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Effects of Trichlorfon on Maternal Estrous Cycle, Oocyte Maturation, and Near-term Fetal Developmental Outcome in Mice

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Abstract: Trichlorfon (TCF) is a widely used broad-spectrum agricultural organophosphate (OP) pesticide. Few studies have evaluated the effects of TCF on reproductive toxicity after low-level exposure, especially after long-term exposure. This study assessed the direct effects of TCF on estrous cycle, oocyte maturation in female mice, and developmental outcome in near-term fetuses after 30 consecutive days of maternal exposure to 2, 10, or 50 mg/kg body weight/d TCF *via* drinking water. Both male and female fetuses in the 50 mg/kg/d TCF-treated group had significantly reduced body weights; but this did not occur in the 2 mg/kg/d and 10 mg/kg/d TCF-treated groups. No difference in oocyte maturation, including the percentages of germinal vesicle breakdown (GVBD) and first polar body (PB1) extrusion, or in estrous cycle was found between the control and TCF-treated groups. No increased incidence of fetal external malformations was observed in the TCF-treated groups. Significant decreases in maternal liver weights occurred in the 10 and 50 mg/kg/d TCF-treated groups in a dose-dependent manner. No significant changes were found in the weight of organs such as the ovaries, thymus, kidneys, spleen, lungs, heart or brain. The lack of effects of 2 mg/kg/d and 10 mg/kg/d TCF on any *in vivo* reproductive and developmental endpoints examined suggest that no TCF reproductive toxicity occurs at exposures less than 10 mg/kg/d.

Key words: Trichlorfon, Low-level exposure, Oocyte maturation, Estrous cycle, Reproduction, Development, Mice

Introduction

Trichlorfon (TCF), an organophosphate (OP) pesticide, has been widely used in agriculture as a broad-spectrum pesticide based on its action as an inhibitor of acetylcholine esterase. Recent studies have demonstrated widespread exposure to OP pesticides among some susceptible populations, including pregnant women and children[1, 2]. Risk of deleterious effects of pre- or post-conception exposure to OP pesticides includes menstrual disorders, sterility, fetal toxicity, abortion, stillbirth, and developmental deficits[3, 4]. TCF was one of the most heavily used OP pesticides in China, ranking fourth for all OP pesticide used in 2005, with an annual production of 13,333 tons[5]. Epidemiological studies have reported that *in utero* exposure to OP pesticides may occur, decreasing fetal growth, shortening the gestational period[6], and increasing congenital malformations[7].

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In addition, SL Farr *et al*. examined the association between pesticide use and menstrual function among 3,103 women living on farms, and found that women who used pesticides experienced longer menstrual cycles and more missed periods compared with women who never used pesticides[8].

In animal experiments, the reproductive effect of TCF has been well documented in rodents following maternal exposure to TCF with one acute dose or during fetal organogenesis at high doses. TCF exerted developmental toxicity at a dose level of 480 mg/kg/d in the CP rat and 400 mg/kg/d in the hamster by gavage during the critical period of organogenesis[9]. Major external and skeletal alterations of fetuses occurred after TCF administration by diet to pregnant rats from days 6 through 15 of gestation in the 432 or 519 mg/kg/d dose group[10]. It is important to note, however, that these studies have mainly focused on the acute effects of relatively high doses of TCF. The lowest dose in this study was 10 times the lowest-observed-effect-level (LOEL) of 0.2 mg/kg bw per day (i.e., 2.0 mg/kg bw per day), which is based on inhibition of erythrocyte acetylcholinesterase activity in humans treated orally[11], and allows exploration of the reproductive toxicity of TCF at low-level exposure. Up until now, little information has been available on the reproductive toxicity of TCF after maternal subacute exposure at dosages as low as 2 mg/kg, which includes the TCF residue levels detected from most vegetables in China[12, 13], and little is known about the effects of TCF on maternal estrous cycle. The present study was designed to provide such information.

In the present study, we hypothesized that maternal exposure to TCF for 30 d might disrupt the estrous cycle and inhibit oocyte maturation, which could cause developmental toxicity in late-gestational embryos, because menstrual cycle and oocyte maturation characteristics have implications for women's fecundability and embryonic development[14, 15]. The experimental design was created to test this hypothesis and provide some information on the pathogenesis of developmental defects that originate from damage to the estrous cycle or oocyte maturation.

Materials and Methods

Animals, doses, and treatments

Randomly-bred, virgin, female, 10–14 wk old ICR mice weighing at least 30 g (range 31.5 to 35.2 g) were used for this study. Male ICR mice (10–14 wk old, weighing at least 30 g) were available for mating. Animals were purchased from the Animal House Center at the Chinese Academy of Sciences (Shanghai, China) and housed in a specific pathogen-free room maintained at 23 ± 2°C with a relative humidity of 50 ± 10%. The room was kept on a 12 h light-dark cycle and animals were allowed to acclimate for at least 2 wk prior to beginning experiments. All mice were fed a standard breeding granulated diet, and food was given *ad libitum* during exposures. Female mice were divided randomly into three experimental groups and one control group (25 in each group) according to TCF dose (purity 97.8%, Sigma-Aldrich, MO, USA). TCF was dissolved in distilled water and administered in drinking water (distilled water) at daily doses of 2, 10, or 50 mg/kg/d. Fresh TCF solutions were prepared daily. Water and TCF ingestion were monitored by weighing bottles each time solutions were changed. Body weights were recorded twice a week during the treatment period. The concentrations of TCF in water were adjusted according to changes in drinking water consumption and body weight. The dams in the control group were provided distilled water only.

The maximum TCF dose was selected based on the current OECD guidelines for *in vivo* assays, which suggest that the maximum dose in rodents should be the greatest dose that does not induce any obvious toxicity to the animals[16]. Preliminary studies demonstrated that 50 mg/kg was the greatest tested dose that met this criterion. Additional TCF doses of 2.0 mg/kg, corresponding to 1/225 of the LD50, and 10 mg/kg in mice were used. All animal experiments were approved by the Animal Research Ethical Committee of Shanghai Jiao Tong University School of Medicine. The following three experiments were conducted.

Estrous cycle monitoring

All animals were examined for changes in the estrous cycle during the last 12 d of the 30-d exposure, involving approximately 2–3 estrous cycles in the mice. Therefore, estrous cycle was evaluated from 19 to 30 d of administration. After 18 d of treatment, the vaginal smear was examined every morning (08:00–09:00 h) as described by Zarrow *et al*[17]. Vaginal smears were taken by instilling 0.9% sodium chloride solution into the vagina of the mouse using a plastic pipette tip, exercising care not to insert the tip in too far and stimulate the cervix. The aspirated water and vaginal secretions were applied to clean glass slides. Smears were dried and stained with 0.1% toluidine blue and viewed with a light microscope to observe cell populations and determine estrous cycle phase. The major normal stages and their characteristics are as follows: Proestrous, showing mainly nucleated epithelial cells, parvus keratinized cells; Estrous, showing large keratinized cells without nuclei; Metestrous, showing approximately equal num-

bers of leukocytes, keratinized cells, and epithelial cells; Diestrus, showing almost exclusively leukocytes.

Oocyte preparation and examination

For evaluation of nuclear maturation of oocytes, injection treatment began on the 27th day and ended on the 30th day of the experiment. All female mice were injected i.p. with 10 IU pregnant mare serum gonadotropin (PMSG) (Sigma-Aldrich, MO, USA) followed 48 h later by injection i.p. of 10 IU human chorionic gonadotropin (hCG) (Sigma-Aldrich, MO, USA) to induce superovulation. At 14 h post-injection, the mice were sacrificed by cervical dislocation. Thus, oocyte maturation was evaluated on day 30. Cumulus-oocyte complexes (COCs) were released by puncturing the ampullae of the oviducts with a needle under a stereomicroscope. The cumulus cell masses surrounding the eggs were removed by brief exposure to 300 IU/ml hyaluronidase in M2 medium. After denuding by gentle repeated aspiration and flushing through a glass capillary, oocytes were incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with BAS under mineral oil at 37°C in a humidified atmosphere of 5% CO_2. The meiotic stage of the oocytes was assessed under an inverted microscope (Nikon, Tokyo, Japan). The oocytes were classified into the following stages: 1) GVBD: healthy oocytes showing GVBD in which the outline of the GV had disappeared but no polar body had been released; 2) PB1: healthy oocytes in which a polar body had formed; and 3) Atypical oocytes showing degenerative changes or atypical morphology, such as cytoplasmic fragmentation or an irregular shape of the vitellus[18].

Developmental outcome assessment

For evaluation of developmental outcome, treatment began at 13 d pre-pregnancy and ended at day 17 of gestation (dg 17). On the 13th day, two females were paired with one male overnight, and the females were examined for the presence of a vaginal plug on the following morning. The day on which a vaginal plug was observed was considered dg 0. The pregnant mice were separated from the male and continuously administered TCF until they were sacrificed by cervical dislocation on dg17. Total treatment time was 30 d, which covered pre- and early pregnancy. Maternal ovaries, thymus, liver, kidneys, spleen, lungs, heart, and brain were dissected, freed from adherent tissue, and weighed to the nearest milligram. Organ weights were expressed per 100 g body weight. The uterine horns were exteriorized through a midline abdominal incision. The intrauterine implantations were classified as live, dead, or resorbed fetuses. Gross macroscopic examinations included all maternal organs, position of the fetus in the uterus, and number of corpora lutea. Each live fetus was removed from the uterus, sexed, weighed, and carefully examined for external anomalies under a dissecting microscope (Leica Microsystems, Wetzlar, Germany). The external malformations examined included exencephaly, cleft palate, abdominal hernia, polydactyl, and hedratresia[19]. Placentae were weighted separately.

Statistical analysis

A litter was considered to be one experimental unit in the statistical analysis. Data were expressed as mean ± S.E.M. Statistical significance between the control and experimental data were subjected to analysis of variance (ANOVA) together with Dunnett's test. The Kruskal-Wallis test was applied when the data could not be assumed to follow a normal distribution. All statistical analyses were performed using SPSS 11.5 for Windows (SPSS Inc., IL, Chicago); $p<0.05$ represented statistical significance.

Results

Estrous cycle

The control mice exhibited regular estrous cycle and normal duration of each phases of estrous cycle. Treatment with TCF could not cause any significant change in mean estrous cycles or durations of proestrus, estrus, metestrus and diestrus when compared with the control group (Table 1).

Nuclear maturation of oocytes

The percentage of oocytes at each stage of meiosis

Table 1. Effect of TCF treatment on estrous cycle and duration of each phase

Treatment (mg/kg/d)	No. of mice	Duration in days (mean ± S.E.M.)				Mean estrous cycle (days)	Percentage of abnormal estrous cycle (%) (>6 d)
		Proestrus	Estrus	Metestrus	Diestrus		
Control	8	1.69 ± 0.28	4.88 ± 0.81	1.56 ± 0.24	3.88 ± 0.92	5.93 ± 0.34	25 (2/8)
2	8	1.63 ± 0.35	4.38 ± 0.96	1.94 ± 0.33	4.06 ± 0.86	6.02 ± 0.47	25 (2/8)
10	8	1.75 ± 0.37	4.50 ± 0.50	2.31 ± 0.33	3.44 ± 0.68	5.54 ± 0.53	37.5 (3/8)
50	8	1.63 ± 0.21	4.81 ± 0.63	1.94 ± 0.24	3.63 ± 0.74	5.60 ± 0.32	37.5 (3/8)

was calculated by dividing the number of oocytes at each stage by the total number of oocytes. In the control, almost 95% of the oocytes (128/137) underwent GVBD spontaneously and almost 65% (91/137) extruded PB1 (Table 2). In comparison with the control, TCF treatment did not significantly inhibit GVBD or extrusion of PB1.

Organ weight

Maternal body weights among all groups were not statistically different. No significant changes were found in absolute and relative weight of organs such as the ovaries, thymus, kidneys, spleen, lungs, heart or brain, or in absolute liver weight between the control and TCF-treated groups. However, relative liver weights in the 10 and 50 mg/kg/d TCF-treated groups were decreased significantly compared with those of the control group. To clarify the relation between TCF dose and relative liver weight, Pearson's correlation was conducted. A significantly negative correlation (r=–0.377, p=0.003) was found, indicating a dose-dependent decrease in relative liver weight.

Developmental outcome

No effects on pregnant female mice from treatment were observed at any of the time intervals during the experimental period. No significant differences in maternal body weights between the controls and TCF-treated groups were found. No dead animals were recorded in any group. In addition, animals treated

Table 2. Effect of TCF treatment on nuclear maturation of oocytes

Treatment (mg/kg/d)	No. of mice	Percentage of GVBD	Percentage of PB1 extrusion	Percentage of atypical oocytes
Control	11	93.43 (128/137)	66.42 (91/137)	6.57 (9/137)
2	12	97.74 (130/133)	63.90 (85/133)	2.26 (3/133)
10	14	98.50 (197/200)	61.00 (122/200)	1.50 (3/200)
50	13	94.81 (128/135)	60.74 (82/135)	5.19 (7/135)

Table 3. Organ weight of female pregnant mice exposed to TCF during organogenesis

Parameter	0 (Control)	Trichlorfon (mg/kg/d)		
		2	10	50
Number (n)	15	15	14	15
Maternal body weight (g)	56.48 ± 1.47	58.24 ± 1.52	57.15 ± 1.51	58.69 ± 1.96
Ovary				
Absolute[a]	0.025 ± 0.001	0.025 ± 0.001	0.026 ± 0.001	0.026 ± 0.001
Relative[b]	0.044 ± 0.002	0.043 ± 0.001	0.046 ± 0.002	0.044 ± 0.002
Thymus				
Absolute[a]	0.069 ± 0.004	0.072 ± 0.003	0.075 ± 0.004	0.076 ± 0.004
Relative[b]	0.120 ± 0.011	0.124 ± 0.007	0.133 ± 0.012	0.132 ± 0.010
Liver				
Absolute[a]	3.627 ± 0.082	3.566 ± 0.109	3.534 ± 0.092	3.543 ± 0.098
Relative[b]	6.680 ± 0.214	6.123 ± 0.191	5.991 ± 0.212*	5.857 ± 0.140**
Kidney				
Absolute[a]	0.770 ± 0.012	0.769 ± 0.013	0.774 ± 0.011	0.798 ± 0.014
Relative[b]	1.364 ± 0.031	1.321 ± 0.032	1.363 ± 0.030	1.412 ± 0.040
Spleen				
Absolute[a]	0.396 ± 0.015	0.382 ± 0.018	0.335 ± 0.018	0.371 ± 0.023
Relative[b]	0.719 ± 0.039	0.657 ± 0.051	0.551 ± 0.048	0.633 ± 0.056
Lung				
Absolute[a]	0.312 ± 0.004	0.315 ± 0.004	0.356 ± 0.013	0.310 ± 0.004
Relative[b]	0.553 ± 0.012	0.541 ± 0.010	0.623 ± 0.040	0.529 ± 0.015
Heart				
Absolute[a]	0.253 ± 0.003	0.256 ± 0.006	0.278 ± 0.011	0.250 ± 0.003
Relative[b]	0.448 ± 0.010	0.439 ± 0.011	0.488 ± 0.023	0.423 ± 0.008
Brain				
Absolute[a]	0.697 ± 0.007	0.690 ± 0.008	0.700 ± 0.008	0.718 ± 0.005
Relative[b]	1.235 ± 0.018	1.185 ± 0.036	1.225 ± 0.035	1.224 ± 0.040

[a]absolute organ weight (g); [b]relative organ weight (g/100 g body weight), mean ± S.E.M.
*p<0.05, **p<0.01 compared to control.

Table 4. Effect of TCF on embryonic development and incidence of external malformations on dg 17

Parameter	0 (Control)	Trichlorfon (mg/kg/d)		
		2	10	50
No. of litters (fetuses) examined	17 (236)	11 (142)	17 (254)	14 (214)
Maternal body weight gain (g)[a]	21.82 ± 2.52	24.37 ± 2.34	21.65 ± 0.96	23.72 ± 3.15
No. of corpora lutea per litter	14.88 ± 0.86	16.09 ± 0.71	16.53 ± 0.75	16.07 ± 0.84
No. of fetuses per litter	13.88 ± 0.74	12.91 ± 1.42	14.94 ± 0.63	15.29 ± 1.31
Implantation rate (%) =(total fetuses/corpora lutea)[b]	94.08 ± 1.87	79.38 ± 7.42	91.48 ± 2.87	93.53 ± 3.47
No. of live fetuses	220	132	241	179
No. of dead or resorbed fetuses	16	10	13	35
Percentage of live fetuses per litter	93.60 ± 2.17	93.30 ± 2.94	95.13 ± 1.70	84.21 ± 5.09
Percentage of dead or resorbed fetuses[b]	6.40 ± 2.17	6.70 ± 2.94	4.55 ± 1.65	15.79 ± 5.09
Male fetuses body weight (g)[b]	1.158 ± 0.030	1.154 ± 0.026	1.077 ± 0.023	1.056 ± 0.030*
Female fetuses body weight (g)[b]	1.123 ± 0.030	1.103 ± 0.019	1.065 ± 0.024	1.011 ± 0.028*
Placenta weight (g)[b]	0.086 ± 0.005	0.078 ± 0.004	0.079 ± 0.001	0.095 ± 0.003
No. of malformed fetuses	2[c]	1[d]	2[e]	0

[a]Maternal body weight gain=Dg 0–17.
[b]Litter basis;
[c]open eyelids=1, badger leg=1;
[d]open eyelids=1;
[e]cleft palate=1, syndactyly=1;
*p<0.05, **p<0.01 compared to control.

with TCF did not show any specific toxic signs related to treatment.

The mean placenta weight and numbers of corpora lutea, implantation, dead fetuses, resorbed fetuses, and live fetuses did not indicate any significant influence of TCF (p>0.05). A significant decrease in both male and female fetal body weights was noted only in the 50 mg/kg TCF-treated group compared with control. Male fetuses were heavier than female fetuses. No increased incidence of external malformations was observed in the TCF-treated groups. Absolute body weights of dams were similar among all groups; maternal body weights gain were not statistically different from controls (Table 4).

Discussion

This study focused on estrous cycle, oocyte maturation, and developmental hazards in mice after TCF exposure for 30 d at dosages ranging from 2 to 50 mg/kg/d. Results demonstrated that both male and female fetal body weights in the 50 mg/kg/d TCF-treated group were significantly decreased without increasing incidence of fetal external malformations. No differences in estrous cycle and oocyte maturation were found between the control and TCF-treated groups. Significant decreases in maternal liver weights occurred in the 10 and 50 mg/kg/d TCF-treated groups in a dose-dependent manner. No significant changes were found in the weights of organs such as the ovaries, thymus, kidneys, spleen, lungs, heart, or brain.

The precise mechanism for the potential adverse fetal growth effects associated with OP pesticides is not known. Fetuses may be more susceptible to OP pesticide toxicity because their organ systems are developing rapidly, and they have lower than adult levels of detoxifying enzymes (paraoxonase or chlorpyrifos-oxonase) that deactivate OP pesticides[20]. OP pesticides and their metabolites in a developing organism may have nonspecific effects on growth regulation, perhaps altering the transport of nutrients or the interaction between receptors and proteins involved in the transduction and production of cyclic AMP, contributing to adverse effects on cellular replication and differentiation[21].

Previous studies have reported an association between developmental defects and menstrual or oocyte function. Troya *et al*. observed that mothers of malformed infants usually had longer menstrual cycles and a longer hypothermic phase during the conceptional cycle when compared with a control group of mothers with normal term infants[22]. Baligar *et al*. reported that the disruption in the estrous cycle may decrease the number of healthy follicles with a concomitant increase in atretic follicles, retarding further development of surviving follicles into the successive follicular stage[23]. Also, Van Blerkom assessed human oocyte function and its role in developmental competence, and showed that oocyte maturation arrest could have negative downstream development con-

sequences[24]. These results indicate that developmental defects might be related to menstrual cycle disorders or oocyte maturation arrest.

Cyclic changes in the vaginal smear indicate ovarian activity, which is controlled by the hypothalamopituitary unit; the phases vaginal cells go through represent parallel changes in the entire reproductive tract and, hence, are diagnostic[25]. Some toxicity studies of OP pesticides, such as methyl parathion[26], monocrotophos[27], and dimethoate[28], on animal estrous cycles have reported inconsistent results. In our study, control mice exhibited regular estrous cycle of 4–5 d. Mice treated with TCF exhibited no significant changes in the mean estrous cycle and duration of each phases. This agrees with the results of Astroff *et al*., which indicated no abnormalities in the estrous cycle of TCF-treated (7.5, 25, or 88 mg/kg/d) adult female SD rats before mating[29].

The basic functional unit of reproduction within the ovary is the oocyte[30]. It is essential for nuclear maturation of oocytes to complete the first meiotic division in preparation for normal fertilization and embryonic development[31]. Our results showed that the first meiotic progression from GVBD to extrusion of PB1 was not significantly affected in mouse oocytes after maternal exposure to TCF. In short, no significant change was found in estrous cycle, nuclear maturation of oocytes, or ovary weight in TCF-treated groups compared with the controls, suggesting there was no evidence of TCF on maternal reproductive toxicity.

In the present study, significant decreases in maternal liver weights occurred in the 10 and 50 mg/kg/d TCF-treated groups in a dose-dependent manner. Yamano *et al*. investigated the hepatotoxicity of OPs, TCF, and dichlorvos (a dechlorinated form of OP), in isolated hepatocytes from untreated control and phenobarbital-pretreated rats. These compounds produced toxic effects on hepatocytes as shown by malondialdehyde production and lactate dehydrogenase leakage in a dose-dependent manner up to 2 mM, dichlorvos was more toxic than TCF. Hepatocytes from phenobarbital-pretreated rats were more sensitive to these OPs than those from control rats[32]. Although the relevant liver enzyme concentrations have not been measured, the decrease in liver weights may be caused by chronic hepatocyte injury from TCF and its metabolite dichlorvos.

In conclusion, no effects of TCF at 2 mg/kg/d and 10 mg/kg/d on *in vivo* reproductive and developmental endpoints suggest that TCF has low risk of causing reproductive toxicity at levels less than 10 mg/kg/d. Maternal liver weights in the 10 and 50 mg/kg/d TCF-treated groups significantly decreased in a dose-dependent manner. A significant decrease in fetal body weight was found for the 50 mg/kg/d TCF-treated group (compared to the control), without concomitant maternal estrous cycle and oocyte maturation disruption. Thus, these data do not support the hypothesis that developmental defects originated from damage to the estrous cycle or to oocyte maturation. However, the significant decreases in fetal body weight and maternal liver weight observed after TCF treatment suggest that potential toxic effects of TCF exposure on fetal growth and maternal liver require additional study.

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