

The Effects of Trichlorfon on Maternal Reproduction and Mouse Embryo Development during Organogenesis

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Abstract: The organophosphate pesticide trichlorfon is a widely used agricultural broad spectrum insecticide. The health effects on a developing fetus of low level exposure to trichlorofon ingested by the mother in contaminated foods are unclear. We assessed reproductive and developmental toxicity in pregnant female mice following gavage-administered exposure to distilled water or to low levels (12.5, 25, or 50 mg/kg body weight/d) of trichlorofon during organogenesis (gestation days 6–15). Following sacrifice on day 17, reproductive outcomes and teratogenesis were assessed. Trichlorfon exposure did not affect maternal weight gain, organ weights, corpora lutea, implantation sites, or reproductive success, nor were external or skeletal abnormalities evident. The lack of effects of trichlorfon on any *in vivo* reproductive and fetuses endpoints above suggested that for trichlorfon, a hazard of reproductive toxicity below 50 mg/kg body weight/d maybe not expected. However, a well-designed epidemiological study is necessary for further risk assessment of human developing fetus exposed to trichlorfon at a lower level.

Key words: Trichlorfon, Low level exposure, Organogenesis, Reproduction, Development, Mice

Introduction

In recent years much attention has focused on the potential for a wide range of pesticides to interact with and disrupt human reproductive health and genetic homeostasis^{1–3}. Pesticide exposure may alter reproductive behavior and contribute to subfecundity, infertility, pregnancy loss, growth retardation, intrauterine fetal death, birth defects, and testicular and ovarian failure^{4–10}.

Trichlorfon is an organophosphate insecticide that is widely used in agriculture as a broad spectrum insecticide based on its action as an inhibitor of acetylcholinesterase. There have been some experimental animal studies on the

reproductive and teratogenic effects of trichlorfon during organogenesis. Trichlorfon was found to be teratogenic and embryotoxic at a dose level of 400 mg/kg body weight/d^{11, 12}. Embryotoxic and teratogenic effects have been observed in Wistar rats after oral administration of 80 mg/kg body weight dose during a critical period of embryogenesis (day 9 of pregnancy)¹³. An epidemiological study conducted in Hungary reported that a cluster of congenital abnormalities and a subcluster of Down syndrome were caused by eating fish recently exposed to a trichlorfon-containing delousing compound¹⁴. That study estimated the initial trichlorfon content of the fish could have been as high as 100 mg/kg body weight. However, little information is available on the reproductive effect of trichlorfon at a daily dosage below

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100 mg/kg body weight.

Human exposure to pesticides through the consumption of treated foods, water, and environmental contact is a health concern, especially in developing countries. In China, a nationwide investigation of food contamination levels including organophosphorus pesticides in 2,000 found every sample to be contaminated with low but measurable amounts of organophosphorus pesticide residues¹⁵). Trichlorfon residue levels ranged from 0–0.148 mg/kg vegetables¹⁶), averaging 1.22 mg/kg with a maximum level as high as 14.5 mg/kg from vegetables¹⁷). It was reported that maternal to fetus transfer of organophosphorus pesticides may occur⁷), decreasing fetal growth⁹) and shortening the gestational period⁸).

The purpose of the present study was to determine potential reproductive and developmental toxicities of trichlorfon at levels ranging from 12.5–50 mg/kg body weight/d and evaluate the relationship of maternal toxicity and fetal development from administration of trichlorfon during the principal stages of organogenesis.

Material and Methods

Animals, doses, and treatments

Randomly-bred, virgin, female ICR mice, 10–14 wk old and weighing at least 30 g were used. The mice were obtained from the Laboratory Animal Center, Shanghai Jiaotong University. All mice (including males for mating) were maintained under the clean barrier system at a room temperature of $23 \pm 2^\circ\text{C}$ and a relative humidity of $50 \pm 10\%$ with a controlled 12 h light-dark cycle for at least 2 wk prior to mating. The mice were fed a standard breeding granulated diet and tap water was supplied ad libitum. The females were paired with a male (ICR mice) (2:1) overnight and were examined for the presence of a vaginal plug the following morning. The day on which a vaginal plug observed was considered day 0 of gestation. All mice were weighed and randomly assigned to four groups of 10–12 mice/group (0, 12.5, 25 and 50 mg/kg body weight/d) with respect to trichlorfon dose, to give approximately equal group mean body weights. Trichlorfon, purchased at a purity of 97.8% from Sigma-Aldrich (St. Louis, MO), was dissolved in distilled water and administered orally by gavage once daily from days 6–15 of gestation. The dams in the control group received an administration of dilute water only. A standard dose volume of 10 ml/kg body weight was used. Dose volumes were calculated according to individual body weight on the first day of treatment and adjusted for body weight at 3 to 4 d intervals. Selected doses were based on the previously determined LD_{50} of trichlorfon in mice⁶). Presently, the lowest dose level was 12.5 mg/kg body weight/d ($1/32 \text{LD}_{50}$) to correspond to the highest report-

ed level of trichlorfon residue in vegetables; other doses were two- and four-fold the lowest dose. All animal experiments were approved by the Medical Ethics Committee of Shanghai Jiao Tong University School of Medicine.

Maternal observations

All animals were observed daily for toxicological effects and mortality. Body weights of each dam were recorded at 0, 6, 10, 13, and 17 d of gestation. Maternal body weight gains were calculated by subtracting the body weight on days 6 and 17 of gestation. All animals were killed by cervical dislocation at day 17 and were examined macroscopically. Gross macroscopic examinations included all maternal organs with emphasis on uterus, uterine contents, position of the fetus in the uterus, and number of corpora lutea. The liver, spleen, kidneys, ovaries, placenta, and thymus were weighed wet. Litter size, sex composition of each litter (number of male offspring/number of total offspring), percent of implantation, and the number of ovarian corpora lutea were also recorded.

Fetal observation

All the mice were sacrificed by cervical dislocation on day 17 of gestation. The uterine horns were exteriorized through a midline abdominal incision. The number and distribution of intrauterine implantations were classified as live, dead, or resorbed fetuses. Each live fetus was removed from the uterus, sexed, weighed, and carefully examined for external anomalies under a Leica-GZ6 dissecting microscope (Leica Microsystems, Wetzlar, Germany). Placentae were weighted separately. The external malformations examined included exencephaly, cleft palate, abdominal hernia, and polydactyl. An alizarin red S staining procedure¹⁸) was used for observation of skeletal anomalies. Briefly, fetuses were maintained in an alizarin red solution (0.004% in 2.0% KOH) for 3 d, kept in a 70% solution of ethanol, glycerin, and benzylalcohol (2: 2: 1) for 3 d, and finally stored in 100% glycerin until use.

Statistical analyses

A litter was considered to be one experimental unit in the statistical analysis¹⁹). Mean and standard deviations of various data were calculated. Data represent mean \pm SD. If the variables can be assumed to follow a normal distribution, the variance (ANOVA) analysis followed by Student's *t*-test, based on a pooled variance estimate, were used for intergroup comparison (i.e. single treatment groups against the control group). The Kruskal-Wallis test was applied when the data could not be assumed to follow a normal distribution. Fisher's Exact

test for 2×2 tables was applied if the variables could be dichotomized without loss of information. All statistical analyses were carried out using SPSS Ver. 11.5 (SPSS, Chicago, IL) and $p < 0.05$ was considered to represent statistical significance.

Results

Maternal and reproductive outcomes

With regard to reproductive parameters, no trichlorfon treatment related symptoms were observed. Absolute body weights of females were similar among all groups throughout the study. Maternal body weights gain were not statistically different from controls (Table 1). The mean number of corpora lutea, implantations, dead fetuses, resorbed fetuses, live fetuses, and sex ratio (Table 1) did not indicate any significant influence of trichlorfon ($p > 0.05$). Likewise, no macroscopic differences between treated and control animals were evident. Absolute and relative organ weights of livers, kidneys, spleens, thymus, ovaries, and placentas did not differ between trichlorfon treated and control animals (Table 2).

Fetal observations

Mean fetal body weights in the 12.5 mg/kg body weight/day group were significantly decreased, although no significant changes were observed in the mid- and high-dose groups (Table 1). Male fetuses were always significantly heavier than female. The lowest sex ratio (number of male offspring/number of total offspring) of 43.3% was observed in the 12.5 mg/kg group, compared to control (50.7%). Sex ratios in the other trichlorfon treatment groups were groups 49.7% in the 25mg/kg

group and 53.0% in the 50 mg/kg group. None of these differences were significant (Table 1).

There was no evidence of a teratogenic response in any group (Table 3), nor were ossification retardation effects including extra ribs, metacarpal bones, or metatarsal bones evident (Table 4).

Discussion

In the present study, we focused on the reproductive and developmental hazards to mice at trichlorfon dosages ranging from 12.5–50 mg/kg body weight/d following maternal exposure during organogenesis. Our results indicated that trichlorfon exposure during organogenesis did not affect maternal weight gain, organ weights, corpora lutea, implantation sites, or reproductive success, nor where external or skeletal abnormalities evident.

In previous animal studies of the potential reproductive, developmental toxicity, as well as mutagenic effects of trichlorfon have been documented^{6, 11–13, 20–23}. Trichlorfon is teratogenic and embryotoxic in rats at 432 mg/kg body weight/d¹¹, and hamsters at 400 mg/kg body weight/d¹², but not at concentrations of 100 and 200 mg/kg body weight/d⁶. In contrast, such effects have been documented in Sprague-Dawley rats using dietary treated with trichlorfon 7.5 and 88 mg/kg body weight/d for 8 wk²³, and in Wistar rats orally exposed to 80 mg/kg at day 9 of pregnancy¹³. It was also reported that 7.5 mg/kg body weight/day trichlorfon-exposed rats, exhibited significantly decreased cholinesterase activities in brain of F0 adult females, and in erythrocyte and brain of F1 adult females²³. The discordance among studies is attributed to difference in experimental protocols such

Table 1. Reproductive and developmental toxicity outcome treated with trichlorfon during organogenesis

parameter	control	trichlorfon		
		12.5 mg/kg	25 mg/kg	50 mg/kg
Litters (embryos)	12 (160)	11 (134)	11 (153)	12 (149)
Litter size	14.25 ± 1.16	13.73 ± 0.72	14.90 ± 0.95	13.82 ± 1.17
Sex ratio (males/total) (%)	50.7	43.3	49.7	53.0
Fetuses body weight	1.14 ± 0.01	1.02 ± 0.01**	1.10 ± 0.01	1.16 ± 0.02
Male	1.15 ± 0.01	1.05 ± 0.01**	1.11 ± 0.02	1.18 ± 0.02
Female	1.13 ± 0.01	1.00 ± 0.02**	1.09 ± 0.01	1.13 ± 0.02
Maternal body weight gain ^a (g)	22.76 ± 1.65	24.33 ± 0.98	24.35 ± 1.08	20.79 ± 2.54
Number of corpora lutea	16.55 ± 0.86	14.64 ± 0.66	17.20 ± 0.61	16.09 ± 0.80
Number of implants	14.25 ± 1.16	13.72 ± 0.71	14.90 ± 0.95	13.82 ± 1.17
% of implantation (%)	84.16 ± 6.68	94.00 ± 2.76	86.73 ± 5.16	85.61 ± 6.29
% of live fetuses (%)	94.05 ± 2.23	89.76 ± 2.73	92.82 ± 2.90	88.38 ± 3.79
% of dead fetuses (%)	0.98 ± 0.66	1.07 ± 0.72	0.67 ± 0.67	1.07 ± 0.72
% of resorbed fetuses (%)	4.97 ± 2.22	9.17 ± 2.29	6.52 ± 2.99	10.55 ± 3.86

** $p < 0.01$ vs. control group (Student *t*-test).

** $p < 0.05$ vs. control group (Student *t*-test).

^a Maternal body weight gain = Dg17 – Dg6.

Table 2. Organs weight at terminal sacrifice of maternal mice exposed of trichlorfon during organogenesis

parameter	control	trichlorfon		
		12.5 mg/kg	25 mg/kg	50 mg/kg
Number (n)	12	11	11	12
Maternal body weight(g)	63.98 ± 7.58	62.69 ± 5.88	66.72 ± 6.42	63.45 ± 6.75
Liver				
Absolute ^a	3.32 ± 0.63	3.44 ± 0.53	3.30 ± 0.51	3.20 ± 0.42
Relative ^b (× 10 ²)	5.17 ± 0.49	5.48 ± 0.52	4.95 ± 0.54	5.04 ± 0.45
Kidneys				
Absolute ^a	0.50 ± 0.07	0.49 ± 0.06	0.51 ± 0.09	0.50 ± 0.06
Relative ^b (× 10 ³)	7.80 ± 1.11	7.80 ± 0.43	7.60 ± 1.42	7.90 ± 0.59
Spleen				
Absolute ^a	0.18 ± 0.09	0.19 ± 0.09	0.19 ± 0.10	0.13 ± 0.04
Relative ^b (× 10 ³)	2.80 ± 1.51	2.90 ± 1.11	2.90 ± 1.69	2.20 ± 0.53
Thymus				
Absolute ^a	0.06 ± 0.07	0.04 ± 0.01	0.04 ± 0.04	0.05 ± 0.04
Relative ^b (× 10 ³)	0.90 ± 0.99	0.60 ± 0.20	0.70 ± 0.58	1.00 ± 1.14
Ovaries				
Absolute ^a	2.72 ± 0.33	2.25 ± 0.34	2.77 ± 0.38	2.36 ± 0.53
Relative ^b (× 10 ²)	4.26 ± 0.43	3.60 ± 0.40	4.17 ± 0.45	3.89 ± 0.68
Placenta				
Absolute ^a	0.13 ± 0.02	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.02
Relative ^b (× 10 ³)	2.00 ± 0.42	2.14 ± 0.18	1.85 ± 0.33	2.12 ± 0.49

^a organ weight (g).^b organ weight/body weight.**Table 3. Incidence and types of external malformation in day 17 fetuses following maternal treatment with trichlorfon during organogenesis**

parameter	control	trichlorfon		
		12.5 mg/kg	25 mg/kg	50 mg/kg
Number of litter (fetuses) examined	12 (160)	11 (134)	11 (153)	12 (149)
Number of litter with malformed fetuses (%)	0 (0)	0 (0)	1 (0.65)	1 (0.67)
Number of malformed fetuses	0	0	1	1
Number of fetuses with cleft palate	0	0	1	1

Table 4. Effects of maternal treatment with trichlorfon on ossification in near-term fetuses

Group	control	trichlorfon		
		12.5 mg/kg	25 mg/kg	50 mg/kg
No. of litters examined	12	11	11	12
No. of fetuses examined	53	47	58	58
No. of thoracic vertebrae	13.00 ± 0.00	12.91 ± 0.46	12.98 ± 0.13	12.98 ± 0.13
No. of caudal vertebrae	4.57 ± 0.56	4.36 ± 0.64	4.53 ± 0.68	4.52 ± 0.53
No. of metacarpal bone	3.87 ± 0.33	3.72 ± 0.45	3.88 ± 0.37	3.82 ± 0.38
No. of metatarsal bone	4.00 ± 0.00	3.98 ± 0.14	3.98 ± 0.13	4.00 ± 0.00
Incidence of extra rib ^a (%)	18.97	25.45	26.42	31.03

^a No. of fetuses with extra rib/no. examined.

as administration routes. Although there have been some studies on the reproductive and developmental toxicities of trichlorfon, they have mainly focused on the effects at levels exceeding 80 mg/kg. Little information is avail-

able on the reproductive effect of lower levels of trichlorfon which was the focus of the current research.

Since the focus of this manuscript is on the potential for adverse health effects of trichlorfon during lower level

in utero exposures, mated female mice were exposed to trichlorfon on days 6–15 of pregnancy, encompassing the sensitive period of organogenesis. Doses in current study were selected based on LD₅₀ of trichlorfon on mice. The selection of 12.5 mg/kg body weight/day (1/36 LD₅₀) as a lower exposure level also covered the highest level of trichlorfon detected in vegetables in China¹⁵).

We found no evidence of changes either in reproductive or in embryotoxic parameters in treatment groups with an exception of fetal body weight in the 12.5 mg/kg body weight/d group, since a significant decrease was found in fetal body weight in the 12.5 mg/kg group only. There are two possible explanations for this observation. Firstly, the least number of male offspring was found in the 12.5 mg/kg group, with the male fetuses being heavier than their female counterparts (Table 1). Secondly, the observed difference is considered to be incidental because of lack of dose response relationship. Therefore, the finding is not considered to be trichlorfon-related.

Trichlorfon is one of the most heavily used organophosphorus pesticides in China, ranking third for all organophosphorus pesticide use in 2000, with an annual production of 10,906 tons²⁴). The long-term potential for adverse health effects of organophosphorus pesticides during low level in utero exposures has raised great concern, especially in developing countries¹⁵). Aside from occupational exposure, lower level exposure to organophosphorus pesticide residues in food has become a global issue particularly in China²⁵). Therefore, it is important to study the long-term potential for adverse health effects during low level in utero exposures. The present results reveal no effects on reproductive indices presented in this study exposed daily during organogenesis. For trichlorfon, a hazard of reproductive toxicity below 50 mg/kg body weight/day (which includes the highest trichlorfon residue levels detected from vegetables in China) maybe not expected. However, additional data such as cholinesterase activities of erythrocyte and brain conducted *in vivo* and *in vitro* for hazard identification is still necessary to confirm our findings, and well-designed epidemiological studies focusing on the effects of long time maternal exposures to trichlorfon at lower doses on developing fetuses are still necessary further for risk assessment.

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