Reproductive Toxicology 31 (2011) 194–199

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Stage-dependent abnormalities induced by the fungicide triadimefon in the mouse☆

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ARTICLE INFO

Article history:
Received 19 April 2010
Received in revised form 18 October 2010
Accepted 22 October 2010
Available online 3 November 2010

Key words:
Triadimefon
Teratogenesis
Stage-specificity
Cleft palate
Cardiac defects

ABSTRACT

Aim of this work is the study of abnormalities induced by the triazole triadimefon (FON) administered to pregnant mice at E8, E9, E10, E11 or E12. Pregnant CD-1 mouse were gavaged with FON 500 mg/kg at the selected stages and sacrificed at term and fetuses morphologically examined and processed for visceral and skeletal analysis. Administration of FON on E8, E10–E12 resulted in fetuses with cleft palate (E8 39% and E12 24% representing the peak of sensitivity, in E8 fetuses associated to severe skull basis abnormalities). Other cranial malformations (fusions abnormalities or agenesis of bones) were observed in E8–E10 groups (E8 the most sensitive with 96% of malformed fetuses). Cardiovascular abnormalities were observed in a stage dependent manner at E8–E10 (22.2, 3.8, 7.8%). As far as craniofacial malformation is concerned, we propose that FON acts on two different stages, involved in early and late craniofacial formation.

1. Introduction

Azoles are antifungals widely used as agrochemicals against mildews and rusts of cereal grains, fruits, vegetables, or clinically against topical or disseminated candidiasis and coccidioidal meningitis (the clinical dosage usually suggested is 100–400 mg/day, but larger doses are recommended for candidaemia, cryptococcal or coccidioidal meningitis; the route of administration is dermal, oral or intravenous injection). Azole derivatives act via the inhibition of CYP enzymes involved during the fungal wall biosynthesis (CYP 51) [1,2]. Depending on their chemical properties, they are used both in agriculture and in clinical therapy. Side effects of azoles (mainly hepatotoxic effects) are mostly due to the inhibition of mammalian CYPs [3] and have been related to affected steroidogenesis [4]. All the tested azoles are teratogenic in experimental animals: dose-related effects have been reported after *in vitro* exposure of postimplantation rodent embryos [5–14] or after exposure of pregnant rats and mice [15–19]. All the studied azoles induce the same malformation pattern: the reported embryonic abnormalities were related to the branchial apparatus (abnormal or fused branchial arches) [5–14]. After external and skeletal analysis of CD-1 mouse fetuses exposed *in utero* on day 8 (E8) to 300 mg/kg *per os* of the agrochemical triadimefon (FON) and examined at term of gestation, cleft palate has been observed in 23% of samples, 87% showed cranio-facial defects reconducible to alteration of skeletal derivatives originating from the embryonic branchial apparatus (middle ear, squamosal, zygomatic, alisphenoid, styloid, pterygoid, mandible abnormalities and fusion of skeletal elements) and axial defects (fusion, homeotic respecification and duplication of segments). Interestingly, 77% of examined treated fetuses showed ectopic cartilage at the level of upper jaw [18]. Similar results were obtained by treating *per os* pregnant rats on day 9.5 *post coitum* with 500 mg/kg FON [19].

Tiboni and Giampietro [17] determined phase specific effects of the clinically used Fluconazole by treating CD-1 mice with single oral dose of 700 mg/kg on gestational day 8, 9, 10, 11, or 12. Gestational day 10 was identified as the phase of maximal sensitivity as indicated by the postimplantation loss and for induction of cleft palate. Middle ear abnormalities were detected after exposure on gestational day 8, while limb defects were characteristic of the group exposed on day 10 of gestation. In addition, the authors identified a dose–response relationship, investigated on gestational day 10, showing a clear dose–response in the induction of postimplantation loss and cleft palate.

The aim of this study is to verify if a stage-specificity relationship is detectable also after *in utero* FON exposure, and to expand knowledge on FON-related effects. For this purpose, pregnant CD-1 mice at different gestational stages (E8, E9, E10, E11 or E12) have been treated with a single oral administration of 500 mg/kg FON, and fetuses examined at term of gestation for external, visceral and skeletal morphology.

☆ Grant sponsor: Ministry of University; grant number: 200605902.

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doi:10.1016/j.reprotox.2010.10.011

Table 1
Maternal and fetal parameters.

	CON	FON 8	FON 9	FON 10	FON 11	FON 12
Pregnant dams	13	9	13	9	8	12
mbw change (0–18)	34.69 ± 4.87	27.78 ± 4.74	27.78 ± 9.65	31.0 ± 04.72	31.75 ± 8.03	28.33 ± 5.72
Litter weight	24.08 ± 2.60	19.56 ± 7.13	18.67 ± 7.00	23.00 ± 2.87	23.50 ± 5.18	21.00 ± 5.73
mbw change without litter	10.62 ± 4.35	8.22 ± 5.19	9.11 ± 3.14	8.00 ± 2.60	8.25 ± 3.11	7.33 ± 2.66
Implantations	13.62 ± 1.56	11.56 ± 4.03	11.56 ± 3.91	14.33 ± 1.50	14.13 ± 3.14	12.83 ± 3.43
Dead fetuses	0.08 ± 0.28	0.11 ± 0.33	0.22 ± 0.67	0.22 ± 0.44	0.13 ± 0.35	0.00 ± 0.00
Resorptions	0.08 ± 0.28	1.33 ± 0.58	0.89 ± 1.17	0.75 ± 0.89	0.75 ± 0.71	1.50 ± 1.38
Live fetuses	13.38 ± 1.80	11.00 ± 4.12	10.44 ± 3.84	13.56 ± 1.42	13.25 ± 3.15	11.33 ± 3.93
Postimplantation loss (%)	1.19 ± 2.92	4.95 ± 6.91	10.14 ± 10.88	5.89 ± 7.03	6.54 ± 4.80	12.81 ± 11.73[a]
Fetal weight	1.44 ± 0.11	1.43 ± 0.14	1.41 ± 0.09	1.33 ± 0.09	1.39 ± 0.07	1.47 ± 0.14

Data expressed as means and standard deviations of the litter means. mbw, maternal body weight.
[a] $p < 0.05$ vs. control.

2. Materials and methods

Virgin CD-1 mice (Charles-River, Calco, Italy), housed in a thermostatically maintained room ($T = 22 \pm 2$ °C; relative humidity 55 ± 5%; light cycle from 7:00 AM to 7:00 PM) with food (4RF21; Charles River Laboratories) and tap water *ad libitum*, were caged overnight with males of proven fertility. The day of the vaginal plug was considered as E0. 10 pregnant mice/group were orally treated by gavage with 500 mg/kg FON (Fluka, Milan, Italy; dissolved in a solution containing 10% acetone in corn oil) on E8, E9, E10, E11 or E12 at 10:00 AM. The dosage was selected on the basis of previous range-finding tests in order to obtain almost 100% of *fetuses* with head abnormalities after dam treatment on E8. Controls were dosed with vehicle alone on E8–E12. Females were checked for signs of toxicity after the treatment until the sacrifice and weighted on E0, at the time of treatment and at term of gestation.

2.1. Analysis at term

After dam sacrifice by CO_2 asphyxia, the entire uterine content was weighed and total implantation, live and dead fetuses, early and late resorptions were recorded. The postimplantation loss index [((total implantation − live fetuses)/total implantation) × 100] was calculated. Live fetuses were then explanted from the uterus, morphologically examined, and weighed. The examined fetuses were processed for visceral examination according to the free-hand razor blade sectioning method proposed by Wilson [20] or for the double skeletal staining of bone and cartilage, carried out according to the method described by Kimmel and Trammell [21], partially modified as previously described [22]. The osseous and cartilaginous tissues were stained using respectively alizarin red and alcian blue reagents. After staining, fetuses were analyzed under a dissecting microscope and the abnormalities were recorded.

2.2. Statistical analysis

Statistical analysis (Student's *t*-test) was performed on mean and standard deviation of maternal body weight (mbw) change (E0–18), litter weight, mbw change without litter (mbw change E0–18 – litter weight) and on mean and standard deviation of postimplantation loss index and fetal weight (using litter as experimental unit). The incidence of fetuses with defects was analyzed both by using fetus and litter as experimental unit. The percentage of fetuses and litters affected was analyzed using the Chi-square test. The level of significance was set at $p < 0.05$.

3. Results

No significant signs of maternal toxicity were detected in the treated groups, as shown by clinical signs and by the weight gain after treatment, even if a not significant trend of weight reduction (without litter) was observable (Table 1). FON exposure induced a general increase of postimplantation loss index, statistically significant on E12 (Table 1).

As far as the external examination is concerned, except for E9 group, FON exposure induced cleft palate in fetuses (Table 2). The timing of exposure was critical to determine this malformation: the peak sensitivity for this malformation was after treatment on E8. Interestingly only 1.1% of fetuses exposed on E9 showed this malformation, while the percentage progressively increased from E10 to E12. At the visceral examination, a stage-related significant increase of cardiovascular abnormalities was observed at E8–E10 (respectively 2.2 and 3.8% of great vessels abnormalities at E8 and E9, 3.1 dextrocardia and 4.7 intraventricular septal defects at E10) treatment dosage groups (Table 2).

After skeletal double staining, the morphological analysis of cranial basis bones revealed a complete disruption of bone architecture only in the group treated with FON on E8: fusion of elements

Table 2
External and visceral examination of fetuses: % of affected fetuses/litters. Statistics was done only on bold parameters.

	CON	FON 8	FON 9	FON 10	FON 11	FON 12
Pregnant dams	13	9	13	9	8	12
Total externally examined fetuses	174	99	94	122	106	126
Cleft plate	**0/0**	**39.4[aa]/88.9[aa]**	**1.1[bb]/0.08[bb]**	**16.4[aa,bb,cc]/33.3[aa,bb,cc]**	**15.1[aa,bb,cc]/75.0[bb,cc,dd]**	**23.8[aa,b,cc]/33.3[aa,bb,cc,ee]**
Total viscerally examined fetuses	85	45	53	64	52	63
Total malformed at visceral examination (cardiac defects)	**0/0**	**2.2/11.1[a]**	**3.8/15.4[aa]**	**7.8[a]/44.4[aa,bb,cc]**	**0/0[b,cc,dd]**	**0/0[b,cc,dd]**
Great vessels abnormalities	0/0	2.2/11.1	3.8/15.4	0/0	0/0	0/0
Interventricular septal defects	0/0	0/0	0/0	4.7/11.1	0/0	0/0
Dextrocardia	0/0	0/0	0/0	3.1/22.2	0/0	0/0

[a] $p < 0.05$ vs. control.
[aa] $p < 0.01$ vs. control.
[b] $p < 0.05$ vs. FON 8.
[bb] $p < 0.01$ vs. FON 8.
[c] $p < 0.05$ vs. FON 9.
[cc] $p < 0.01$ vs. FON 9.
[d] $p < 0.05$ vs. FON 10.
[dd] $p < 0.01$ vs. FON 10.
[e] $p < 0.05$ vs. FON 11.
[ee] $p < 0.01$ vs. FON 11.