Collaborative work on evaluation of ovarian toxicity

4) Two- or four-week repeated dose study of 4-vinylcyclohexene diepoxide in female rats

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(Received December 10, 2008)

ABSTRACT — To determine the optimal administration period for evaluation of ovarian toxicity of 4-vinylcyclohexene diepoxide (VCD), VCD was intraperitoneally administered to female Sprague-Dawley rats at 0 (Control), 5, 20 and 80 mg/kg once a day for 2 or 4 weeks (2- or 4-week study). To identify small follicles, serial sections of the ovaries were stained with routine hematoxylin and eosin (HE) and proliferating cell nuclear antigen (PCNA) immunohistochemistry. In the 4-week study, decrease in small follicles was observed in the ovaries at 20 and 80 mg/kg. In the 2-week study, the same change was also observed at 80 mg/kg. Identification of small follicles using PCNA-stained slides was easier than that using HE-stained slides. In conclusion, histopathological findings in the ovaries are important for evaluation of female reproductive toxicity of VCD, and ovarian toxicity of VCD can be detected by administration for 2 weeks at an appropriate dose level. Furthermore, PCNA immunohistochemistry is effective for evaluation of small follicle destruction in chemical-induced ovarian toxicity.

Key words: 4-Vinylcyclohexene diepoxide, Ovarian toxicity, Rats, Histopathology, PCNA

INTRODUCTION

As part of a collaborative study on toxicity related to female fertility, we performed experiments using 4-vinylcyclohexene diepoxide (VCD), the occupational chemical known to be an ovarian toxicant. VCD is a metabolite formed by epoxidation of 4-vinylcyclohexene (VCH), and is used industrially as a diluent for other epoxides (IARC, 1976). It has been reported that VCD administration to female rats and mice decreased small follicles of the ovaries (Smith *et al.*, 1990). VCD is known to be an ovarian toxicant targeting small follicles directly (Hoyer and Sipes, 1996) and the mode of action (MOA) of their ovarian toxicity is acceleration of the natural process of apoptosis in small follicles (Springer *et al.*, 1996). This acceleration is known to be associated with activation of the Bcl-2 family (Hu *et al.*, 2001a, 2001b) and mitogen-activated protein kinase (MAPK) family (Hu *et al.*, 2002) in small follicles. Mayer *et al.* (2002) reported that administration of VCD (80 mg/kg/day, i.p. for 30 days) to female rats caused premature ovarian failure by 360 days after dosing. Haas *et al.* (2007) reported that VCD-treated female mice can be used to model infertility in perimen-

opausal women. Chemicals that deplete ovarian follicles are of toxicological concern because the mammalian ovary contains a finite number of oocyte-containing follicles. This finite pool cannot be regenerated, and thus its depletion can result in premature ovarian failure (early menopause in women; Hooser *et al.*, 1994; Mayer *et al.*, 2002, 2004).

The present study was designed to determine whether a 2- or 4-week administration period can detect histopathological findings in a repeated dose toxicity study to compare potential ovarian toxicity with female reproductive function in a fertility study. In the repeated dose toxicity study, VCD was intraperitoneally administered to female Sprague-Dawley rats at 0 (Control), 5, 20 and 80 mg/kg once a day for 2 or 4 weeks. To identify small follicles, serial sections of the ovaries were stained with routine hematoxylin and eosin (HE) and proliferating cell nuclear antigen (PCNA) immunohistochemistry. PCNA immunohistochemistry is well established as a tool for evaluation of follicles in the ovaries (Muskhelishvili *et al.*, 2005). In the female fertility study, VCD was intraperitoneally administered to female rats at dose levels of 0, 5, 20 and 80 mg/kg from 2 weeks before mating to day 7 of preg-

nancy, that is for at least 3 weeks, including mating period (Kodama *et al.*, 2009).

MATERIALS AND METHODS

Animals and husbandry

Female Sprague-Dawley rats (Slc:SD), 5 weeks-old, were purchased from Japan SLC, Inc. (Shizuoka, Japan). The rats were individually housed in a room maintained under controlled conditions of temperature ($23 \pm 3^\circ\text{C}$) and relative humidity ($50 \pm 20\%$), with a 12-hr light/dark cycle (7:00–19:00) in a barrier system. A standard commercial diet (FR-2, Funabashi Farms CO., Ltd., Chiba, Japan) and tap water were available *ad libitum*. After 1-week acclimatization, the rats were allocated randomly to 4 groups ($n = 10$ in each group) based on their body weights in both studies.

This study conformed with the “Guide for animal experiments in Toa Eiyo Ltd.”.

Chemical and administration

The chemical structure of VCD is shown in Fig. 1. VCD was obtained from Fluka (Buchs, Switzerland). VCD was dissolved in sesame oil (Sigma-Aldrich, St. Louis, MO, USA) to a final volume of 2.5 ml/kg for each dose. Control rats received the same dose volume of sesame oil.

The study design is shown in Fig. 2. VCD was intraperitoneally administered to rats at 0 (Control), 5, 20 and 80 mg/kg once a day for 2 or 4 weeks (2- or 4-week study). Dosage levels were selected based on the results of the previous study (Smith *et al.*, 1990).

Clinical examination

All rats were observed daily for mortality and clinical signs, and weighed twice a week. Vaginal smears were

taken daily to determine the estrous cycle length in the last week of the 2-week study and in the last 2 weeks of the 4-week study.

Pathological examination

All rats were sacrificed under anesthesia the day after 2- or 4-week administration and examined macroscopically. At sacrifice, the ovaries, vagina, uterus, adrenal glands, pituitary gland, liver and kidneys were weighed and their weights relative to the final body weight were calculated. All the tissues were fixed in 10% neutral-buffered formalin. At trimming, the bilateral ovaries were transversally halved to observe maximum area of the ovary under a light microscope. All the tissues were embedded in paraffin, sectioned and stained with HE. To identify small follicles, PCNA immunohistochemistry was performed using serial sections stained with HE. The PCNA immunohistochemical procedure was according to Muskhelishvili *et al.* (2005), and used anti-PCNA antibody (clone PC10, DAKO, Glostrup, Denmark). Histopathological examination was performed under a light microscope. The follicles of the ovaries were classified into three groups: small (Type 1 to 3a), medium (Type 3b to 5a) and large (Type 5b to 8) according to the classification of ovarian follicles reported by Pedersen and Peters (1968). The criteria for follicular reduction are shown in Table 1.

Statistical analysis

The means and standard deviations were calculated for body weights, estrous cycle length and absolute/relative organ weights. To determine whether a difference between the control group mean and each dose group mean was significant when the variance was homogeneous in Bartlett's test (Bartlett, 1937). Each dose group mean was compared with that of the control group by Dunnett's test

Fig. 1. Chemical structure of 4-vinylcyclohexene diepoxide.

Fig. 2. Study design.

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(Dunnett, 1995). When the variance was not homogeneous, Dunnett's mean rank paired comparison test was performed.

Significance levels of 5% and 1% were applied.

RESULTS

Clinical examination

There was no mortality in either study. There were no drug-related changes in clinical signs, body weight or estrous cycle length in either study.

Pathological examination

There were no abnormalities in absolute/relative organ weights or macroscopic examination except for peritonitis in either study. Peritonitis was considered to be induced by intraperitoneal injection of vehicle (sesame oil) and

not to be a drug-related change since it was observed at the same level in all groups.

The results of histopathological examination of the ovaries are summarized in Table 2. Identification of small follicles using PCNA-stained slides was easier than that using HE-stained slides. Although small follicles of the ovaries were frequently observed in a control rat (Fig. 3), decrease in small follicles was observed at 20 and 80 mg/kg in the 4-week study (Fig. 4). The degree of the change at 80 mg/kg was remarkable, and few follicles were detected in 3 animals (Table 2). In the 2-week study, the same change was also observed at 80 mg/kg. The degree of the change at 80 mg/kg in the 2-week study was comparable to that at 20 mg/kg in the 4-week study. The incidence and grade of the change were observed in a dose- and duration-dependent manner. No other histopathological findings without the reduction of small fol-

Table 1. Criteria for follicular reduction in 2- or 4-week study of 4-vinylcyclohexene diepoxide

Finding	Number of follicles		
	Grade: +	Grade: ++	Grade: +++
Unilateral ovary			
Decrease in small follicles	4-6	2-3	0-1
Bilateral ovaries			
Decrease in small follicles	11-15	6-10	0-5

Table 2. Histopathological findings of ovaries in 2- or 4-week study of 4-vinylcyclohexene diepoxide

2-week study												
Dose (mg/kg)	0			5			20			80		
No. of animals	10			10			10			10		
No. examined	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10
Decrease in small follicles	0	0	0	0	0	0	0	0	0	2	2	3
Slight	—	—	—	—	—	—	—	—	—	1	0	1
Moderate	—	—	—	—	—	—	—	—	—	0	1	2
Severe	—	—	—	—	—	—	—	—	—	1	1	0
4-week study												
Dose (mg/kg)	0			5			20			80		
No. of animals	10			10			10			10		
No. examined	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10
Decrease in small follicles	0	0	0	0	0	0	2	2	2	7	8	9
Slight	—	—	—	—	—	—	2	2	1	3	3	2
Moderate	—	—	—	—	—	—	0	0	1	1	4	4
Severe	—	—	—	—	—	—	0	0	0	3	1	3

R: right ovary, L: left ovary, W: whole body, -: no noteworthy findings

Fig. 3. In a control rat, small follicles were frequently observed. PCNA immunohistochemistry.

Fig. 4. Decrease in small follicles of a female rat administered with 4-vinylcyclohexene diepoxide at 80 mg/kg for 4 weeks. PCNA immunohistochemistry.

licles were detected in the ovaries and the other organs examined.

DISCUSSION

In the present study, we conducted the 2- or 4-week repeated dose toxicity study of VCD in rats to determine

the optimal administration period concerning the effects on ovarian histopathological findings. Moreover, the no observed adverse effect level (NOAEL) for the repeated dose toxicity study focused on the ovaries, and that of the female fertility study was also compared.

In the repeated dose toxicity study, there were no drug-related changes in any examination, except for a decrease

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in small follicles of the ovaries. It has also been reported that there were no effects on the estrous cycle and organ weights (ovary, uterus, liver, kidney) in immature (30 days) rats dosed with VCD (80 mg/kg/day, i.p. for 30 days) (Flaws *et al.*, 1994). In histopathological examination, decrease in small follicles was observed at 80 mg/kg and 20 mg/kg or above in the 2- and 4-week study, respectively. Kodama *et al.* (2009) also reported that the same change was observed at 80 mg/kg of VCD in the female fertility study. The present data were consistent with a previous study that VCD caused a decrease of small follicles in the ovaries (Smith *et al.*, 1990). Thus, we consider that decrease in small follicles of the ovaries induced by VCD is the most sensitive parameter for evaluating ovarian toxicity, therefore, the NOAEL for the repeated dose toxicity study focused on the ovaries was 20 mg/kg and 5 mg/kg in the 2- and 4-week study, respectively.

A female is born with a finite number of small follicles and, once destroyed, no more can be generated. Therefore, exposure to chemicals that target small follicles can impair reproductive function and cause premature menopause in women (Hooser *et al.*, 1994; Mayer *et al.*, 2002, 2004). If any deviation from the normal small follicle condition is found on routine histopathological examination, it might be an indication of ovarian toxicity, and more precise histopathological examination, including PCNA immunohistochemistry, should be added. Muskhelishvili *et al.* (2005) reported that in rat formalin-fixed, paraffin-embedded ovary sections follicles of all degrees of maturity can be visualized by PCNA immunohistochemistry, and follicles are easily distinguished from ovarian background with the ability to detect and identify primordial follicles being enhanced. Picut *et al.* (2008) reported that counting of primordial and primary follicles using PCNA-stained slides is preferable to that using HE-stained slides. In the present study, identification of small follicles using PCNA-stained slides was easier than that using HE-stained slides. Therefore, PCNA immunohistochemistry is effective for evaluation of small follicles destruction in chemical-induced ovarian toxicity.

In the female fertility study, decreased ovary weights, decreased the number of implanted embryos, decreased implantation rate and increased preimplantation loss rate as observed at 80mg/kg of VCD. Therefore, the NOAELs for general toxicity in females, for the reproductive function in females and for the development of the next generation in females were considered to be 20 mg/kg, 20 mg/kg and more than 80 mg/kg, respectively (Kodama *et al.*, 2009). The histopathological findings of the ovaries in the 2-week repeated dose toxicity study were considered to be the same sensitive endpoint as female fertility

parameters in the female fertility study for the detection of female reproductive toxicity of VCD.

In conclusion, histopathological findings in the ovaries are important for evaluation of female reproductive toxicity of VCD, and ovarian toxicity of VCD can be detected by administration for 2 weeks at an appropriate dose level. Furthermore, the histopathological findings in the ovaries in the repeated dose toxicity study performed at an appropriate dose level would predict alterations in female reproductive function in the female fertility study.

ACKNOWLEDGMENTS

The authors would like to thank Y. Sato, F. Yoneyama, Y. Ogino, T. Yoneda and K. Mori for technical assistance.

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