Food and Chemical Toxicology 123 (2019) 106–112

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Reproductive and developmental toxicity study of caffeic acid in mice

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ARTICLE INFO

Keywords:
Caffeic acid
Thrombocytopenia in pregnancy
Developmental toxicity
Reproductive toxicity
Teratogenicity

ABSTRACT

Caffeic acid is an antioxidant commonly used to promote hematopoiesis and hemostasis. However, little is known about its systemic safety profile in reproduction and development. Here, we focused on the reproductive and developmental toxicity of caffeic acid in F0 female mice and F1 offspring. In the three-segment study, the F0 female mice were continuously exposed to 0, 0.15, 5 or 150 mg/kg/day of caffeic acid by gavage. We found that 5 mg/kg/day and 150 mg/kg/day of caffeic acid affected implantation of embryos when administered before gestation day 6. In addition, 150 mg/kg/day of caffeic acid affected fetal weight gain. No maternal toxicity, fetal teratogenesis or post-natal effects on pup development were observed. The no-observed-adverse-effect-level was 0.15 mg/kg/day for pregnant mice under the conditions of this study.

1. Introduction

Caffeic acid (CFA, trans-3,4-dihydroxycinnammic acid) is a plant growth regulator abundantly found in plants (Park, 2009). It is also an intermedium of many plant compounds such as chlorogenic acid, cynarin and coumarins (Jiang et al., 2005; Li et al., 2004; Pellati et al., 2005). Currently, CFA is a self-developed drug commonly used to promote hematopoiesis and hemostasis in China. In the 1970s, the pharmaceutical research group of Nanjing College of Pharmacy discovered that CFA has hemostatic, leukogenic, thrombopoietic, choleretic and anti-hyperlipidemic effects (Xu et al., 1980). In recent years, CFA has been used to treat various causes of thrombocytopenia and leukocytopenia in China, such as myelosuppression after radiotherapy or chemotherapy (Jiang and Hao, 2008; Pang et al., 2012), aplastic anemia (Song et al., 2009) and primary immune thrombocytopenia (Lin et al., 2009; Qin et al., 2015).

Incidence of thrombocytopenia in pregnancy ranges from 6 to 10% (Danaee et al., 2014; McCrae, 2010), which is four times that of non-pregnant females. Gestational thrombocytopenia and primary immune thrombocytopenia (ITP) are two main causes (Schwartz, 2000). Given the risks associated with thrombocytopenia in pregnancy, physicians have paid great attention to the treatment over the past decade. The most commonly used regimens include steroids, thrombopoietin receptor agonists, and intravenous immunoglobulin (Cines and Levine, 2017). These therapies have mild to moderate adverse effects on the mother and fetus (Boehlen, 2006; Cines and Levine, 2017). The need for a reliable and safe thrombopoietic agent for thrombocytopenia in pregnancy is urgent. CFA may have the potential in treating thrombocytopenia in pregnancy.

In 1980, Xu et al. performed an in vivo study with tritium-labeled CFA and found that only a small quantity passed through the placental barrier (Xu et al., 1980). A few years later, the same group reported the anti-implantation activity after CFA administration, and explored the relevant mechanisms (Zheng and Xu, 1986; Zheng et al., 1987, 1988). CFA significantly reduced the level of plasma progesterone in mice in early pregnancy, possibly through luteolysis caused by an anti-gonadotrophic effect, and then interfered with implantation. The anti-implantation activity of CFA could be reversed by administration of human chorionic gonadotropin, dopamine antagonists or megestrol acetate (Zheng and Xu, 1986).

Though researchers have explored the anti-implantation activity of CFA, to date there have been no systemic preclinical studies of reproductive and developmental toxicity, which prevents conduction of clinical trials in pregnant and lactating women. We investigated the

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https://doi.org/10.1016/j.fct.2018.10.040
Received 9 August 2018; Received in revised form 30 September 2018; Accepted 14 October 2018
Available online 23 October 2018

reproductive and developmental toxicity of CFA in a three-segment mouse study.

2. Materials and methods

All experimental procedures comply with the Technical Guidelines for non-Clinical Safety Study of Drugs, Technical Guidelines for Reproductive Toxicity Study of Drugs and Good Laboratory Practice of China Food and Drug Administration. All animal experiments comply with the ARRIVE guidelines and were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition, revised 2011) (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011). All animal procedures were performed under the approval of the Animal Ethics Committee of Shandong University School of Medicine.

The fertility toxicity study, prenatal developmental toxicity study and reproductive toxicity study were conducted in accordance with ICH Harmonised Guideline (2017): Detection of Toxicity to Reproduction for Human Pharmaceuticals S5 (R3) (ICH, 2017).

2.1. Preparation of CFA

The bulk drug of CFA was provided by Dezhou Deyao Pharmaceutical Co., Ltd. (batch number: 1101150908; Dezhou, China), and the effective component was CFA with a purity of 99.7%. After the bulk drug was fully ground, CFA was dissolved in 0.5% carboxymethyl cellulose sodium (Sigma Aldrich; St. Louis, MO, USA). Before the start of the study, the dose level concentrations, homogeneity and stability of CFA in 0.5% carboxymethyl cellulose sodium aqueous solution at concentrations of 15 μg/mL, 500 μg/mL and 15000 μg/mL were analyzed using high-performance liquid chromatography-tandem mass spectrometry (HPLC; Agilent 1260 Infinity II, Agilent Technologies; Wilmington, DE, USA). The suspensions were confirmed stable after 4-h storage at room temperature. The concentrations of CFA in the dosing solutions were analyzed at the first and last preparation, and were verified to be 97.1–104.3% of the target. Suspensions were prepared daily before use. The bulk drug of CFA and feeds were stored in a dry place at room temperature, protected from light.

2.2. Fertility toxicity study in females

This section was designed to assess the disturbances of CFA on maternal reproductive functions, mating behavior, fertilization and embryo implantation by exposure from premating to implantation (14 days before mating-gestation day 5(G5)).

2.2.1. Animals

Eighty virgin female ICR mice (7 weeks old, 25–31 g) and 80 male ICR mice for mating (10 weeks old, 33–40 g) were used. Mice were acclimatized for one week before dosing. Animals were housed individually in standard polycarbonate cages under standardized conditions with a controlled temperature of 19–25 °C, a relative humidity of 40–70% and a 12 h dark/12 h light cycle. Each female mouse was identified with an ear tag. Mice were offered with a sterilized commercial feed pellets (Jiangsu Xietong Medical Bio-engineering Inc.; Nanjing, China) and sterilized tap water ad libitum.

2.2.2. Dosing

Doses were determined according to a previous dose-finding study. Female mice were randomly distributed into four groups and received the following doses of CFA by gavage: 0 mg/kg/day (control), 0.15 mg/kg/day (low-dose), 5 mg/kg/day (mid-dose) or 150 mg/kg/day (high-dose). Control mice received 0.5% carboxymethyl cellulose sodium solution. CFA concentrations used in corresponding experimental groups were 15 μg/mL (low-dose), 500 μg/mL (mid-dose) and 15000 μg/mL (high-dose). Volumes were calculated and adjusted based on the most recent weight (10 mL/kg). Body weight was measured every three days. Dosing began 14 days (two complete estrus cycles) before mating and continued until G5. No drugs were administered to male mice.

2.2.3. Mating

One male and one female were randomly chosen and placed together in a cage during the dark period. The mating period lasted for two weeks. During this period, daily vaginal smears were examined for the presence of sperm at 8:00 AM. The presence of sperm in the vaginal smear and/or a mating plug were considered evidence of successful mating, and the day was recorded as G0. Copulation index (no. of females copulated/no. of pairs × 100%) was calculated after the mating period.

2.2.4. Maternal observations

Female mice were observed at least once daily during CFA treatment period for activities, gait, behavior and other clinical signs (changes in skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions) in order to assess the health status (Burkholder et al., 2012; Falk et al., 2017). If any mouse showed extreme pain or distress, it would be terminated and underwent gross pathological examination and histopathological analysis of its organs and tissues. Body weight was measured every three days. Feed consumption was monitored weekly.

2.2.5. Necropsy of F0 female mice

Female mice were terminated with chloral hydrate by gavage on G18 and then dissected for gross anatomy within half an hour. The morphology, color, border, size, texture and section of important organs, including heart, liver, spleen, kidneys and reproductive organs were inspected. The body weight without the uterus was recorded. Histopathological evaluations were performed on hematoxylin-eosin stained tissue sections for any abnormal organs. The numbers of corpora lutea, implantations, viable fetuses and absorbed fetuses were recorded, and gender was determined. Conception and abortion were recorded. Fertility index was calculated (no. of females pregnant/no. of females copulated × 100%).

2.3. Prenatal development toxicity study

This section aimed to detect adverse effects of CFA on the pregnant female and development of the embryo and fetus during the period of major organogenesis (G6-G15).

2.3.1. Animals

In the present study, a total of 80 successfully mated female ICR mice (10 weeks old, 32–38 g) were used. Mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were housed individually in standard polycarbonate cages under conditions described in 2.2.1.

2.3.2. Dosing

Doses were the same as in the fertility toxicity study (2.2.2). Each mouse received the dose by gavage from G6 to G15.

2.3.3. Maternal observations and necropsy

Female mice were observed as described in 2.2.4. F0 female mice were then terminated with chloral hydrate gavage, dissected and observed on G18 following the methods described in 2.2.5.

2.3.4. Fetal analyses

Fetuses were weighed and observed by anatomic microscope to check for external malformations, including encephalocele, cleft lip, short limb, polydactyly, oligodactyly, short tail, kinked tail, and so on.

Table 1
General examination of F0 females in fertility toxicity study.

	Control	Low-dose	Mid-dose	High-dose
No. of females	20	20	20	20
No. of deaths before/during mating	0	0	1[a]	0
No. of deaths during pregnancy	0	0	0	0
No. of females copulated	19	18	19	20
Copulation index (%)	95%	90%	100%	100%
Initial body weight (g)	29.53 ± 1.22	28.49 ± 2.02	28.87 ± 1.89	29.47 ± 1.06
Body weight gain (g)				
Days 1–7 before CFA administration	0.92 ± 1.15	1.64 ± 1.49	1.67 ± 1.56	1.15 ± 1.10
Days 1–14 of CFA administration	5.80 ± 2.89	4.83 ± 2.37	5.26 ± 2.23	5.45 ± 1.74
Days 0–6 of pregnancy	1.95 ± 1.28	1.34 ± 1.06	1.32 ± 1.19	1.16 ± 1.31
Days 6–15 of pregnancy	10.16 ± 2.07	9.27 ± 2.41	7.06 ± 4.67*	3.72 ± 4.20**
Days 15–18 of pregnancy	7.06 ± 3.01	6.77 ± 3.17	4.58 ± 4.00*	1.61 ± 2.96**
Feed consumption (g/day/mouse)				
Days 6–7 before CFA administration	8.05 ± 2.20	7.89 ± 1.60	8.16 ± 2.12	7.75 ± 2.07
Days 6–7 of CFA administration	8.00 ± 1.83	8.22 ± 2.02	7.68 ± 1.53	8.30 ± 1.38
Days 6–7 of pregnancy	7.95 ± 2.09	8.83 ± 2.41	8.53 ± 2.46	8.65 ± 2.48
Days 13–14 of pregnancy	8.26 ± 1.88	8.61 ± 1.79	8.32 ± 1.86	8.40 ± 1.54

*$p < 0.05$, **$p < 0.01$ vs. control.
[a] One female (No.4, mid-dose) died at day 3 of CFA administration due to dosing accident, not CFA-related.

For the visceral examination, half of the fetuses in each litter were examined using the method of Staples for presence of dysplasia or malformation (Kang et al., 1986; Staples, 1974). The remaining half of the fetuses were examined for skeletal anomalies. The skeleton was stained with Alizarin red (Isoherranen et al., 2003; Nelson et al., 1984) and we recorded the number and type of variations and malformations.

2.4. Reproductive toxicity study

In this section, we generally investigated the adverse effects of CFA on maternal fertility, embryo implantation, embryo-fetal development and postnatal development of pups by exposure of the female throughout the study (14 days before mating-day 21 of lactation period).

2.4.1. Animals

Eighty virgin female ICR mice (7 weeks old, 27–32 g) and 80 male ICR mice for mating (10 weeks old, 32–41 g) were used. Mice were acclimatized for one week before dosing. Animals were housed and fed as described above (2.2.1). Mating was as described in 2.2.3.

2.4.2. Dosing

Doses were the same as in the fertility toxicity and prenatal development toxicity studies (2.2.2). Each mouse was administered the drug or control by gavage persistently from 14 days (two complete estrus cycles) before mating until day 21 of lactation period. Volumes for gavage were adjusted according to the most recent weight. No drugs were administered to male mice.

2.4.3. Maternal observations

Female mice were observed as described in 2.2.4. In addition, physical and behavioral abnormalities were observed in dams after delivery. Parturition was observed to calculate the gestation index (no. of females with parturition/no. of females copulated × 100%).

2.4.4. Viability and developmental state of F1 mice

The day on which parturition was completed was designated as post-natal day 0 (P0). The number of live births, stillbirths and malformed pups was recorded after delivery. Gender was determined by gross examination of live pups. The F1 mice were weighed every three days. The time of eye opening and tooth eruption was recorded. Physiological status and behaviors (surface righting reflex, cliff avoidance, negative geotaxis, auditory startle reflex and visual placing reflex) were observed. The number of live pups on P4 and P21 were counted. The viability index (no. of live pups/no. of pups delivered × 100%) on P0, P4 and P21 were calculated.

2.5. Statistical analysis

All measured parameters were calculated and expressed as mean ± standard deviation or percentage. For all numerical values, homogeneity of variances was tested using the Bartlett's test. Homogeneous data were analyzed using one-way ANOVA, followed by the Dunnett's multiple comparison test to compare the test groups with the control group. In case of heterogeneity of variances, groups were compared using Kruskal-Wallis non parametric ANOVA followed by Newman-Keuls multiple comparison test. For the comparison of classification measurements, the Fisher's exact test ($n < 100$) or chi-square test with Yates' correction for continuity ($n \geq 100$) was employed. Probability of 0.05 ($p < 0.05$) was used as the criterion of significance.

3. Results

3.1. Fertility toxicity study in females

3.1.1. Maternal observations

No abnormality was noted in appearance, behavior and other clinical signs of the F0 female mice in all experimental groups. No significant difference was found in feed consumption between groups. The body weight of F0 females in the low-dose group increased steadily and weight gain was comparable to that in control group. However, mean body weight gain of F0 females in mid- and high-dose groups was significantly reduced (G6-15, G15-G18). No significant difference in the copulation index was found between CFA-treated groups and the control group (Table 1).

One female mouse in the mid-dose group (No. 4) died at the third day of CFA administration, before mating. Dissection revealed foam-like liquid in the airway, indicating that mis-operation of gavage caused death, not relevant to CFA treatment.

3.1.2. Reproductive evaluation of F0 females by necropsy on G18

No obvious lesions or structural abnormalities were found. No significant differences were noted between CFA-treated groups and the control group with respect to body weight without uterus, fetal sex ratio

Table 2
Maternal reproductive evaluation by necropsy of F0 females on G18 in fertility toxicity study.

	Control	Low-dose	Mid-dose	High-dose
No. of pairs	20	20	19	20
No. of females copulated	19	18	19	20
No. of females pregnant	19	18	13	5
Fertility index (%)	100%	100%	68%*	25%**
Body weight without uterus (g)	42.69 ± 4.03	41.58 ± 3.50	40.63 ± 3.07	40.48 ± 3.05
No. of corpora lutea	13.21 ± 2.55	13.22 ± 2.07	13.63 ± 1.69	13.20 ± 2.42
No. of implantations	12.58 ± 2.41	12.89 ± 2.00	6.32 ± 5.16**	2.70 ± 4.73**
Pre-implantation losses[a] (%)	4.18 ± 8.85	2.36 ± 4.02	53.32 ± 37.65**	79.21 ± 36.94**
No. of live fetuses per litter	12.42 ± 2.37	12.72 ± 1.97	6.32 ± 5.16**	2.65 ± 4.64**
No. of dead fetuses per litter	0.05 ± 0.22	0 ± 0	0 ± 0	0 ± 0
No. of early resorptions	0.05 ± 0.22	0 ± 0	0 ± 0	0.05 ± 0.22
No. of late resorptions	0.05 ± 0.22	0.17 ± 0.50	0 ± 0	0 ± 0
Post-implantation losses[b] (%)	1.21 ± 2.88	1.20 ± 3.51	0 ± 0	0.42 ± 1.82
Sex ratio (males/females)	0.92	1.12	1.14	0.96
Fetal body weight (g)	1.56 ± 0.17	1.54 ± 0.16	1.54 ± 0.15	1.56 ± 0.16

*$p < 0.05$, **$p < 0.01$ vs. control.
[a] Pre-implantation loss per litter = [(corpora lutea − implantation sites)/corpora lutea] × 100%.
[b] Post-implantation loss per litter = [(implantation sites − viable fetuses)/implantation sites] × 100%.

or fetal body weight. In the mid- and high-dose groups, fertility indexes were lower than control. The number of implantations was also statistically lower and pre-implantation losses were significantly higher in mid- and high-dose CFA-treated groups than control. The reduced implantations and increased losses resulted in fewer viable fetuses, while the number of corpora lutea showed no difference. We found no CFA-related differences in post-implantation losses, dead fetuses or absorbed fetuses (Table 2).

3.2. Prenatal development toxicity study

3.2.1. Maternal observations

No clinical signs indicative of toxicity were observed during the course of this study. No abnormalities were noted in appearance or behavior of the F0 female mice in all experimental groups. Body weight gain and feed consumption showed no difference between groups (Table 3).

3.2.2. Reproductive evaluation of F0 females by necropsy on G18

No obvious abnormalities in the morphology or structure of heart, liver, spleen, kidneys and reproductive organs were noted. The numbers of corpora lutea, implantations, viable fetuses, dead fetuses and absorbed fetuses in CFA-treated groups were comparable with those in control group (Table 4). There were no CFA-related changes in pre-implantation losses or post-implantation losses. In one female in the high dose group, we found no live fetuses. The post-implantation loss was 100%, all of which were early absorptions.

3.2.3. Fetal teratology data

Fetal body weight was significantly lower in the high-dose group than control (Table 5), while no significant differences were noted between low- or mid-dose group and control. We found no significant differences in external malformations or variation of fetuses in CFA-treated groups compared to control. Visceral examinations revealed no significant differences in the incidence of malformations or variations (Table 5). One case of cleft palate was observed in the high-dose group. Visceral variations commonly seen in fetal mice included small thymus, enlarged nasal cavity, dilated lateral brain ventricles, dilated renal pelvis, etc. For skeletal examinations, the incidences of malformations and variations were within historical control limits in all groups with no significant differences (Table 5). One case of bipartite sternum was observed in the high-dose group. Skeletal variations commonly found in fetal mice included supernumerary ribs, incomplete temporal bone ossification, supraoccipital perforation, etc.

3.3. Reproductive toxicity study

3.3.1. Maternal observations

No abnormalities were noted in appearance, behavior or other clinical signs of the F0 female mice both before and after delivery. No significant differences were found in the copulation index between groups. No abnormalities were found in lactation of dams in any group. No significant differences were observed in body weight between groups before CFA treatment. However, the body weight of CFA-treated dams was significantly lower in high-dose group compared to control during gestation (G6-G15, G15-G20). After delivery, no significant differences were found in body weight of F0 females. The gestation index in high-dose group was significantly lower than control (Table 6).

3.3.2. General observation of F1 offspring at birth

The number of live births was statistically reduced in mid- and high-dose groups compared to control. Multiple doses of CFA had no distinct

Table 3
General examination of F0 females in prenatal development toxicity study.

	Control	Low-dose	Mid-dose	High-dose
No. of females copulated	20	20	20	20
No. of deaths during pregnancy	0	0	0	0
Initial body weight (g)	35.71 ± 1.03	35.97 ± 1.22	36.28 ± 0.93	36.16 ± 1.47
Body weight gain (g)				
Days 0–6 of pregnancy	1.36 ± 0.61	1.54 ± 0.88	1.96 ± 1.56	1.90 ± 1.46
Days 6–15 of pregnancy	9.17 ± 2.28	8.84 ± 2.14	7.98 ± 3.07	8.97 ± 2.30
Days 15–18 of pregnancy	6.92 ± 5.35	6.45 ± 2.86	7.31 ± 5.28	5.20 ± 4.33
Feed consumption (g/day/mouse)				
Days 6–7 of pregnancy	7.70 ± 1.75	7.90 ± 1.59	8.00 ± 2.05	8.90 ± 2.29
Days 13–14 of pregnancy	8.50 ± 1.93	8.45 ± 1.93	7.75 ± 2.00	8.30 ± 1.69

Table 4
Maternal reproductive evaluation by necropsy of F0 females on G18 in prenatal development toxicity study.

	Control	Low-dose	Mid-dose	High-dose
No. of females copulated	20	20	20	20
No. of females pregnant	20	20	19	20
Fertility index (%)	100%	100%	95%	100%
Body weight without uterus	41.91 ± 2.04	42.01 ± 2.86	42.25 ± 2.66	42.71 ± 2.45
No. of corpora lutea	12.20 ± 2.42	11.80 ± 2.34	12.53 ± 2.20	12.25 ± 2.62
No. of implantations	11.80 ± 2.34	11.45 ± 2.36	12.16 ± 3.31	12.10 ± 2.49
Pre-implantation losses[a] (%)	2.27 ± 4.85	2.92 ± 5.61	7.58 ± 21.65	0.98 ± 3.07
No. of live fetuses per litter	11.60 ± 2.31	11.35 ± 2.39	12.00 ± 1.95	11.30 ± 3.62
No. of dead fetuses per litter	0.10 ± 0.30	0.05 ± 0.22	0.05 ± 0.22	0.10 ± 0.44
No. of early resorptions	0.10 ± 0.30	0.05 ± 0.22	0.05 ± 0.22	0.05 ± 0.22
No. of late resorptions	0.11 ± 0.31	0.20 ± 0.51	0.05 ± 0.22	0.05 ± 0.22
Post-implantation losses[b] (%)	2.44 ± 3.82	0.92 ± 2.76	1.19 ± 2.78	6.65 ± 21.81
Sex ratio (males/females)	0.97	1.06	1.11	0.97

[a] Pre-implantation loss per litter = [(corpora lutea − implantation sites)/corpora lutea] × 100%.
[b] Post-implantation loss per litter = [(implantation sites − viable fetuses)/implantation sites] × 100%.

influence on the number of stillbirths or malformed pups. The sex ratio of live pups was not markedly altered by CFA. The viability index of pups at birth in each CFA-treated group was comparable to control. The mean body weight of pups at birth was significantly lower in the high-dose group than control (Table 7).

3.3.3. *Developmental observation of F1 offspring*

After birth, the body weight of F1 mice increased rapidly, and no significant differences were noted between groups at the end of the lactation period (Table 7).

We found no significant differences in the time of eye opening or tooth eruption of F1 mice. Parameters reflecting nerve function (surface righting reflex, cliff avoidance, negative geotaxis, auditory startle reflex and visual placing reflex) in CFA treated groups were not significantly different from control. The viability indexes on P4 and P21 in CFA-treated groups were comparable to those in the control group (Table 7).

4. Discussion

In this study, we investigated the reproductive and developmental toxicity of CFA in mice. Results indicated that 5 mg/kg/day and 150 mg/kg/day of CFA affected embryo implantation, and 150 mg/kg/day of CFA affected fetal weight gain. We observed no maternal toxicity, fetal teratogenesis or post-natal effects on pup development.

The pre-clinical model was chosen based on information from the literature. Since the anti-implantation activity by CFA can be reversed by exogenous administration of dopamine antagonists in mice, CFA was considered to have an effect on the dopamine system. Given that rats depend on prolactin as the primary hormone for establishment and maintenance of early pregnancy, which limits their application in dopamine agonists related researches (China Food and Drug Administration, 2006; ICH, 2017), a mouse model was chosen for our study.

We conducted preliminary experiment in order to determine appropriate doses for the mouse model. In our preliminary experiment, CFA was administered by gavage to F0 females from two weeks before mating to the end of lactation period at 0 mg/kg/day, 0.15 mg/kg/day, 1.5 mg/kg/day, 15 mg/kg/day, 150 mg/kg/day or 1500 mg/kg/day. Concentrations were selected for three reasons. First, the clinical dose of CFA in patients is 15 mg/kg/day (assuming an average body weight of 60 kg). Second, the median lethal dose (LD50) via gavage is 4850 mg/kg (Zhang and Shen, 1994). Third, the median effective dose (ED50) in anti-implantation activity is approximately 4.26 mg/kg/day (Zheng et al., 1987). The preliminary experiment revealed no significant changes in reproductive parameters of F0 females or developmental parameters of F1 mice at 0.15 mg/kg/day. A gestation index of

Table 5
External, internal and skeletal examination of fetuses on G18 in prenatal development toxicity study.

	Control	Low-dose	Mid-dose	High-dose
No. of live fetuses	232	227	228	226
Fetal body weight (g)	1.53 ± 0.14	1.53 ± 0.15	1.51 ± 0.13	1.37 ± 0.19**
No. of fetuses examined externally	232	227	228	226
No. of external malformations (litters)	0 (0)	0 (0)	0 (0)	0 (0)
No. of external variations (litters)				
Kinked tail	1 (1)	0 (0)	0 (0)	0 (0)
No. of fetuses examined viscerally	121	117	120	119
No. of visceral malformations (litters)				
Cleft palate	0 (0)	0 (0)	0 (0)	1 (1)
No. of visceral variations (litters)				
Small thymus	1 (1)	0 (0)	0 (0)	5 (3)
Enlarged nasal cavity	7 (3)	1 (1)	1 (1)	2 (2)
Dilated lateral brain ventricles	0 (0)	0 (0)	0 (0)	1 (1)
Dilated renal pelvis	1 (1)	0 (0)	1 (1)	0 (0)
No. of fetuses examined skeletally	111	110	108	107
No. of skeletal malformations (litters)				
Bipartite sternum	0 (0)	0 (0)	0 (0)	1 (1)
No. of skeletal variations (litters)				
Supernumerary ribs	3 (3)	1 (1)	2 (2)	3 (2)
Incomplete ossification of temporal bone	1 (1)	0 (0)	0 (0)	0 (0)
Supraoccipital perforation	0 (0)	0 (0)	1 (1)	0 (0)

*p < 0.05, **p < 0.01 vs. control.

Table 6
General examination of F0 females in reproductive toxicity study.

	Control	Low-dose	Mid-dose	High-dose
No. of females	20	20	20	20
No. of deaths before/during mating	0	0	0	0
No. of deaths during pregnancy	0	0	0	0
No. of females copulated	20	19	20	20
Copulation index (%)	100%	95%	100%	100%
No. of females with parturition	17	18	14	6
Gestation index (%)	85%	95%	70%	30%**
Initial body weight (g)	29.38 ± 1.27	28.77 ± 1.02	29.68 ± 1.15	29.74 ± 1.12
Body weight gain (g)				
Days 1–7 before CFA administration	1.88 ± 1.12	1.12 ± 1.55	1.21 ± 1.56	1.65 ± 0.66
Days 1–14 of CFA administration	3.42 ± 2.44	3.80 ± 1.98	4.36 ± 2.56	4.36 ± 1.34
Days 0–6 of pregnancy	1.92 ± 1.63	1.65 ± 1.09	2.15 ± 1.56	1.62 ± 1.77
Days 6–15 of pregnancy	8.19 ± 3.77	8.63 ± 2.59	6.09 ± 3.21	4.12 ± 3.99**
Days 15–20 of pregnancy	13.64 ± 6.79	17.17 ± 5.98	10.24 ± 8.30	4.66 ± 7.43**
Days 3–21 of lactating period	1.83 ± 4.63	−0.62 ± 3.23	0.37 ± 3.38	0.33 ± 3.13
Feed consumption (g/day/mouse)				
Days 6–7 before CA administration	7.80 ± 1.99	7.68 ± 1.53	7.95 ± 1.39	8.15 ± 2.30
Days 6–7 of CA administration	8.40 ± 2.39	7.63 ± 1.54	7.75 ± 1.80	7.35 ± 1.23
Days 6–7 of pregnancy	8.40 ± 2.28	8.42 ± 2.14	7.65 ± 1.87	8.15 ± 2.01
Days 13–14 of pregnancy	8.80 ± 1.54	8.84 ± 1.68	7.85 ± 1.73	7.70 ± 2.20
Days 6–7 of lactating period	9.40 ± 2.84	8.00 ± 1.25	8.20 ± 2.38	8.50 ± 2.24
Days 13–14 of lactating period	8.45 ± 1.70	8.21 ± 1.87	8.55 ± 2.14	7.80 ± 1.44

*p < 0.05, **p < 0.01 vs. control.

Table 7
Development of F1 pups in reproductive toxicity study.

	Control	Low-dose	Mid-dose	High-dose
No. of litters	17	18	14	6
No. of live pups delivered	231	218	105	40
No. of stillbirths	1	0	1	0
No. of pups with malformations	0	0	0	0
Sex ratio of live pups (males/females)	1.01	1.02	0.91	1.22
Viability index (%)				
Day 0 of lactation	99.57%	100.00%	99.06%	100.00%
Day 4 of lactation	88.79%	81.19%	87.74%	92.50%
Day 21 of lactation	80.60%	72.94%	71.70%	87.50%
Body weight of pups during lactation (g)				
Day 0 of lactation	1.96 ± 0.30	1.96 ± 0.26	1.99 ± 0.21	1.73 ± 0.18**
Day 4 of lactation	2.97 ± 0.45	3.04 ± 0.35	2.90 ± 0.39	2.59 ± 0.24**
Day 21 of lactation	12.91 ± 1.56	12.81 ± 0.96	12.59 ± 1.05	12.62 ± 0.51
Eye opening (day)	15.63 ± 1.27	15.93 ± 1.31	15.33 ± 1.05	15.70 ± 1.11
Tooth eruption (day)	10.6 ± 0.81	10.65 ± 0.83	10.53 ± 0.72	10.33 ± 0.66
Surface righting reflex (day)	6.55 ± 0.85	6.35 ± 0.95	6.30 ± 0.97	6.50 ± 0.88
Negative geotaxis (day)	7.60 ± 1.52	7.45 ± 1.72	7.55 ± 1.43	8.09 ± 1.22
Cliff avoidance (day)	6.60 ± 0.93	6.40 ± 0.81	6.30 ± 0.72	6.69 ± 0.96
Visual placing reflex (day)	16.60 ± 0.93	16.30 ± 0.72	16.65 ± 0.95	16.34 ± 0.76
Auditory startle reflex (day)	16.65 ± 0.95	17.00 ± 1.01	16.90 ± 1.01	16.91 ± 1.01

*p < 0.05, **p < 0.01 vs. control.

30% (3/10) was observed at 150 mg/kg/day. At 1500 mg/kg/day, mortality reached 37.5% (3/8), which did not comply with the requirement of the reproductive toxicity test (< 10%) and the dose was not considered for further evaluation (Moore et al., 2013). Accordingly, we chose to administer 0.15 mg/kg/day, 5 mg/kg/day and 150 mg/kg/day in our formal experiment.

In the fertility toxicity study, the weight gain of F0 females in the mid- and high-dose groups was significantly lower than control. However, when we measured body weight without the uterus, we found no significant differences. This suggests the differences in body weight gain did not result from maternal toxicity. Instead, the reduced weight gain likely occurred because of the low pregnancy rate, evidenced by the lower fertility index, fewer implantations, and higher pre-implantation losses compared to control. We found no post-implantation losses, dead fetuses, or absorbed fetuses on G18, indicating that mid- and high-dose CFA mainly affected embryo implantation.

In the prenatal development toxicity study, fetal body weight was evidently reduced in the high-dose group, suggesting that high-dose CFA affected fetal development. The incidences of malformations or variations in external, visceral and skeletal examinations were within historical control limits and were not significantly different between CFA-treated groups and control. Thus, CFA did not display notable teratogenic effect on fetal mice.

As for the reproductive toxicity study, the body weight of CFA-treated dams in the high-dose group was not significantly different before CFA treatment and after delivery. However, a statistical decrease was noted during gestation (G6-G15, G15-G20). Given the low gestation index, the decrease of body weight in the high-dose groups during gestation was maybe attributed to the low pregnancy rate of F0 females. The body weight of pups was reduced in the high-dose group at birth, then increased rapidly over time and no significant difference was observed in the late development of pups. We infer that high-dose CFA

only affected prenatal development of fetuses. There were no apparent CFA treatment related changes in the time of eye opening or tooth eruption, or the development of nerve function. Thus, CFA administration to dams had little effect on pup development during the post-natal period.

Researchers have investigated the effects of CFA on early pregnancy since the 1980s. After CFA administration, luteolysis was induced with a declined plasma level of progesterone, which resulted in the anti-implantation activity (Zheng and Xu, 1986). However, studies showed that CFA had no significant effect on progesterone synthesis (Zheng and Xu, 1986). Besides, CFA had very low affinity with estrogen and progesterone receptors in mice and did not affect the uptake of estrogen, progesterone or other steroid hormones in the uterus (Zheng and Xu, 1986; Zheng et al., 1987, 1988). The specific mechanisms of anti-implantation effect of CFA still need to be explored.

In our study, CFA exposure to female mice had no significant effect on maternal toxicity, fetal teratogenesis or pup development. Only implantation and fetal body weight were affected, and the latter became comparable to control over time. Therefore, we speculate that CFA may be safely used in pregnant women who have presented successful implantation. Since human chorionic gonadotropin (hCG) is the first hormonal message from the placenta to the mother and it is detectable in maternal blood two days after implantation (Fournier, 2016), hCG positive can be used as an evidence for successful implantation. However, further pre-clinical reproductive toxicity and genotoxicity tests need to be conducted before the clinical trials of CFA on pregnant women.

5. Conclusions

In conclusion, CFA demonstrated anti-implantation activity during early pregnancy in mice at 5 mg/kg/day and 150 mg/kg/day. In addition, 150 mg/kg/day of CFA affected fetal weight gain. CFA administration at 0.15 mg/kg/day, 5 mg/kg/day and 150 mg/kg/day had no maternal toxicity, fetal teratogenesis or post-natal effects on pup development. The no-observed-adverse-effect level (NOAEL) of CFA for pregnant female mice under the conditions of this study was 0.15 mg/kg/day.

Conflicts of interest

Nothing to declare.

Acknowledgements

The authors wish to acknowledge the assistance of Shuang Wang for editing the manuscript; Yanling Mu, Tao Sun and Yanfeng Liu for providing professional guidance in performing animal experiments. This work was supported by grants from National Natural Science Foundation of China (No. 81770114, No. 81600091, No. 81570103, No.81500094, No. 81470284, No. 81470285), Major Research Plan of Natural Science Foundation of Shandong Province (ZR2016QZ008), State Key Clinical Specialty of China for Hematological Diseases and Taishan Scholar of Shandong Province.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.10.040.

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