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Two generation reproduction and teratogenicity studies of feeding diaveridine in Wistar rats

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Abstract

Diaveridine (DVD), belonging to diaminopyrimidines, has been widely used as a bacteriostatic agent. To evaluate the reproductive toxicity and teratogenic potential of DVD, different concentrations of DVD were administered to Wistar rats by feeding diets containing 0, 23, 230, 1150 and 2000 mg/kg, respectively. Each group consisting of 18 males and 25 females (F_0) was treated with different concentrations of DVD through a 13-week period prior to mating and during mating, gestation, parturition and lactation. At weaning, 20 males and 25 females of F_1 generation weanlings per group, were selected randomly as parents for the F_2 generation. Selected F_1 weanlings were exposed to the same diet and treatment as their parents. In the F_0 and F_1 generation of 1150 and 2000 mg/kg diet groups, body weights, feed efficiency, weight gain of pregnant rats, the litter and the average number of live fetus and fetus body weight were significantly decreased. In the highest dose group, uterine wall contraction, uterine cavity narrow and uterine tumors were observed. Combined with the 2nd generation of reproduction test to investigate the teratogenic toxicity of DVD in Wistar rats, the pregnant rats were subjected to caesarean section on 20th gestational days for examination. At 1150 and 2000 mg/kg groups, litter weights, body weights, body length, tail length of fetus and number of viable fetuses were significantly decreased. There were no obvious external, skeletal and visceral effects in all groups. The no-observed-adverse-effect level for reproduction/development toxicity of DVD was 23 mg/kg diet (about 2.3~2.8 mg/kg b.w./day).

Keywords: Reproductive toxicity; Teratogenicity; Diaveridine; Diaminopyrimidines; Wistar rats

Abbreviations: ADP, aditoprim; ANOVA, analysis of variance; BQP, baquiloprim; b.w., body weight; DHFR, dihydrofolate reductase; DVD, diaveridine; GD, gestational day; H&E, hematoxylin and eosin; NOAEL, no-observed-adverse-effect level; OMP, ormetoprim; TMP, trimethoprim.

1. Introduction

As a class of synthetic bacteriostatic agents, diaminopyrimidines are also known as dihydrofolate reductase (DHFR) inhibitors and are usually in combination with sulfonamides to improve efficiency of bacterial inhibition or killing.[1-3] Baquiloprim (BQP), trimethoprim (TMP), ormetoprim (OMP) and diaveridine (DVD) have been used in human and veterinary medicine for treatment and prevention of disease for many years.[4] DVD, 5-(3,4-Dimethoxy-benzyl) -2,4- pyrimidinediamine (CAS No.5355-16-8, $C_{13}H_{l6}N_4O_2$), was widely used with sulfa or sulfa-methoxy-quinoline pyrimidine blend premix to improve the antibacterial effects of sulfonamides (Fig. 1).

<Insert Fig. 1 here>

Diaminopyrimidines were suspected to have potential reproductive and developmental toxicity. It was reported that the serious maternal toxicity was observed at 45 and 65 mg/kg b.w./day in the reproduction studies of BQP, respectively.[5] BQP was also found to result in cleft palate at 30 mg/kg b.w./day in the two-generation study for BQP.[5] TMP was reported to increase the risk of certain birth defects in infants.[6,7] Another study showed that TMP was also found to be responsible for fetal malformations in rats at 300 mg/kg b.w..[8,9] Though DVD was widely used as DHFR inhibitor to improve efficiency of bacterial inhibition, no risk assessment on its reproductive and developmental toxicity was reported.

Recent studies have found that DVD had genetoxicity.[10,11] In Ames test, DVD was mutagenic in strain TA100 after metabolic activation with hamster S_9 mix.[10] In cultured Chinese hamster CHL cells in the absence of a metabolic activation system, DVD induced structural chromosome aberrations. DNA damage in liver, kidney, lung, and spleen cells was also observed in a test of single oral administration of DVD by using the *in vivo* comet assay. Together with other genetoxic tests, DVD was concluded to be genotoxic to mammalian cells *in vitro* and *in vivo*.[11] However, despite many years of usage in food producing animals, the toxic characteristics of DVD, including its major toxic effects on the reproduction and development, still remain unknown. Because of no enough toxicology studies, such as reproductive and development studies according to the relative toxicology guidelines, it is impossible to evaluate the risk of DVD. For a long-term perspective, increased use of DVD without comprehensive safety evaluation is questionable, especially because of the concerns related to the potential impact on human health of the proposed use in food producing animals. Therefore, it is necessary to identify the adverse effects using control studies in laboratory animals, such as the reproductive and developmental toxicity studies of DVD.

The present study was, therefore, designed to evaluate the two generation reproduction and teratogenicity of DVD in Wistar rats with a wide range of doses. The present study could provide the effects of long-term treatment of drugs on animal

reproductive organ and fertility. The achievements would provide scientific information for further risk evaluation of DVD in food animals.

2. Materials and methods

2.1. Test substance

Diaveridine (DVD, purity 98%) was purchased from Wuhan Yuancheng Science and Technology Development Co. Ltd. (Wuhan, PR China). All other reagents were of analytical grade.

2.2. Animals and diet preparation

Male and female specific pathogen-free Wistar rats (*Rattus norvegicus*, 5-6-wk old) were procured from Center of Laboratory Animals of Hubei Province, Wuhan, P.R. China. The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Huazhong Agricultural University, Wuhan, PR China). Males and females were acclimated to the laboratory for 7 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. The animals, five per cage per sex, were kept in an environment maintained at 20~25 ℃, relative humidity of 40~70% and a 12-h light: 12-h dark cycle. Beddings (Hardwood shavings) were used after sterilization by autoclaving and were renewed every three days. Feed and tap water were provided *ad libitum*. After quarantine, rats were 6-7-wk old were assigned to different groups by means of randomization stratified by body weight (both sexes), such that the body weights by gender of all groups were homogeneous by statistical analysis at the beginning of the study. Mated females were housed individually in shoebox cages containing nesting material throughout gestation and

lactation. Use of animals in this study was in accordance with NIH Publication 85-23 "Guide for the Care and Use of Laboratory Animals".[12]

2.3. Two generation feeding reproduction study and teratogenic study

Rats were randomly assigned to five groups, and DVD was administered to rats by feeding in diet with the concentrations of 0, 23, 230, 1150 and 2000 mg/kg diet (Rat Maintenance Feed, Ground Fine from the Center of Laboratory Animals of Hubei Province, Wuhan, PR China) throughout the whole period (including exposure, mating, gestation, parturition and lactation periods).

The dosage of 23 mg/kg diet was selected as the minimal dose, according to the fact that it is the intended concentration in clinical application. 1150 and 2000 mg/kg dietary level DVD inhibited feed intake and body weight of the rats and was used as the high dose in the sub-chronic study. The medium dosage of 230 mg/kg is within 10 times of the minimal dose (23 mg/kg) according to the U. S. Food and Drug Administration Toxixological Principles for the Safety Assessment of Food Ingredients. IV C 9 a. and IV.C.9.b.[13,14] This study was conducted in compliance with FDA Good Laboratory Practice Regulations (Part 58 of 21 CFR).[15]

For mating, two females and one male were placed into one cage overnight. Successful mating was ascertained by the presence of sperm in the vaginal smear, and the following first 24 h was designated as day 0 of gestation. Mated females were housed singly in clear polycarbonate cages with stainless steel wire lids and were allowed tap water and feed *ad libitum*.

2.3.1 Study design of the two generation feeding reproduction study

The two generation feeding reproduction study was performed according to FDA Toxicological principles for the safety assessment of food ingredients. IV.C.9.b. Guidelines for Reproduction Toxicity Studies.[13] Total 215 rats were randomly divided into five groups (18 male rats and 25 female rats /group). Before mating, rats (F_0) were exposed to the different diets for 10 weeks. After that, one male and two females were placed into one cage for mating. After mating, F_0 male rats were euthanized. Then, the pregnant F_0 rats (24, 22, 23, 22 and 22 rats for control, 23, 230, 1150 and 2000 mg/kg DVD groups, respectively) produced pups (F_1). F_1 pups were randomly selected on day 4 after birth, 12 males and 24 females of F_1 pups per group were selected randomly as parents for the F_2 generation. Other unselected pups in F_1 litters were euthanized on day 21 after birth and then necropsied. After weaning of the F_1 pups, F_0 female rats were used to pathological examination. Selected F_1 weanlings were exposed to the same diet and treatment as their parents. After 10 weeks exposed to the different diets and mating, the pregnant F_1 rats (26, 25, 24, 23 and 21 rats for control, 23, 230, 1150 and 2000 mg/kg DVD groups, respectively) produced pups (F_2). F_2 pups were necropsied on day 21 after birth as well as their parents.

2.3.2 Study design of the teratogenic study

The teratogenic study was performed according to FDA Toxicological principles for the safety assessment of food ingredients. IV.C.9.b. Guidelines for Developmental Toxicity Studies.[14] Five groups (20 male rats and 30 female rats /group) were used in

the teratogenic study. Before mating, rats were exposed to the different diets for 12 weeks. Then, two females and one male were placed into one cage overnight for mating. Successful mating was ascertained by the presence of sperm in the vaginal smear, and the following first 24 h was designated as day 0 of gestation. Mated females were housed singly in clear polycarbonate cages with stainless steel wire lids and were allowed tap water and feed *ad libitum*. The pregnant rats (27, 26, 24, 22 and 20 rats for control, 23, 230, 1150 and 2000 mg/kg DVD groups, respectively) were subjected to cesarean section on gestational day (GD) 20 for teratogenic examination.

2.3.3 Parental data in the teratogenic study

Routine cage-side observations were performed on all animals twice a day throughout the study for general signs of toxicologic and pharmacologic effects, morbidity and mortality, general appearance, and behavior. An expanded set of clinical evaluations, performed outside of the cage, was performed on all animals twice before test material administration and weekly during the study period to detect neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. Feed intakes of rats were measured weekly. Maternal body weights were measured on GD 0, 7, 14, and 20. Maternal body weight gains were calculated. Throughout gestation, all pregnant rats were observed daily for mortality, morbidity, general appearance and behavior. Females were sacrificed by carbon dioxide asphyxiation and subjected to external and internal macroscopic examination at the scheduled termination (GD 20), and all males were euthanized and examined

similarly.

2.3.4 Cesarean Section of the teratogenic study

The dams were euthanized by carbon dioxide asphyxiation on gestation day 20. The ovaries and uterus of each female were removed and the status of all implantation sites, i.e., live and dead fetuses, early and late resorptions, and total implantations was examined. Live fetuses were weighed individually. All live fetuses were sexed, weighed, and inspected for external malformations and malformations including cleft palate. Approximately one-half of the live fetuses in each litter were randomly selected for either skeletal or visceral examination. The skeletal evaluation of 85% ethanol-fixed fetuses was performed after staining the skeleton with Alizarin Red S and clearing with the potassium hydroxide solution by the modified Dawson's (1926) method.[16] The remaining live fetuses in each litter were fixed in Bouin's solution prior to dissection. A freehand razor sectioning technique [17] was adapted to detect internal malformations of the head and abdomen, and Nishimura's method (1974) was used to detect malformations of the thorax.[18]

2.3.5 Parental data in the reproduction study

Throughout the study, each animal was observed at least twice daily. The first observation was a thorough clinical examination. The second involved observing the animals through the cages. Relevant behavioral changes and all signs of toxicity, morbidity, or mortality were recorded.

Estrous cycle length and normality were observed daily by vaginal smears for all F_0 and F_1 females during a minimum of three weeks before mating and during cohabitation. The duration of gestation was calculated from day zero of pregnancy. The conception rate was calculated, and the equation was (number of pregnancies/number of mating females) ×100. Feed intakes of rats were measured weekly. Throughout gestation, all pregnant rats were observed daily for mortality, morbidity, general appearance, and behavior. Females were sacrificed by carbon dioxide asphyxiation and subjected to external and internal macroscopic examination at the scheduled termination (GD 20 or pups weaned), and all males were euthanized and examined similarly.

From all F_0 and F_1 generation males selected for mating from the control and all dose levels, sperm samples obtained from the right cauda epididymis per testis were collected and then placed in Dulbecco's phosphate buffered saline (maintained at approximately 37 ℃) with 10 mg/ml bovine serum albumin. After10-min incubation, sperm motility was determined under light microscopy (Olympus BX 41, Japan). Sperm (minimum 200 per sample) samples from the cauda epididymis were examined as a fixed wet preparation and classified as either normal (both head and midpiece appear normal) or abnormal (*i.e.*, fusion, isolated heads, misshapen heads and/or tails).[19] The total number of sperm in the left cauda epididymis was also enumerated.

2.3.6 Growth of offspring in the reproduction study

In each litter, the number of pups, stillbirths, live births, and the presence of gross

anomalies were examined as soon as possible after delivery. Dead pups were necropsied and observed for possible gross defects and the cause of death. The neonates were carefully observed, and their sex and weight were noted on postnatal days zero (day of birth), four, seven, fourteen, and twenty one.

2.3.7 Pathology examinations in the teratogenic study and the reproduction study

Histopathological examination was performed on control and treatment groups. All the reproductive organs, including testis, epididymis, prostate, uterus and ovaries were examined macroscopically, and gross lesions were recorded when animals were anesthetized. The tissues from each animal, with the exception of testis, were preserved in 10% neutral-buffered formalin and slides prepared for histopathological examination. Routine paraffin embedding technique was used for histological examination of tissue sections. Sections of 5 μm thickness stained with hematoxylin and eosin (H&E) were examined under light microscopy (Olympus BX 41, Japan) for morphological changes. As a necessary step to determine a no-observed-adverse-effect-level (NOAEL) in target organs, other tissues such as liver and kidneys from treatment groups were also examined histologically.

2.3.8 Calculations of drug intake and feed efficiency

Dose levels (mg/kg b.w./day) were calculated by using the nominal concentration of drugs in the diet, the mean daily feed consumption, and the body weight for the week. The equation was (mean weekly feed consumption of each rat/7) / (the body

weight for the week) ×nominal concentration of drugs in the diet. The equation of feed efficiency was (mean body weight gain / feed consumption) ×100%.

2.4. Statistical analyses

For prebreed data, Levene's test was performed to determine whether the groups had equal variances. If the variances were homogeneous, a one-way analysis of variance (ANOVA) was carried out. If not, they were analyzed by the Kruskal-Wallis non-parametric ANOVA.[20] If either of the tests showed a significant difference among the groups, the data were analyzed by the multiple comparison procedure of the Dunnett's post hoc test or Mann-Whitney U test.

Continuous data such as maternal body weight, food consumption, fetal body weight, sperm numbers, body length and tail length were subjected to ANOVA, and Dunnett's multiple comparison tests were conducted when analytic results were significant. Incidence data such as the gender ratio, external, skeletal and visceral variations, the proportions of litters with malformations and developmental variations were compared using a chi-square test and Fisher's exact test. Sperm motility and sperm morphology were analyzed with Kruskal-Wallis test with Mann-Whitney U test.[21] The unit of comparison was the pregnant dam or the litter for data of the gestational period and thereafter. A difference was considered statistically significant at $p < 0.01$ or $p < 0.05$.

3. Results

3.1. Clinical observations in both teratogenic study and the reproduction study

No test-material-related changes were seen in mortality, clinical signs, and macroscopic examinations throughout the study, other than roughened hair and thin body at the 2000 mg/kg DVD group. Dams administered 23 mg/kg DVD at different days of gestation showed no obvious abnormal appearance and behavior.

3.2. Body weight, food consumption, food efficiency and DVD intakes in the reproduction and teratogenic studies

The body weights of F_0 and F_1 male and female rats before mating were presented in Fig. 2. Body weights of F_0 and F_1 male and female rats were significantly decreased from 1 to 13 weeks at 1150 and 2000 mg/kg DVD groups when compared with controls. Significant decreases of body weights from 2 to 13 weeks and 3 to 10 weeks were noted in F_0 female and male rats of 230 mg/kg DVD group, respectively. Body weights of F_1 male and female rats were significantly decreased from 3 to 13 weeks and 4 to 13 weeks at 230 mg/kg DVD group when compared with controls, respectively. There were no significant changes in body weights at 23 mg/kg DVD group when compared with controls in both F_0 and F_1 male and female rats.

Body weight gain of pregnant rats in the reproduction and teratogenicity during 0~20 days at pregnancy period in F_0, F_{1a} and F_{1b} at 1150 and 2000 mg/kg DVD diet groups was significantly lower than the concurrent control group (Table 2). There were no significant changes in body weight in other groups.

Food consumption of F_0 females decreased significantly during most of the

experimental period at 1150 and 2000 mg/kg DVD groups when compared with controls (Fig. 2). Significant decrease in food consumption of F_0 males in the first, second, third, 9th and 10th weeks at 1150 and 2000 mg/kg DVD groups was noted. There was a significant decrease in food consumption of F_0 males in the 4th, 5th and 6th weeks at 230 mg/kg DVD group. There was significant increase in food consumption of F_0 females in the 6th and 8th weeks at 23 and 230 mg/kg DVD groups and F_1 males in the 4th and 6th weeks at 23 mg/kg DVD group when compared with controls. Similarly, there were significant decrease in food consumption in F_1 females in the first, second, third, 4th, 6th, 7th, 8th, 9th and 10th weeks at 2000 mg/kg DVD group and in the first, second, third, 4th and 13th weeks in F_1 males at 1150 mg/kg DVD group. Significant decrease in food consumption in F_1 males in the first, third, 4th, 6th, 7th, 8th, 9th and 13th weeks at 1150 and 2000 mg/kg DVD groups.

There was a significant decrease in food efficiency in F_0 females in the first, second, 4th and 7th weeks at 2000 mg/kg DVD group and in the second, third, 4th and 7th weeks at 1150 mg/kg DVD group and in the second, 4th and 7th weeks at 230 mg/kg DVD group. Significant decrease in food efficiency in F_0 males in the first, third, 5th, 8th and 12th weeks at 1150 and 2000 mg/kg DVD groups and the first and 12th weeks at 230 mg/kg DVD group was found. There was a significant decrease in food efficiency in F_1 females in the first, second, 8th and 10th weeks at 2000 mg/kg DVD group and the first and 9th weeks at 1150 mg/kg DVD group. Significant decrease in food efficiency in F_1 males in the second, third, 6th and 11th weeks at 2000 mg/kg

DVD group and in the 8th and 10th weeks at 1150 mg/kg DVD group was noted. While there was a significant increase in food efficiency in F_1 males in the third and 10th weeks at 230 mg/kg DVD group.

DVD intakes per kg bodyweight per day achieved in the study were calculated based on the food intakes and bodyweights. They were shown in Table 1.

<Insert Fig. 2 here>

<Insert Table. 1 here>

<Insert Table. 2 here>

3.3. End points of female and male reproductive toxicity

Compared with the concurrent control group, no statistically significant differences were noted in treated parental animals of both generations for conception rate, survival rate of birth, survival rate of lactation and sex ratio (Table 3).

There were no significant differences in the number of spermatids per testis, the sperm motility, sperm morphology and sperm numbers in treated males of both F_0 and F_1 generations when compared with controls.

3.4. Examination of the pup in the reproduction test

In the reproduction test, body weights of F_1 and F_2 pups on days 0, 4, 7, 14 and 21 after birth and average number of live fetus/litter of F_1 and F_2 were significantly

decreased at 1150 and 2000 mg/kg DVD groups when compared with control group (Table 3).

3.5 Examination of the fetus in the teratogenicity test

No obvious appearances, visceral and skeletal malformations in fetuses were noted in any groups in both two generation teratogenic tests. At 1150 and 2000 mg/kg DVD groups, compared with controls, fetus weight, fetal body lengths and tail lengths decreased significantly in the teratogenicity test. Significant decrease in number of live fetus and number of nidation was noted at 2000 mg/kg DVD group. While a significant increase in tail length at 23 and 230 mg/kg DVD groups was also found (Table 4).

3.6 Pathological findings (F_0 and F_1)

Histopathology was conducted on control and treatment groups (Fig. 3). Reproductive organs of F_0 parents, including uterus, ovaries, vagina, testis, epididymis, prostate, and spermatophore were all checked carefully and examined under light microscopy for morphological alterations, and no significant changes were found. In each dose group, no significant changes were found in male genital pathology tests. The uterine wall contraction, narrow uterine cavity and uterine tumors were observed in some females at the highest dose group.

<Insert Fig. 3 here>

3.7 Organ index (F_0)

At sacrifice, the body weights and organ weights of F_0 rats were carefully weighed. Relative body weights of liver, kidneys, adrenals and brain in females and males and testis in male rats at 1150 and 2000 mg/kg DVD groups and relative body weights of kidneys, adrenals and brain in females at 230 mg/kg DVD group were significantly increased when compared with controls. The absolute organ weights of liver, spleen, thymus, heart, lungs, kidneys and uterus in females, and the absolute organ weights of liver, spleen, thymus, heart, lungs and kidneys in males at 1150 and 2000 mg/kg DVD groups and the absolute organ weight of testis at 2000 mg/kg DVD group were significantly decreased (Table 5).

4. Discussion

In the present study, the two-generation reproductive toxicity study and teratogenic test were firstly performed to further evaluate the potential effects of DVD on reproduction and development of rats, which provided the information about adverse effects of DVD on parents and their developing fetuses.

In the two generation reproduction and teratogenicity tests, at the DVD 1150 and 2000 mg/kg diet groups, body weight and food intake of F_0 and F_1 male and female rats were significantly inhibited, indicating that the direct toxic effect of DVD or the reduced food consumption might result in the significant decrease of rat body weight.

In the present study, maternal toxicity was found at 1150 and 2000 mg/kg DVD diet groups, which resulted in a significant loss of body weight in female rats compared with control group. There were no statistically significant differences were

noted in treated parental animals of both generations for conception rate, survival rate of birth, survival rate of lactation and male ratio. While the body weights of F_1 pups on days 0, 7, 14 and 21 and F_2 pups on days 0, 7, 14 and 21 at 1150 and 2000 mg/kg DVD groups were significantly decreased when compared with control group. It indicated that high dose exposure of DVD could induce the developmental inhibition on pups in both generations. It was reported that a higher dose of cyadox and quinocetone administered to Wistar rats could result in the significant decrease of the body weights of pups in the two generation reproduction and teratogenicity.[22,23] In the present study, it was presumed that a constant exposure of DVD to F_0 and F_1 females induced a worse maternal toxicity in high dose groups, which resulted in worse developmental conditions in their pups.

Folic acid is important for normal development of the fetus and placenta.[24] Folic acid deficiency on pregnant rats might result in depressed feed consumption and produced smaller sized litters with lower birth weights and poor survival rate.[25] BQP, TMP, DVD and ADP work as DHFR inhibitors, suggesting that they might result in reproductive and developmental toxicity to females. The absent adverse effects in concurrent folic acid added group in the reproduction study of BQP made it more convincing.[5] However, there were no obvious external, skeletal or visceral malformations in fetuses in the teratogenic test of BQP. In the reproduction studies of BQP, serious maternal toxicity was observed at 45 and 65 mg/kg b.w./day, respectively.[5] TMP was reported to block the conversion of folate to its more active

metabolites,[26] and might increase the risk of certain birth defects on infants [7]. In the reproductive and teratogenic study for TMP, it was found that TMP did not show any substance-related effects on male or female fertility, mating activity and implantation rate at 420 mg/kg b.w.. However, TMP was observed to result in the significant decrease of pup weights and malformations (micrognathia, cleft palate and phocomelia) in teratogenic study at the similar dose.[6] TMP was also found to be responsible for fetal malformations in rats at 300 mg/kg b.w.,[8,9] which indicated that there might be a threshold teratogenic dose of TMP. Similarly, the incidence of cleft palate was observed at 30 mg/kg b.w./day in the two-generation study for BQP.[5] However, both with oral doses of 0, 3, 10 and 30 mg BQP/kg b.w./day, only foetotoxicity was found at an oral dose of 30 mg/kg b.w./day in both species in teratogenic studies of BQP in rats and rabbits.[5] There were no signs of teratogenicity or mutagenicity and the foetotoxicity induced by BQP was thought to be related to maternal toxicity.[5] The apparently conflicting reports of teratogenic effects of BQP on offspring were presented between the two-generation and teratogenicity studies at the dose of 30 mg/kg b.w./day, suggesting that administration of BQP for a long time in the two-generation study might result in the teratogenic effects. In the present study, the significant decrease in average number of live fetus per litter in both (F_1 and F_2) generations and teratogenicity study indicated reproductive difficulties existed in F_0 and F_1 adults at high dose group. Furthermore, the pathology findings, such as the uterine wall contraction, narrow uterine cavity and uterine tumors in some females at

2000 mg/kg DVD group, and the significant decrease of the absolute organ weights of uterus in females at 2000 mg/kg DVD group was noted, indicating that DVD might have reproductive toxicity to Wistar rats. In the present teratogenicity study, there were significant decreases in the number of nidation, the number of liver fetus, uterus and fetus weight, fetal body weight, placenta weight, fetal body length and fetal tail length at the high dose group indicating that high dose exposure of DVD could induce the certain embryo toxicity. The highest dose of DVD (213.5~262.9 mg/kg b.w./day) was about 7~8 times of 30 mg BQP/kg b.w./day and no signs of teratogenicity were noted, indicting that DVD might be more safe than that of BQP. However, TMP could result in malformations at about 420 mg/kg b.w., indicating that the dose threshold for the teratogenic toxicity of DVD might exist and the highest dose used in the present study or the administration time was not long enough to result in teratogenicity. Future study on a higher dose or longer administration time of DVD could be carried out to investigate whether the thresholds of teratogenic dose or time were existed.

Histological examination showed that no significant effects on the reproductive systems were noted in males at the highest dose DVD group. However, it was found that uterine tumors were observed in the females at the 2000 mg/kg DVD groups. This might be one impossible reason that little size of the litter was appeared in the high dose group when compared with controls. We presumed that DVD might change the endocrine and estrogen in the female reproductive system, and result in the combination of gametes or fertilized eggs can not be normal implantation. The 1150

and 2000 mg/kg diets of DVD in rats produced mild maternal toxicity, but the pups did not produce significant teratogenicity, indicating the DVD had slight reproductive and development toxicities. However, it still needs to pay more attentions to its reproductive and development toxicities because it was widely used in food producing animals.

In summary, the results of the teratogenicity and two generation feeding reproduction studies described here provide a more comprehensive toxicity profile of DVD. High dose level of 2000 mg/kg DVD (about 213.5~262.9 mg/kg b. w./day) depressed the development of the fetus and fertility of rats. The no observed adverse effect level (NOAEL) for reproduction/development toxicity of DVD was 23 mg/kg diet which is approximately equal to 2.3 ~ 2.4 mg/kg b. w./day in male and 2.4 ~ 2.8 mg/kg b. w./day in female rats.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Legend

Fig. 1 Chemical structure of diaveridine (DVD).

Fig. 2 Mean body weights (A), food efficiency (B), mean food intakes (C) of F_0 and F_1 rats following dietary exposure to diaveridine for the periods prior to mating in the reproduction and teratogenicity tests. (●) Control group; (■) Diaveridine group (23 mg/kg); (▲) Diaveridine group (230 mg/kg); (▼) Diaveridine group (1150 mg/kg); (♦) Diaveridine group (2000 mg/kg).

Fig. 3 Selected microphotographs of uterus at 10X and 40X. (A) Control uterus of F_0 and F_1 (10X)；(B) Uterus of F_1 females at the 1150 and 2000 mg/kg diaveridine groups (10X). The uterus gland atrophy and the uterine cavity to shrink were marked with arrows；(C) Control uterus of F_0 and F_1 females (40X)；(D) Uterus of F_1 females at the 1150 and 2000 mg/kg diaveridine groups (40X). The multinucleated cell morphology of different sizes of the uterus tumor cells was marked with arrows.

Table 1 Mean daily diaveridine intakes for the entire experimental period in two generation reproductive and teratogenic toxicity test (Mean±SD)

Drug dosage (mg/kg diet)	Rats' intake of DVD (mg/kg b.w. /day)			
	F_0 female	F_0 male	F_1 female	F_1 male
Control	0	0	0	0
DVD23	2.4±0.6	2.3±0.8	2.8±0.8	2.4±0.8
DVD230	24.9±5.7	23.2±7.5	28.5±7.1	23.5±7.9
DVD1150	124.2±22.8	118.8±36.0	155.0±34.7	122.3±40.9
DVD2000	220.2±33.9	213.5±50.6	262.9±88.1	222.2±70.5

Note: DVD, Diaveridine; DVD23, 23 mg/kg diet; DVD230, 230 mg/kg diet; DVD1150, 1150 mg/kg diet; DVD2000, 2000 mg/kg diet.

Table 2 Body weight gain of pregnant rats in the reproduction and teratogenicity during 0~20 days at pregnancy period (Mean±SD)

Dose (mg/kg diet)	Female (n)			Body weight gain (0~20d)		
	F_0	F_{1a}	F_{1b}	F_0	F_{1a}	F_{1b}
Control	24	26	27	134.2±19.4	132.0±17.9	124.9±20.1
DVD23	22	25	26	132.6±17.9	142.5±18.4	129.5±22.6
DVD230	23	24	24	127.2±19.5	133.3±17.7	120.2±23.5
DVD1150	22	23	22	80.0±18.4**	92.0±14.6**	88.0±18.4**
DVD2000	22	21	20	77.8±20.4**	80.3±13.6**	72.3±19.7**

Note: DVD, Diaveridine; DVD23, 23 mg/kg diet; DVD230, 230 mg/kg diet; DVD1150, 1150 mg/kg diet; DVD2000, 2000 mg/kg diet.

* Significantly different from control group at $p < 0.05$. **Significantly different from control group at $p < 0.01$.

Table 3 Reproduction toxicity following maternal exposure to diaveridine by dietary administration (Mean±SD)

Group	No. of female	No. of pregnancy	Conception rate (%)	Average No. of live fetus	Survival rate of birth (%)	Survival rate of lactation (%)	Day 0 fetus weigh (g)	Day 4 fetus weigh (g)	Day 7 fetus weigh (g)	Day 14 fetus weight (g)	Day 21 fetus weigh (g)	Male ratio (%)
							F_1					
Control	25	23	92.0	13.2±1.8	99.4±1.9	100	6.5±0.7	10.8±1.7	19.0±1.7	32.1±2.0	47.3±5.8	49.76±17.8
DVD23	25	22	88.0	12.9±2.1	99.1±2.3	100	6.7±0.6	12.4±1.2**	18.9±1.5	32.5±2.3	49.8±4.8	50.20±11.2
DVD230	25	23	92.0	13.0±3.4	99.4±1.8	100	6.3±0.7	11.7±1.5*	17.9±2.4	32.6±2.4	48.9±4.5	51.75±16.8
DVD1150	25	22	88.0	9.0±3.3**	98.6±3.6	99.4±2.7	5.7±0.8**	10.6±1.7	13.3±1.6**	23.4±2.2**	32.9±5.1**	46.88±21.99
DVD2000	25	22	88.0	8.4±3.3**	99.1±2.9	98.8±3.8	5.6±0.9**	10.5±1.4	11.1±2.5**	20.8±3.2**	27.4±5.6**	48.89±21.12
							F_2					
Control	30	26	86.7	13.6±2.2	99.3±2.1	100	6.2±0.7	12.0±0.8	19.1±1.9	38.9±2.4	60.4±3.4	47.37±12.33
DVD23	30	25	83.3	13.7±1.7	99.4±2.1	100	6.6±0.7	11.8±1.0	19.4±1.8	38.8±3.5	59.8±3.0	44.93±11.05
DVD230	30	24	80.7	12.5±1.9	99.3±2.4	99.4±2.7	6.1±0.4	11.8±1.4	19.0±1.7	36.7±2.6	58.9±3.4	45.69±12.67
DVD1150	30	23	76.6	10.7±3.0**	99.1±2.9	100	5.9±0.4*	9.1±1.0**	13.5±1.2**	24.7±1.3**	36.2±2.6**	48.42±16.07
DVD2000	30	20	66.7	8.8±2.3**	98.9±3.6	99.4±2.7	5.8±0.4*	8.7±1.2**	11.9±1.7**	20.9±2.6**	30.1±5.0**	47.57±15.54

Note: DVD, Diaveridine; DVD23, 23 mg/kg diet; DVD230, 230 mg/kg diet; DVD1150, 1150 mg/kg diet; DVD2000, 2000 mg/kg diet.

* Significantly different from control group at $p < 0.05$. **Significantly different from control group at $p < 0.01$.

Table 4 Teratogenicity toxicity in rat fetuses following maternal exposure to diaveridine by dietary administration (Mean±SD)

Group	No. of Female	No. of pregnancy	No. of nidation	No. of live fetus	No. of dead fetus (%)	Absorb fetus	Uterus and fetus weight (g)	Fetus weight(g)	Placenta weight (g)	Body length (cm)	Tail length (cm)	Male rate (%)
Control	30	26	14.0±3.6	13.7±3.3	0.02	0	113.0±19.6	6.5±0.6	0.74±0.15	4.7±0.4	1.7±0.1	48.49±13.54
DVD23	30	26	15.0±3.8	14.6±3.6	0.02	0	111.8±19.5	6.6±0.7	0.75±0.13	4.8±0.3	1.8±0.2*	46.39±12.61
DVD230	30	25	14.0±3.6	13.8±3.5	0.01	0	108.5±13.8	6.6±0.6	0.72±0.15	4.7±0.4	1.8±0.1*	47.33±17.24
DVD1150	30	22	13.5±1.6	12.7±1.7	0.05	0	96.6±9.4*	5.3±0.6**	0.60±0.11**	4.6±0.2*	1.7±0.2	49.45±16.94
DVD2000	30	20	10.5±3.2*	9.7±2.8**	0.07	0	75.3±14.8**	5.0±0.7**	0.63±0.11**	4.4±0.3**	1.6±0.1**	48.81±16.45

Note: DVD, Diaveridine; DVD23, 23 mg/kg diet; DVD230, 230 mg/kg diet; DVD1150, 1150 mg/kg diet; DVD2000, 2000 mg/kg diet.

* Significantly different from control group at $p < 0.05$. **Significantly different from control group at $p < 0.01$.

Table 5 Organ weights of F_0 rats for treatment period in the reproduction test (Mean±SD)

Organs	Control	DVD23	DVD230	DVD1150	DVD2000	Control	DVD23	DVD230	DVD1150	DVD2000
	Absolute weights (g)					Relative weights (g/100g)				
F_0 Female										
Liver	10.84±1.13	10.85±0.97	10.29±0.95	9.42±0.86*	9.32±0.90**	3.50±0.32	3.66±0.26	3.67±0.42	3.87±0.28*	3.98±0.29**
Spleen	0.70±0.11	0.70±0.10	0.71±0.11	0.57±0.08*	0.53±0.05**	0.23±0.03	0.24±0.04	0.25±0.04	0.24±0.03	0.23±0.02
Thymus	0.34±0.10	0.31±0.07	0.25±0.06*	0.21±0.10**	0.20±0.06**	0.11±0.03	0.11±0.02	0.09±0.02	0.09±0.05	0.09±0.03
Heart	1.22±0.11	1.17±0.12	1.07±0.12*	0.91±0.11**	0.94±0.10**	0.39±0.03	0.39±0.03	0.38±0.03	0.37±0.04	0.40±0.03
Lungs	1.70±0.10	1.65±0.08	1.62±0.12	1.38±0.12**	1.31±0.12**	0.55±0.02	0.56±0.02	0.58±0.05	0.57±0.05	0.56±0.04
Kidney	2.15±0.13	2.05±0.16	2.13±0.20	1.90±0.20**	1.90±0.16**	0.70±0.04	0.69±0.06	0.76±0.06*	0.78±0.08*	0.81±0.07**
Adrenal	0.093±0.020	0.096±0.017	0.099±0.012	0.092±0.008	0.091±0.014	0.030±0.006	0.033±0.006	0.035±0.004*	0.038±0.005**	0.039±0.005**
Ovary	0.24±0.04	0.22±0.03	0.22±0.02	0.17±0.03	0.17±0.03	0.08±0.01	0.07±0.01	0.08±0.01	0.07±0.02	0.08±0.01
Uterus	0.65±0.11	0.62±0.15	0.55±0.12	0.45±0.10**	0.46±0.09**	0.21±0.03	0.21±0.04	0.20±0.04	0.19±0.04	0.20±0.05
Brain	1.34±0.09	1.34±0.08	1.34±0.08	1.31±0.08	1.29±0.09	0.44±0.04	0.45±0.04	0.48±0.04*	0.55±0.04**	0.56±0.05**
F_0 Male										
Liver	12.73±1.09	12.68±0.88	12.37±0.83	11.39±1.11*	10.97±1.28**	2.49±0.13	2.47±0.11	2.48±0.12	2.68±0.16*	2.75±0.14**

Spleen	0.96±0.09	0.91±0.11	0.90±0.12	0.77±0.08**	0.71±0.13**	0.19±0.02	0.18±0.02	0.18±0.02	0.18±0.02	0.18±0.03
Thymus	0.53±0.08	0.53±0.06	0.53±0.08	0.42±0.05**	0.38±0.06**	0.10±0.02	0.10±0.01	0.11±0.02	0.10±0.01	0.09±0.01
Heart	1.77±0.19	1.88±0.16	1.78±0.25	1.48±0.14**	1.43±0.12**	0.35±0.03	0.37±0.03	0.36±0.03	0.34±0.02	0.35±0.03
Lungs	1.92±0.16	1.92±0.12	1.90±0.15	1.64±0.10**	1.57±0.13**	0.38±0.02	0.37±0.02	0.38±0.01	0.38±0.02	0.38±0.02
Kidney	2.94±0.24	2.96±0.21	2.91±0.23	2.64±0.20*	2.58±0.32*	0.58±0.03	0.58±0.03	0.58±0.03	0.61±0.04*	0.63±0.04**
Adrenal	0.079±0.009	0.073±0.009	0.074±0.009	0.076±0.008	0.074±0.009	0.016±0.001	0.014±0.002	0.015±0.002	0.018±0.002*	0.018±0.002**
Testis	6.43±0.40	6.46±0.46	6.26±0.53	6.07±0.43	5.90±0.42*	1.26±0.10	1.26±0.07	1.26±0.09	1.41±0.11**	1.45±0.10**
Brain	1.49±0.07	1.52±0.05	1.50±0.07	1.46±0.11	1.48±0.07	0.29±0.02	0.30±0.02	0.30±0.03	0.34±0.03**	0.36±0.03**

Note: DVD, Diaveridine; DVD23, 23 mg/kg diet; DVD230, 230 mg/kg diet; DVD1150, 1150 mg/kg diet; DVD2000, 2000 mg/kg diet.

* Significantly different from control group at $p < 0.05$. **Significantly different from control group at $p < 0.01$.

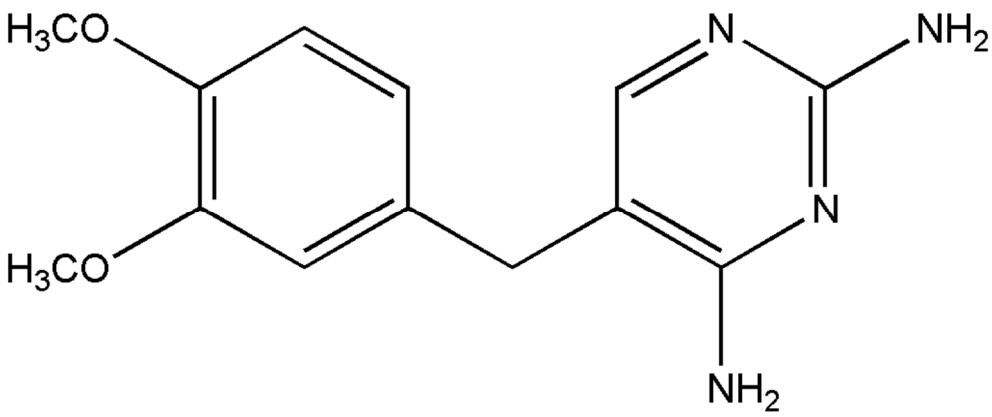

Fig. 1 Chemical structure of diaveridine (DVD).
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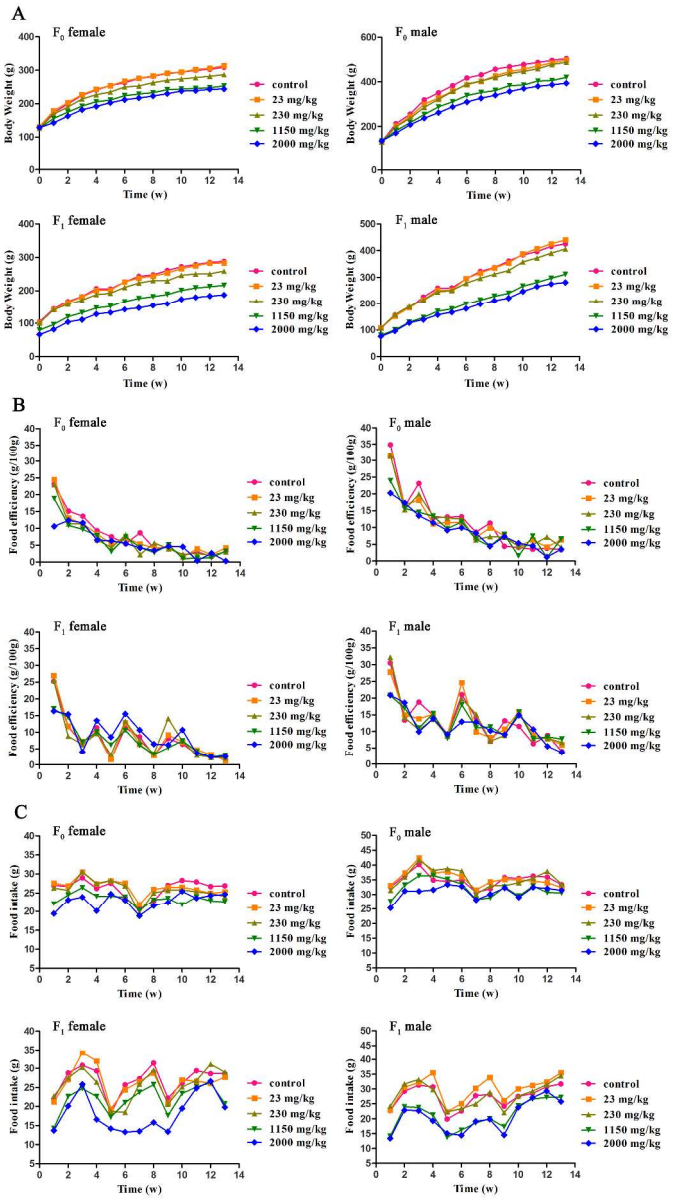

Fig. 2 Mean body weights (A), food efficiency (B), mean food intakes (C) of F0 and F1 rats following dietary exposure to diaveridine for the periods prior to mating in the reproduction and teratogenicity tests. (●) Control group; (■) Diaveridine group (23 mg/kg); (▲) Diaveridine group (230 mg/kg); (▼) Diaveridine group (1150 mg/kg); (♦) Diaveridine group (2000 mg/kg).
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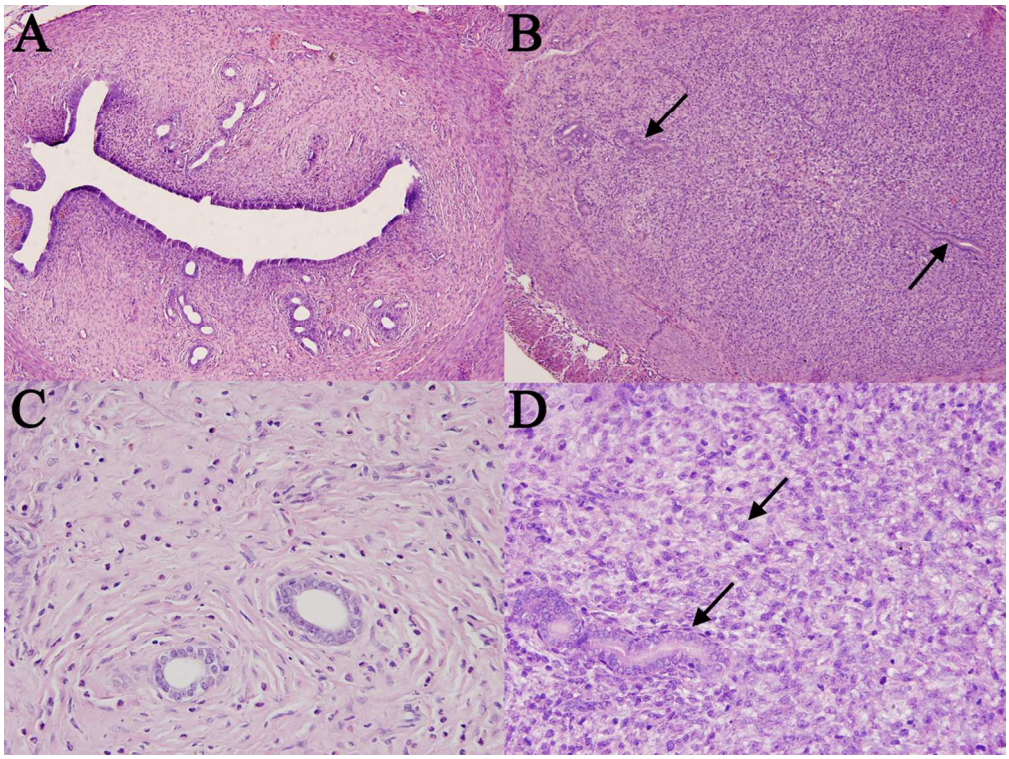

Fig. 3 Selected microphotographs of uterus at 10X and 40X. (A) Control uterus of F0 and F1 (10X) ; (B) Uterus of F1 females at the 1150 and 2000 mg/kg diaveridine groups (10X). The uterus gland atrophy and the uterine cavity to shrink were marked with arrows ; (C) Control uterus of F0 and F1 females (40X) ; (D) Uterus of F1 females at the 1150 and 2000 mg/kg diaveridine groups (40X). The multinucleated cell morphology of different sizes of the uterus tumor cells was marked with arrows.
115x86mm (300 x 300 DPI)