, 2004). Major concerns are related to the possibility of inadvertent exposure during initial phases of pregnancy. Letrozole is a potent embryo toxicant in rodents. We recently used rats to analyze the possible noxious effects elicited by letrozole on pregnancy outcome and found that exposure during organogenesis to a dose equivalent to that used in human therapy, kills about 50% of embryos and causes minor morphological abnormalities of the axial skeleton in the 40% of term fetuses (Tiboni et al., 2008).

Since it is known that preventive approaches must be based on a clear understanding of underlying etiological mechanism, the present study was initiated to gain mechanistic insights on letrozole-mediated developmental toxicity. Letrozole acts by inhibiting the cytochrome P450 enzyme aromatase which is responsible for the last step in estrogen biosynthesis, catalyzing the aromatization of androgens into estrogens (Haynes et al., 2003). It may thus possible that estrogen deprivation plays a role in the induction of embryopathic effects. To address this hypothesis, we tested the capacity of estrogen replacement to prevent letrozole-induced developmental toxicity.

Materials and Methods

Animal housing and breeding

Male and nulliparous female Spagye-Dawley rats from our breeding colony were used. Animals were housed individually in standard plastic cages with stainless steel covers, had wood shavings as bedding, and were kept in a bio-clean 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 a.m.) was used to designate gestation Day 0.

**Agents**

Letrozole (Femara®) is a product of Novartis (Basel, Switzerland). All the other chemicals used in the study were purchased from Sigma (Milan, Italy). Tablets of letrozole containing 2.5 mg of active drug were dissolved in 10% propylene glycol and added to drinking water to reach the selected concentration according to the previously described methodology (Tiboni et al., 2008). Estradiol cyclopentylpropionate (ECP) was dissolved in sesame oil and injected subcutaneously in a volume of 0.2 ml.

**Experimental procedures**

Institutional and national guidelines for the care and use of laboratory animals were followed. Pregnant rats were exposed to a daily oral dose of letrozole at 0.04 mg/kg from gestation Days 6–16 (a gestational phase encompassing organogenesis). This treatment regimen was previously found to significantly affect rat embryo development (Tiboni et al., 2008). Animals were also co-exposed to ECP given subcutaneously at 0 (vehicle) 0.5, 1 or 2 µg/rat concentrations during the same gestational period. The control group received vehicles only. Doses of ECP were identified on the basis of a preliminary study showing that a dose of 3 µg/rat was toxic for the embryo, causing a marked increase in post-implantation loss. The resulting five experimental groups consisted of 8–14 pregnant rats each, with an overall number of 57. During the treatments, animals were monitored daily by visual inspection for water consumption and for the occurrence of 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; placental weight and number and type of external morphological abnormalities. The fetuses from each uterus were selected alternately 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).

**Statistical analysis**

Continuous data were compared using Student’s t-test or ANOVA and post hoc Student–Newman–Keuls test for multiple comparisons. Bimodal data were compared using the χ² test. Differences were considered statistically significant when P < 0.05.

**Results**

No detectable clinical signs of maternal toxicity resulted from the single or combined administration of letrozole and ECP. The effects of the concomitant exposure to letrozole and ECP on maternal and litter parameters are shown in Table I. Letrozole alone or in co-administration to ECP at any dosage, did not affect maternal body weight parameters, including maternal body weight at term gestation, maternal absolute body weight (maternal body weight at term minus gravid uterine weight) and uterine weights (with or without contents), in comparison to the control group. As expected, letrozole exposure resulted in a significant increment of embryonic mortality, with the post-implantation loss (including embryonic and fetal loss) increasing from the control value of 3.5–41%. Co-treatment with ECP at doses of 0.5 or 1 µg/rat provided protection from the embryo lethal effects associated with letrozole treatment, lessening post-implantation loss to percentages (7 and 8%, respectively) that approximated and did not significantly differ from the control level. Co-exposure with ECP at 2 µg/rat resulted in a post-implantation loss (15%) that was significantly lower in comparison to the letrozole group, but significantly higher in comparison to controls. Areas of uterus hemorrhage surrounding the gestational sac were seen in letrozole alone exposed animals, but never in animals co-treated with ECP at any dose. No differences in fetal sex ratio were observed among the experimental groups. Likewise, there were no treatment-related effects on the mean fetal body weight/litter. The mean placental weight/litter of the letrozole group (expressed as g ± SEM) was significantly higher in comparison to the control group (1.12 ± 0.05 versus 0.56 ± 0.07, respectively). Co-treatment with ECP at 1 or 2 µg/rat, but not with 0.5 µg/rat, restored placental weight to control values.

In line with previous data (Tiboni et al., 2008), developmental exposure to letrozole induced abnormalities of the axial skeleton. Types and frequencies the skeletal defects are shown in Table II. Axial skeletal anomalies were noted only in 5% of control fetuses, but in 51.5% of fetuses exposed to letrozole. Administration of ECP did not alter the sensitivity of the developing skeleton to letrozole, resulting in per fetus incidences of skeletal defects of 56.5, 52.4 and 50.0% after co-treatment with ECP at 0.5, 1 or 2 µg/rat, respectively. Anomalies, found in the thoracic and lumbar segments, mainly consisted of bipartite vertebra, bipartite ossification of centrum, dumbbell shaped vertebra and especially dumbbell ossification of centrum and wavy ribs. Two cases of brachydactyly, due to hypoplasia of distal phalanges of the hind-limbs, were observed in fetuses exposed to letrozole plus ECP at 0.5 µg/rat (not shown).

**Discussion**

Letrozole is toxic for developing rat conceptuses, causing increased prenatal mortality and minor anomalies of the axial skeleton at doses of that are equivalent to or lower than the daily recommended human dose. In consideration of the pharmacological action of aromatase inhibitors, the present study was undertaken to test whether estrogen replacement can prevent the deleterious effects induced by letrozole on rat pregnancy. Drugs were administered from gestation Days 6–16, a gestational phase approximately corresponding to 3–10 weeks of gestation in the human. The single letrozole administration induced a developmental toxic response that mirrored, both quantitatively and qualitatively, previous observations (Tiboni et al., 2008). Reflecting the vital role played by estrogen in pregnancy maintenance, ECP resulted in highly effective prevention of pregnancy loss associated with letrozole administration. Maximal protection was already visible after treatment with the lower ECP dosage (0.5 µg/rat), reducing the level of post-implantation loss to a frequency close to that recorded in the control group. The observation that estrogen can protect from letrozole-induced pregnancy loss is in
line with data gained 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 estradiol as replacement therapy. It was found that letrozole exposure periods ranging from 5 to 69 days caused a significant increase in pregnancies ending in abortion, and that incidence of abortion was completely prevented by estradiol co-administration. An intriguing finding of the present study was that the maximal ECP dose administered protected from embryolethality less than lower dosages. In fact, letrozole-associated post-implantation loss was 41% and decreased to 7 and 8% with ECP at 0.5 and 1 μg/rat, respectively, but only to 15% with ECP at 2 μg/rat. We interpret this apparent incongruity in dose-dependency as the consequence of an emerging embroyotoxicity caused by supra-physiological estrogen levels. It is worthwhile to remember that, as reported in the materials and methods section, a preliminary study conducted to select ECP testing dosages revealed overt embryo lethal effects involving the majority of the litter following administration of ECP at levels of 3 μg/rat. The concept that supraphysiological estrogen levels can increase embryonic mortality is also consistent with literature data (Dreisbach, 1959; Haddad and Ketchel, 1969; Sarkar et al., 1986; Matsuura et al., 2004).

Focusing on fetal phenotype, the study revealed that ECP co-administration, at any dosage, was ineffective in preventing the skeletal anomalies caused by letrozole. Although it cannot be ruled out that lack of skeletal response reflects the incapacity of maternal ECP treatment to restore proper estrogen levels in the embryonic compartment, it seems feasible that a mechanism other than estrogen ablation is behind the altered skeletal morphology. The spectrum of the observed skeletal anomalies appears to reflect a delay in the ossification process (Carney and Kimmel, 2007). Fetal ossification is highly dependent on maternal physiologic factors, and maternal toxicity has been consistently associated in developmental toxicity studies with...
skeletal anomalies compatible with delayed ossification (Carney and Kimmel, 2007). Besides maternal toxicity, it is also possible that altered ossification reflects a primary and specific effect on the process. This possibility is more likely when skeletal alterations occur in the absence of maternal toxic effects, and/or in association with a specific pattern which is not consistent with a generalized delay (Carney and Kimmel, 2007). In the case of letrozole, the lack of maternotoxicity and absence of signs of generalized ossification delay seem to favor this latter possibility. Searching, from a speculative standpoint, for a possible mechanism accounting for the morphological response, it is notable that letrozole treatment has been shown to significantly increase total circulating insulin-like growth factor 1 (IGF-1) response, it is notable that letrozole treatment has been shown to significantly increase total circulating insulin-like growth factor 1 (IGF-1) response. This is not the first study reporting that antenatal exposure to an aromatase inhibitor is associated with increased placental weight in rats. This effect has also been noted after exposure to anastrozole at doses of 0.1 mg/kg/day or more (Arimidex, prescribing information, 2008), and exemestane at doses of 10, 50, 250 or 810 mg/kg (Beltrame et al., 2001). The estrogen inhibition which determines increased placental weight is occurring outside the placental site, considering that rodent placentas appear not to express aromatase (Terashima et al., 1991). Additional study is needed to ascertain the basis for interference with placental growth mediated by aromatase inhibitors.

Results provided by this study are consistent with the concept that estrogen deprivation plays a key role in letrozole-mediated embryolethality. Estrogen synthesis inhibition does not seem to account for all the adverse effects induced by letrozole on rat gestation.

### References


Submitted on May 4, 2009; resubmitted on June 26, 2009; accepted on July 2, 2009.