Toxicol. Res.
Vol. 29, No. 1, pp. 27-34 (2013)
Open Access http://dx.doi.org/10.5487/TR.2013.29.1.027
pISSN: 1976-8257 eISSN: 2234-2753

Toxicological Research
Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Embryotoxicity and Toxicokinetics of the Antimalarial Artesunate in Rats

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(Received October 31, 2012; Revised December 10, 2012; Accepted January 24, 2013)

This study was conducted to investigate the potential embryo-fetal toxicity and toxicokinetics of the antimalarial agent artesunate (ARTS) in Sprague-Dawley rats. Pregnant rats were administered ARTS daily from gestational day 6~15 via oral gavage, at test doses of 0, 2, 4, or 8 mg/kg (22 females per group). The fetuses were examined for external, visceral, and skeletal abnormalities on gestational day 20. With regard to the dams, there were no deaths, treatment-related clinical signs, changes in body weight, or food intake in any of the treatment groups. There were no treatment-related gross findings at necropsy in any treatment group. In the 8 mg/kg group, there was a decrease in gravid uterine weight and in the weight of female fetuses. There was also an increase in fetal deaths (primarily late resorptions) and an increase in post-implantation losses (37%) at 8 mg/kg. An increase in the incidence of visceral and skeletal variations at 4 and 8 mg/kg was observed. These defects included minor changes in the appearance of the kidney and thymus, as well as absent ribs or thoracic vertebrae. Toxicokinetics were assessed in a parallel study, using 4 mated females per group. Using liquid chromatography-mass spectrometry (LC-MS) analysis, the concentration of ARTS and its metabolite dihydroartemisinin (DHA) were quantified in plasma from rats on gestational days 5, 6, 10, and 15. Amniotic fluid was assayed for ARTS and DHA on gestational day 15. There was evidence of rapid conversion of ARTS to the metabolite DHA in maternal plasma, since ARTS could not be consistently detected in plasma at the three doses tested. ARTS and DHA were not detected in amniotic fluid at gestational day 15, indicating limited placental transfer of the two agents. The embryo-fetal no-observable-adverse-effect level (NOAEL) of the test item was considered to be 8 mg/kg/day for dams, and 2 mg/kg/day for embryo-fetal development.

***Key words*:** Artesunate (ARTS), Antimalarial agent, Embryotoxicity, Toxicokinetics, Rats

INTRODUCTION

Human malaria is caused by four species of obligate intracellular protozoans of the genus *Plasmodium* including *P. falciparum*. Infections of pregnant women can be lethal, making immediate drug theraphy mandatory even when the potential for teratogenicity is known (1). Malaria during pregnancy is also associated with adverse consequences for the conceptus including intrauterine death, stillbirth, preterm labor, and low birth weight (2). The mortality and morbidity associated with malaria is on the rise worldwide due to the development of resistance to existing therapies (3).

Artemisinin and its derivatives such as artesunate (ARTS) and artemether have become an integral component of malaria treatment protocols in Asian and African countries for over 15 years especially against chloroquine resistant *falciparum* malaria (4). Artemisinin is a naturally occurring sesquiterpene lactone containing an endoperoxide group. This drug was first extracted from a Chinese herb *Artemisia annua* (QINGHAO). The free acid of ARTS is a lipophilic substance, very slightly soluble in water (5).

ARTS is a hemisuccinate ester of DHA and is highly effective in the treatment of malaria. It is clinically useful in the treatment of acute malaria and is particularly effective against chloroquine resistant strains of *falciparum* malaria. Similar to other derivatives of artemisinin, ARTS is rapidly converted to its active metabolite DHA *in vivo* which is responsible for the antimalarial action.

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List of abbreviation: ARTS: artesunate, DHA: dihydroartemisinin, d: day, GD: gestational day

There are two possible mechanisms through which artesunate may exert its antimalarial effect (6): (1) suppression of the production or activity of antioxidant enzymes in the erythrocyte; or (2) enhanced production of activated oxygen species in erythrocytes, leading to increased levels of oxygen radicals. Although several studies have been examined to investigate the adverse effects of antimalarial agents (7,8), limited data have been published on the animal or human embryo-fetal effects of Artesunate. Recently, the teratogenic effect of Artesunate has been published by Clark and colleagues (9).

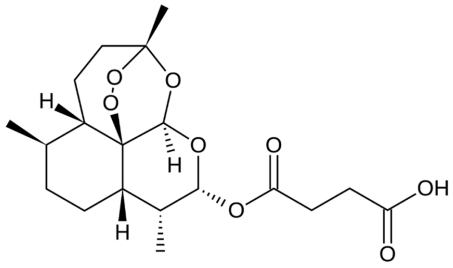
The present study was conducted in Sprague-Dawley rats to investigate the adverse effects of ARTS on pregnant dams and embryo-fetal development when administered during the organogenesis period. In addition, toxicokinetics were followed in a parallel study since it is important to assess the ability of the compound to penetrate the placenta in order to assess the exposure of the drug to the developing fetus.

MATERIALS AND METHODS

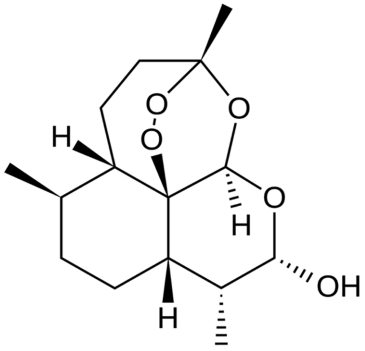
Animal maintenance and mating procedure. Sprague-Dawley rats (Orient Bio Inc, 143-1, Sangdaewon-Dong, Jungwon-Gu, Sungnam-si, Gyeonggi-Do, Korea) were kept under SPF (specific pathogen free) conditions at a constant day/night cycle as 08:00 h~20:00 h light. Standard laboratory rodent diet (PMI Nutritional International, Inc., 505 North 4th Street Richmond, IN 47374, USA) and sterilized water were available ad libitum. For mating, two females were placed into the cage of one male overnight and the first 24 h period following the mating procedure was designated as day 0 of pregnancy if vaginal sperms were detected. This experiment was conducted in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All procedures were approved by our Institutional Animal Care and Use Committee (IACUC).

Test substance. Artesunate, a white or light yellowish crystal powder, was provided by Shin Poong Pharm. Co., Ltd. (Seoul, Korea). The chemical structure of artesunate is depicted in Fig. 1. High-dose solution was prepared before treatment once every 6 days because the stability of prepared test article for 6 days was confirmed. Artesunate was dissolved in 0.5% methylcellulose (MC) solution. Dosing solutions for the lower dose groups were prepared by stepwise dilution of the high-dose solution.

Drug treatment. The application volume was 10 ml/kg. The daily application volume was calculated according to the body weight on days 6, 9 and 12 of gestation. Artesunate was administered by gavage to rats from days 6 to 15 of gestation.



Artesunate (AS)



Dihydroartemisinin (DHA)

Fig. 1. Chemical structure of artesunate (ARTS) and its active and major metabolite, dihydroartemisinin (DHA).

Experimental groups. Four groups were constructed: artesunate 2, 4, 8 mg/kg/d, and a vehicle control. Twenty-two mated females were used in each group.

Dose range finding study. Dosages of 3, 9, 27, and 54 mg/kg/d were given in a oral pilot study to 6 pregnant dams per group. The doses of over 27 mg/kg showed complete resorption of implanted embryos. At 9 mg/kg, about 50% of embryos were resorbed. Based on these results, 8 mg/kg/d was selected for the highest dose in the definitive study. Doses of 4 and 2 mg/kg were selected as middle and low doses, respectively, using a common ratio of 2. A vehicle control group was also added.

Observation of dams. Clinical signs of toxicity, body weight change, and food consumption were examined in the pregnant females. Dams were weighed on day 0, 6, 9, 12, 15 and 20 of gestation and individual food consumption was measured on day 1, 7, 10, 13, 16 and 20 of gestation. They were subjected to autopsy on day 20 of gestation. The following organs were weighed at necropsy: brain, adrenal gland, liver, spleen, kidney, heart, and ovary.

Caesarean section. On day 20 of gestation, all pregnant females were euthanized by carbon dioxide. The numbers of implantation sites, corpora lutea, live fetuses, dead fetuses and resorptions were recorded. Placenta were

weighed. All live fetuses were weighed, sexed and evaluated for externally visible abnormalities. Alternate fetuses were selected for either skeletal or visceral examination and fixed in 5% formalin or Bouin's fluid, respectively. The evaluation of skeletal abnormalities was performed after clearing the fixed fetuses with potassium hydroxide solution, and staining the skeleton with Alizarin Red S (10). For visceral examinations, we adapted Wilson's technique (11) for the head and abdomen, and Nishimura's method (12) for the thorax. External, visceral, and skeletal findings were classified as malformations and variations. We have used the terminology suggested in an internationally developed glossary of terms for structural developmental abnormalities in common laboratory mammals (13).

Design of toxicokinetics and sampling. Plasma and amniotic fluid ARTS and DHA concentrations were determined to be used as a measure of drug exposure.

Embryo-fetal development toxicokinetic study of ARTS in rats. Four groups were constructed: artesunate 2, 4, 8 mg/kg/d, and a vehicle control. Four mated females were used in each group. All pregnant animals received single daily oral administrations of test article formulations at the dose levels of 0, 2, 4 and 8 mg/kg from GD 6 through GD 15. Blood samples (0.8 ml) were collected from *venae caudales* using anticoagulant (1.0 mg sodium fluoride and 3.0 mg potassium oxalate per ml blood) on GD 5 and at 1 hour post-dose on GD 6, 10, and 15. The amniotic fluid was taken by Caesarean section on GD 15.

LC-MS analysis of ARTS and DHA. The ARTS and its primary metabolite, DHA concentrations in maternal plasma and amniotic fluid were quantified based on the sensitive and specific LC-MS method with 1 ng/ml of quantification (14). ARTs and DHA were separated using an isocratic elution on Synergi Max-RP HPLC column (75 mm × 4.6 mm, 4 μm, Phenomenex, USA) using an acetic acid (0.1%)-methanol-acetonitrile (38 : 15.5 : 46.5, v/v) as a mobile phase at a flow rate of 1 ml/min.

Statistical analysis of data. The unit of comparison was the litters. Continuous data variables such as body weight of dams, food consumption, litter size, and number of live fetuses were evaluated by one-way analysis of variance (ANOVA), and Dunnett's multiple comparison test was conducted when analytic results were significant. Nonparametric data such as fetal deaths were analyzed by Kruskal-Wallis test. The sex ratio was analyzed using a chi-square test. In addition, the incidence of external, visceral and skeletal malformations in fetuses was recorded as percents. It was compared using the Fisher's exact probability test. A difference was considered statistically significant at $p < 0.05$.

RESULTS

Effects on dams. There were no deaths in any treatment group. Loss of fur was observed in one female of the 2 mg/kg group on Days 5 to 13 of gestation and in one female of the 8 mg/kg group on Days 5 to 20 of gestation.

Table 1. Changes in body weights of dams treated with artesunate (ARTS) from gestational day (GD) 6 to day 15[a]

DOSE: (mg/kg)	0	2	4	8
No. of dams	19	21	22	22
Gestational day 0	267.1 ± 21.3	267.4 ± 22.8	266.5 ± 22.6	267.2 ± 25.6
Gestational day 6	299.7 ± 24.4	301.0 ± 25.5	300.8 ± 27.7	300.6 ± 24.9
Gestational day 9	311.3 ± 23.8	315.1 ± 26.1	313.5 ± 24.4	313.4 ± 24.2
Gestational day 12	328.0 ± 26.0	330.3 ± 27.6	328.1 ± 26.4	331.4 ± 26.1
Gestational day 15	347.3 ± 27.1	346.7 ± 28.6	344.6 ± 29.5	345.7 ± 28.4
Gestational day 20	425.0 ± 34.2	424.8 ± 37.9	407.6 ± 45.7	396.0 ± 39.6

[a]Mean ± SD.

Table 2. Changes in food consumption of dams treated with ARTS from GD 6 to day 15[a]

DOSE: (mg/kg)	0	2	4	8
No. of dams	19	21	22	22
Gestational day 1	19.5 ± 2.8	22.0 ± 11.2	19.5 ± 3.8	18.1 ± 5.5
Gestational day 7	25.9 ± 8.4	25.8 ± 5.5	24.3 ± 2.8	25.1 ± 3.7
Gestational day 10	28.0 ± 11.3	24.5 ± 4.3	25.8 ± 3.0	26.1 ± 4.1
Gestational day 13	29.5 ± 7.1	29.5 ± 5.2	27.9 ± 3.9	27.8 ± 3.3
Gestational day 16	30.6 ± 13.9	28.6 ± 2.9	27.0 ± 4.1	26.9 ± 3.3
Gestational day 20	28.5 ± 4.5	29.1 ± 3.3	27.3 ± 3.7	27.2 ± 4.1

[a]Mean ± SD.

There were no statistically significant differences between the vehicle control and treatment groups in the maternal body weight and corrected maternal body weight (Table 1). There was no statistically significant difference in the food consumption of dams during the gestation period (Table 2). At necropsy of the dams on Day 20 of gestation, yellow-white capsulation of spleen and adhesion of left kidney to spleen were observed in one animal each of the 2 mg/kg group. Atrophy of spleen and congestion of the thymus were observed in one animal each of the 8 mg/kg group. However, no treatment-related gross findings were observed in the 4 mg/kg group. At necropsy of the dams on Day 20 of gestation, although there were no changes in body weights or absolute organ weights, minor shifts in opposing directions resulted in increased relative kidney weights at 4 mg/kg and in liver, spleen, heart and left and right kidney weights at 8 mg/kg compared with controls. However, the magnitude of change is small, of the order of 3% for the relative kidney weight differences.

Effects on embryo-fetal development. No statistically significant differences were observed in the number of corpora lutea, implantations, placental weights and sex ratio in any treatment group, compared with those of the vehicle control group. However, a statistically significant decrease in the gravid uterine weight and a statistically significant increase in the number of fetal deaths (mostly late resorptions) and the rate of post implantation losses were observed in the 8 mg/kg group. Two females of the 4 mg/kg group and 5 females of the 8 mg/kg group exhibited complete resorption of implanted embryos at scheduled Caesarean section. However, there were no statistically significant differences, when compared with those of the vehicle control group. The litter size of the treatment groups did not differ significantly from the vehicle control group. A significant decrease in the weight of female fetuses was observed in the 8 mg/kg group, compared with those of the control group. There was one external malformed fetus showing anasarca, short snout, club food, domed head and absent eye bulge in the 4 mg/kg group (Table 3). One fetus (0.8%) of the 4 mg/kg group showed visceral malformation, namely a ventricular septum defect. The occurrences of visceral malformations as the number of fetuses and litters compared well between the groups. The number of fetuses with visceral variations were 11 (8.3%), 23 (15.6%), 29 (24.4%) and 24 (25.3%) in the vehicle control, 2, 4 and 8 mg/kg groups, respectively. The occurrences of visceral variations in the 4 and 8 mg/kg groups were statistically significantly higher than those of the vehicle control group. However, the number of litters with variations compared well between the groups. Dilated renal pelvis was observed in 3, 3, 2 and 1 fetuses in the vehicle control, 2, 4 and 8 mg/kg groups, respectively. Dilated ureter in 6, 8, 16 and 6 fetuses, and misshapen thymus in 4, 16, 14 and 17 fetuses were observed. Enlarged ventricular chamber, enlarged atrial chamber and small kidney were observed in 1 fetus of the 4 mg/kg group and misshapen heart was seen in 2 fetuses of the 4 mg/kg group (Table 4). The number of fetuses with skeletal malformations were 1, 3 and 1 fetuses in the vehicle control, 4 and 8 mg/kg groups. There were no significant differences in the number of fetuses and litter with malformations between the groups. Absent rib and absent thoracic vertebra

Table 3. Caesarean section data of dams treated with ARTS from GD 6 to 15

DOSE: (mg/kg)	0	2	4	8
No. of dams	19	21	22	22
Gravid uterine weight (g)	82.8 ± 12.3	81.2 ± 12.3	64.1 ± 26.6	52.2 ± 31.7**
Corpora lutea	15.9 ± 1.73	15.8 ± 1.78	16.1 ± 1.95	15.9 ± 2.20
Implantations	15.1 ± 2.15	15.2 ± 1.73	14.5 ± 3.07	14.5 ± 2.87
Fetal deaths	0.5 ± 0.84	0.7 ± 0.80	3.2 ± 4.53	5.6 ± 6.39**
Early resorption	0.5 ± 0.77	0.6 ± 0.74	1.3 ± 3.14	1.0 ± 1.20
Late resorption	0.1 ± 0.23	0.0 ± 0.22	1.9 ± 3.78	4.6 ± 6.83
% post-implantation loss[a]	3.4 ± 5.18	4.7 ± 5.61	19.9 ± 31.13	37.3 ± 40.45**
Litters totally resorbed	0	0	2	5
Live fetuses (M/F)	135/140	143/163	121/129	100/98
Litter size	14.5 ± 2.12	14.6 ± 2.20	11.4 ± 4.91	9.0 ± 6.16
Sex ratio (Male/Female)	0.96	0.88	0.93	1.02
Fetal weight (g): Male	3.9 ± 0.59	3.7 ± 0.21	3.6 ± 0.38	3.6 ± 0.34
Female	3.7 ± 0.52	3.5 ± 0.20	3.5 ± 0.35	3.3 ± 0.41*
Placental weight (g)	0.48 ± 0.05	0.47 ± 0.05	0.47 ± 0.04	0.52 ± 0.07
Fetuses with external anomaly (%)	0	0	1(0.4)[b]	0

[a]Post-implantation loss = {(implantations-live fetuses)/implantations} × 100.
[b]Anasarca, short snout, club foot, domed head, and absent eye bulge.
*: Significant difference from the control group ($p < 0.05$).
**: Significant difference from the control group ($p < 0.01$).

Table 4. Visceral findings in fetuses from dams treated with ARTS from GD 6 to 15

DOSE: (mg/kg)	0	2	4	8
Litters examined	19	21	20	16
Fetuses examined	133	147	119	95
Fetuses with malformations (%)	0	0	1(0.8)	0
Litters affected	0	0	1(5.0)	0
Ventricular septum defect	0	0	1	0
Fetuses with variations (%)	11(8.3)	23(15.6)	29(24.4)**	24(25.3)**
Litters affected	9(47.4)	12(57.1)	17(85.0)	14(87.5)
Dilated renal pelvis	3	3	2	1
Dilated ureter	6	8	16	6
Misshapen thymus	4	16	14	17
Enlarged ventricular chamber	0	0	1	0
Enlarged atrial chamber	0	0	1	0
Misshapen heart	0	0	2	0
Small kidney	0	0	1	0

**: Significant difference from the control group ($p < 0.01$).

were observed in 3 fetuses of the 4 mg/kg group, respectively. Supernumerary phalanx, short 12th rib and short 13th rib were observed in 1 fetus of the vehicle control, 4 and 8 mg/kg groups, respectively. The number of fetuses with skeletal variations were 3 (2.1%), 31 (19.5%), 27 (20.6%) and 23 (22.3%) in the vehicle control, 2, 4 and 8 mg/kg groups, respectively. The number of fetuses with variations in the treatment groups were statistically significantly increased, when compared with those of the vehicle control group. However, the number of litters with variations compared well between the groups. Bipartite ossification of sternebrae was observed in 2 fetuses of the 8 mg/kg group. Bipartite ossification of thoracic centrum was seen in 1, 3 and 2 fetuses in the vehicle control, 4 and 8 mg/kg groups, respectively. Full supernumerary rib, misshapen thoracic centrum and wavy rib were observed in 1 fetus each of the 8 mg/kg group. Misshapen lumbar arch was seen in 3 fetuses of the 4 mg/kg group and misshapen sacral arch in 4 fetuses of the 2 mg/kg group. Misshapen sternebrae were observed in 1, 5, 7 and 6 fetuses in the vehicle control, 2, 4 and 8 mg/kg groups, respectively. Short supernumerary ribs in 1, 20, 8 and 5 fetuses in the vehicle control, 2, 4 and 8 mg/kg groups, respectively. Unossified thoracic centrum was seen in 3, 4 and 9 fetuses in the 2, 4 and 8 mg/kg groups. The incidences of skeletal retardation were 22 (15.5%), 35 (22.0%), 13 (9.9%) and 25 (24.3%) in the vehicle control, 2, 4 and 8 mg/kg groups, respectively. There were no statistically significant differences in the number of fetuses and litters with retardation between the groups. Dumbbell ossification of the thoracic centrum was seen in 21, 32, 9 and 18 fetuses in the vehicle control, 2, 4 and 8 mg/kg groups, respectively. Dumbbell ossification of the lumbar centrum was seen in 2 fetuses of the 2 mg/kg group. Incomplete ossification of the pubis was observed in 1, 2 and 8 fetuses in the vehicle control, 4 and 8 mg/kg groups, respectively. Incomplete ossification of interparietal bone was observed in 1, 1 and 3 fetuses in the 2, 4 and 8 mg/kg groups, respectively. Incomplete ossification of parietal was observed in 2 fetuses of the 8 mg/kg group. Incomplete ossification of supraoccipital bone was observed in 1 fetus of the 4 mg/kg group. Incomplete ossification of the thoracic arch was seen in 1 fetus of the 8 mg/kg group and incomplete ossification of the ischium was seen in 1 fetus each of the 4 and 8 mg/kg groups. There were no statistically significant differences in the number of ossification centers of sternebrae, metacarpals, metatarsals, phalanges, cervical vertebrae and sacro-caudal vertebrae between the groups (Table 5).

Toxicokinetic data. ARTS and DHA were not detected in the plasma taken from animals in the vehicle control group. A plot of the mean maternal DHA plasma concentrations (1 hour after dosing) for gestation Days 5 through 15 was shown in Fig. 2. Exposure to ARTS, as determined by the 1 hour post-dosing DHA plasma concentration, remained relatively constant from gestation Day 6 to 15. Mean maternal plasma DHA concentrations on gestation Day 15 increases with doses up to 4 mg/kg, then reaches an apparent plateau, but this pattern could also be consistent with an approximate linear relationship over the concentration range studied because of variability in the plasma concentrations (Fig. 3). Neither artesunate nor DHA was detected in any of the vehicle control specimens. In addition, artesunate and DHA were not detected in amniotic fluid samples in the three treatment groups. The limit of detection of the LC-MS assay is 1 ng/ml.

DISCUSSION

The present study was conducted to investigate the potential embryo-fetal toxicity of ARTS in Sprague-Dawley rats. No treatment-related changes in clinical signs, body weights,

Table 5. Skeletal findings in fetuses from dams treated with with ARTS from GD 6 to 15

DOSE: (mg/kg)	0	2	4	8
Litters examined	19	21	20	17
Fetuses examined	142	159	131	103
Fetuses with malformations (%)	1(0.7)	0	3(2.3)	1(1.0)
Litters affected	1(5.3)	0	1(5.0)	1(5.9)
Absent rib	0	0	3	0
Absent thoracic vertebra	0	0	3	0
Supernumerary phalanx	1	0	0	0
Short 12th rib	0	0	1	0
Short 13th rib	0	0	0	1
Fetuses with variations (%)	3(2.1)	31(19.5)**	27(20.6)**	23(22.3)**
Litters affected	3(15.8)	12(57.1)	15(75.0)	8(47.1)
Bipartite ossification of sternebra	0	0	0	2
Bipartite ossification of thoracic centrum	1	0	3	2
Misshapen lumbar arch	0	0	3	0
Misshapen sacral arch	0	4	0	0
Misshapen sternebra	1	5	7	6
Misshapen thoracic centrum	0	0	0	1
Full supernumerary rib	0	0	0	1
Short supernumerary rib	1	20	8	5
Unossified thoracic centrum	0	3	4	9
Wavy rib	0	0	0	1
Fetuses with retardations (%)	22(15.5)	35(22.0)	13(9.9)	25(24.3)
Litters affected	9(47.4)	15(71.4)	8(40.0)	12(70.6)
Dumbbell ossification of lumbar centrum	0	2	0	0
Dumbbell ossification of thoracic centrum	21	32	9	18
Incomplete ossification of pubis	1	0	2	8
Incomplete ossification of interparietal	0	1	1	3
Incomplete ossification of parietal	0	0	0	2
Incomplete ossification of supraoccipital	0	0	1	0
Incomplete ossification of thoracic arch	0	0	0	1
Incomplete ossification of ischium	0	0	1	1
No. of ossification centres				
Sternebra	4.9 ± 0.66	5.0 ± 0.30	4.8 ± 0.80	4.6 ± 0.72
Metacarpals in both forelimbs	6.7 ± 0.67	6.8 ± 0.50	6.8 ± 0.74	6.7 ± 0.73
First phalanges in both forelimbs	2.0 ± 3.00	1.4 ± 2.04	1.4 ± 1.76	0.6 ± 1.16
Metatarsals in both hindlimbs	8.0 ± 0.08	8.0 ± 0.07	7.9 ± 0.33	7.8 ± 0.49
First phalanges in both hindlimbs	1.0 ± 2.45	0.3 ± 0.86	0.2 ± 0.66	0.3 ± 1.05
Cervical vertebra	0.1 ± 0.29	0.1 ± 0.21	0.1 ± 0.15	0.1 ± 0.16
Sacral and caudal vertebra	7.8 ± 0.53	7.9 ± 0.30	7.6 ± 0.50	7.2 ± 1.18

**: Significant difference from the control group ($p < 0.01$).

food consumption or organ weights of dams were observed at any of the doses tested. Loss of fur was observed in 1 animal each of the 2 and 8 mg/kg groups. The incidence was low. Therefore, it was considered to be an incidental finding.

At necropsy of the dams on Day 20, a yellow-white encapsulation of spleen, adhesion of left kidney to spleen, atrophy of spleen and congestion of the thymus were observed in one animal each of the 2 and 8 mg/kg groups, respectively. The incidence was low and not dose-related. Therefore, it was not considered to be treatment-related.

At Caesarean section on Day 20, gravid uterine weight was decreased in the 8 mg/kg group, which is associated with the increases in the incidences of the fetal deaths and the post-implantation losses (37%). Clark and colleagues, (9) have reported similar embryolethality and post implantation losses (34% at 10 mg/kg) but without further increase at higher dose levels (16.7 mg/kg, 31%). The decreases in the weight of female fetuses observed in the 8 mg/kg group may possibly be related to fetal toxicity induced by artesunate.

An external abnormality in one fetus (anasarca, short snout, club foot, domed head and absent eye bulge) observed in the 4 mg/kg group is classified as a major anomaly, but the incidence was very low and within the normal range (15). In addition, it was not dose-related and therefore, it was considered to be an incidental finding.

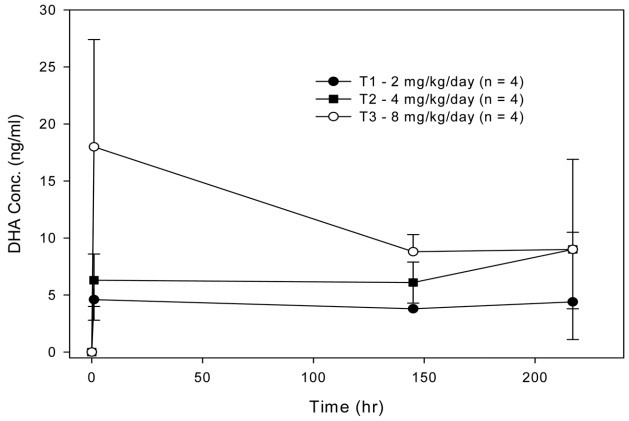


Fig. 2. Plot of mean maternal DHA plasma concentration vs. time for rat embryo-fetal toxicokinetic study.

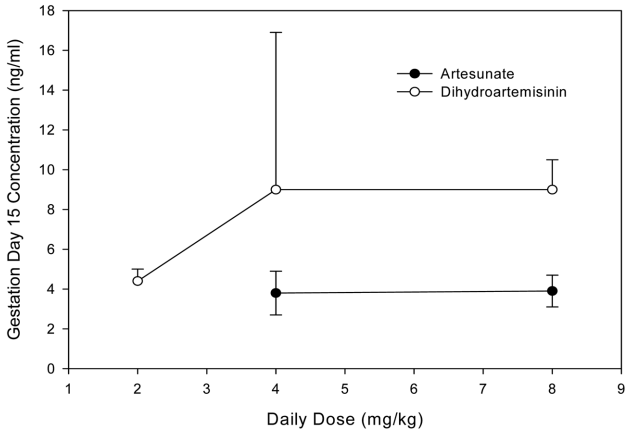


Fig. 3. Plot of mean (± SD) maternal ARTS and DHA plasma concentrations on gestation day 15 vs. ARTS dose in pregnant female rats.

An increase in the incidence of fetal visceral and skeletal variations observed in the 4 and 8 mg/kg groups occurred in a dose-dependent manner, which suggests that fetal development was adversely affected by artesunate. However, increased skeletal variation rate observed in the 2 mg/kg group is due to spontaneously occurred high incidence of short supernumerary ribs. Therefore it was not considered to be treatment-related. The occurrences of fetal visceral and skeletal abnormalities compared well between the groups. They were within the normal range and common for the SD rats (15) and they are therefore considered to be of a spontaneous nature. Clark and colleagues, (9) reported cardiovascular and skeletal defects, which were not confirmed in this study. Explanations of the discrepancy might include strain of rat, dosing form, dosing level (6, 10 and 16.7 mg/kg) or dosing period. Among these factors, the difference in the dosing level seems to be the most probable one. There was no maternal or developmental toxicity in the 2 mg/kg group.

In this study of daily oral administration of 2, 4 and 8 mg/kg of ARTS to pregnant rats from GD 6 through 15, relatively constant maternal drug exposure was produced as measured by plasma concentrations post-dosing. In addition, this study showed a linear increase in DHA with increasing dose up to 4 mg/kg of ARTS and a plateau in the exposure at the 4 and 8 mg/kg treatment groups. ARTS and DHA could not be detected in amniotic fluid at the three dose levels, indicating limited penetration of ARTS and DHA across the placenta of the pregnant rat.

It is uncertain whether development toxicity, accompanied by maternal toxicity, was directly or indirectly induced in developmental toxicity studies (16). In the present study, significant developmental toxicity by ARTS was observed in the 4 and 8 mg/kg groups. Therefore, ARTS might be a selective developmental toxicant and might act directly on the embryo, even though exposure levels to ARTS or DHA could not be detected. ARTS is much more effective as an embryolethal agent than as a teratogen.

Regarding the mechanism of fetal toxicity of ARTS, it was recently reported that embryonic erythroid cells are the primary target of ARTS fetal toxicity in the rat embryo, preceding lethality and changes to other target organs. Thus, anaemia/hypoxia may be causal in ARTS-induced embryotoxicity in rats (17). The developmental toxicity of ARTS might be mediated via production of free radicals within the developing embryo or associated structures with the consequent formation of reactive oxygen species and irreversible modification of vital cellular biomolecules (18).

Pregnant women in the first trimester exposure to artemisinin-based combination therapy from 1989 to 2009 did not show embryonic deaths, and this was considered to result from the difference in the period of early red blood cell formation in the rodent and human (19). In the pre-clinical study conducted with the rodent, animals are treated with ARTS for full organogenesis period, but pregnant women with malaria infection are treated for only two or three days, indicating that short term exposure of ARTS can not cause embryonic death if it does not cover a full period of sensitive red blood cell formation.

In conclusion, the administration of ARTS to pregnant rats resulted in an increased incidence of fetal morphological variations at a daily dose level of 4 mg/kg, decreased gravid uterine and fetal weights, and increased fetal deaths, post implantation losses and fetal morphological variations at a daily dose level of 8 mg/kg. Under the present experimental conditions, the no-observable-adverse-effect level (NOAEL) of the test item is considered to be 8 mg/kg per day for dams and 2 mg/kg per day for embryo-fetal development.

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