ment of female fertility and embryo-fetal development must be completed prior to the inclusion of WOCBP using birth control in any type of clinical trial because the current repeated-dose toxicity studies have been considered inadequate for evaluating female reproductive function. On the other hand, the assessment of embryo-fetal development must be completed prior to the Phase I trials in WOCBP and the female fertility studies prior to the Phase III trials in the EU. In the USA, WOCBP may be included in early, carefully monitored clinical studies without reproduction toxicity studies as long as steps are taken to minimize the risk of pregnancy while assessment of female fertility and embryo-fetal development must be completed before WOCBP using birth control are enrolled in Phase III trials.

Accordingly, the National Institute of Health Sciences (NIHS) and the Japan Pharmaceutical Manufacturers Association (JPMA) have organized a collaborative study to evaluate whether ovarian toxicities can be detected by repeated-dose general toxicity studies in rats. In the collaborative study, NIHS and 18 pharmaceutical companies of JPMA have now conducted a validation study intended to examine whether a 2-week testing period is adequate to detect the ovarian toxicity of various Mode of action (MOA) chemicals and whether careful histopathological examination of the female reproductive organs would be a good tool to assess female reproductive function. In addition, a female fertility study was also conducted to compare the results with those of the ovarian histopathological findings.

The results of the studies conducted by individual companies will be reported by those companies elsewhere. This report is a summary of the assembled data.

MATERIALS AND METHODS

Eighteen JPMA member companies participated in this cooperative investigation and a total of 17 compounds were evaluated. Details of the participating companies, substances used, animal strains, routes of administration and duration of treatment are summarized in Table 1.

Each company was requested to include the following minimum requirements in each protocol. Modifications to the minimum requirements, however, were allowed according to each company's situation. The experimental design of the female fertility study is shown in Fig. 1.

1) Animal strain: The strains used in this collaborative study were SD, Wistar: Hannover or F344 for the repeated toxicity studies, and SD or Wistar: Hannover for the female fertility studies.

2) Number of animals: At least 10 animals per dose were used in both the repeated toxicity and the fertility studies.

3) Age at treatment initiation: The rats were treated with a test substance until 6 weeks of age for the repeated toxicity studies and until 10 weeks of age for the female fertility study.

4) Test substances: The test substances were selected by the individual participating companies from 4 categories (hormone analogues, primordial follicle damaging agents, metabolite imbalance inducers, and endocrine imbalance inducers) depending on published or in-house data.

5) Dosage selection: The highest dosage of the repeated toxicity and female fertility studies was selected based on the doses that caused any ovarian toxicity in published or in-house data. In principle, the same dosage was selected in both studies.

6) Observations: The clinical signs, estrous cycle and body weight were checked during both studies.

7) Histopathological examination in the repeated toxicity study: After weighing the reproductive and related organs (ovaries and uterus) and representative ones related to the test articles, these tissues and additional ones including the vagina and mammary gland were fixed in 10% neutral buffered formaldehyde solution and routinely processed for histopathological analysis. Both ovaries from all the animals were transversely dissected or cut to provide the maximum ovary area for visualization using light microscopy. After fixation, the ovaries were embedded in paraffin wax, sectioned at 4-5 μm and stained with hematoxylin and eosin (HE) for the histopathological examination. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) was also performed on serial sections of the ovaries based on the work of Muskhelishvili *et al.* (2005). The sections stained with PCNA were mainly used for the identification of primordial or primary follicles.

8) Terminology used for histopathological analysis in the ovary: Follicles were classified into small, medium and large based on Pedersen's classification (Pedersen and Peters, 1968). Corpora lutea were classified into corpora lutea formed within one estrous cycle after the most current ovulation, and previous ones formed before the current one estrous cycle. The characteristics of the follicles and corpora lutea at each estrous cycle stage referred to Yoshida *et al.* (2009). The terminology and histopathological changes observed in the validation studies were standardized by toxicological pathologists participating in several face-to-face meetings held by JMPA and NIHS. After completion of the examination by each company, a peer review was conducted with Midori Yoshida, Divi-

Table 1. Summary of collaborative study -General toxicity study-

Category	Chemical	Dose levels (mg/kg/day)	Route	Animal strain	Results	Detection of ovarian toxicity	Company and reference
Hormone analogues	Medroxyprogesterone acetate (Progesterone)	0.4, 2.0 and 10 mg/kg/day for 2 and 4 weeks	PO	Crl:CD (SD)	Irregular estrous cycle and decreased ovary weight at 2.0 mg/kg and greater in the 2- and 4-week-treated groups. Atretic large follicles, decreased current and previous/large or previous/small corpora lutea at the same doses in the 2- and 4-week treated groups at histopathological examination.	Possible by both 2- and 4-week repeated toxicity study	ASKA Pharmaceutical Co., Ltd. Ohtake *et al.* (2009)
	Mifepristone (Progesterone receptor antagonist)	0.8, 4, 20 and 100 mg/kg/day for 2 and 4 weeks	PO	Crl:CD (SD)	Persistent estrus as seen in the vaginal smears. Multiple cysts in the ovaries at necropsy. Increases in luteinized cysts and enlargement of previous corpora lutea at 20 mg/kg or more for 2 or 4 weeks at the histopathological examination of the ovaries	Possible by both 2- and 4-week repeated toxicity study	Kissei Pharmaceutical Co., Ltd. Tamura *et al.* (2009)
	Tamoxifen (Estrogen receptor antagonist)	0.005, 0.03 and 0.2 mg/kg/day for 2 and 4 weeks	PO	Wistar Hanover Crl:WI (HAN)	Increases in the large atretic follicles, increases in the interstitial cells and absence of currently formed corpus lutea in the ovary at 0.2 mg/kg/day in the 2-week study and at 0.03 and 0.2 mg/kg/day in the 4-week study at histopathological examination. Estrogenic and antiestrogenic reactions in the uterus. Mucinous degeneration in the vagina.	Possible by both 2- and 4-week repeated toxicity study	Banyu Pharmaceutical Co., Ltd. Tsujioka *et al.* (2009)
Primordial follicle damaging agents	4-vinylcyclohexene diepoxide (Group 2B carcinogen)	0, 5, 20 and 80 mg/kg/day for 2 and 4 weeks	IP	Slc:SD	A decrease of small follicles at 80 mg/kg in the 2 weeks study at histopathological examination. In the 4 weeks study, the increased incidence of the decrease at 20 and 80 mg/kg with dose-dependent manner. Estrous cyclicity was not disturbed both studies.	Possible by both 2- and 4-week repeated toxicity study	Toa Eiyo Ltd. Ito *et al.* (2009)
	Busulfan (Antineoplastic alkylating agent)	0.1, 0.5 and 1.5 mg/kg/day for 2 or 4 weeks	PO	Crl:CD (SD)	No pathological changes in the 2-week study. In the 4-week study, a decrease in small follicles observed histopathologically in one rat even at 0.5 mg/kg and in 4 rats at 1.5 mg/kg. PCNA immunohistochemistry of the follicles confirmed the above decrease in the number of small follicles at 1.5 mg/kg.	Possible by a 4-week repeated toxicity study	Kyorin Pharmaceutical Co., Ltd. Sakurada *et al.* (2009)
	Cisplatin (Antineoplastic)	0.25, 0.5, 1.0 and 2.0 mg/kg/day for 2 weeks, or those of 0.125, 0.25 and 0.5 mg/kg/day for 4 weeks	IP	Crl:CD (SD)	Controversial results in estrous cyclicity (Irregular estrous cycling at 2-weeks treatment group, and normal cycling at 4-weeks group). A decrease in large follicle, an increase in atresia of medium and large follicles, and/or a decrease in currently formed corpus luteum at 1.0 and 2.0 mg/kg for 2 weeks at histopathological examination. Decreases in small and/or large follicles and an increase in atresia of large follicle at 0.25 and 0.5 mg/kg for 4 weeks.	Possible by both 2- and 4-week repeated toxicity study	Toyama Chemical Co., Ltd. Nozaki *et al.* (2009)

Table 1. (Continued)

Category	Chemical	Dose levels (mg/kg/day)	Route	Animal strain	Results	Detection of ovarian toxicity	Company and reference
Primordial follicle damaging agents	Cyclophosphamide (Antineoplastic Alkylating agent)	5, 10 and 20 mg/kg/day for 2 or 4 weeks	PO	Crl:CD (SD)	No pathological changes in the 2-week study. In the 4-week study, decrease of absolute ovarian weights at 20 mg/kg/day. Atrophy of corpora lutea at 20 mg/kg/day for 4 weeks at histopathological examination.	Possible by a 4-week repeated toxicity study	Otsuka Co., Ltd. Sato M. *et al.*
Metabolism imbalance inducers	Anastrozole (Antineoplastic, Aromatase inhibitor)	0.01, 0.1, 1 and 50 mg/kg/day for 2 and 4 weeks	PO	CrjDu: F344	Large abnormal atretic follicles, follicular cysts, a decrease in the corpus luteum and the depletion of currently formed corpus luteum at 1 and/or 50 mg/kg groups of both the 2-week and 4-week studies in a histopathological examination of the ovaries. Irregular estrous cycle.	Possible by both 2- and 4-week repeated toxicity study	Daiichi-Sankyo Co., Ltd. Shirai *et al.* (2009)
	Di(2-ethylhexyl) adipate (DEHA)	200, 1,000 and 2,000 mg/kg/day for 2 and 4 weeks	PO	Crl:CD (SD)	Abnormal estrous cyclicity. Increase in atresia of large follicle, decrease in currently formed corpus luteum and follicular cyst at 1,000 mg/kg and higher at histopathological examination.	Possible by both 2- and 4-week repeated toxicity study	Kowa Company, Ltd. Wato *et al.* (2009)
	Di(2-ethylhexyl) phthalate (DEHP)	300, 1,000 and 3,000 mg/kg/day for 2 and 4 weeks	PO	Crl:CD (SD)	Vacuolation of stromal cells in the groups receiving 300 mg/kg or more and an increased of large atretic follicle in groups at 1,000 mg/kg or higher at histopathological examination of the ovaries in both repeated dose toxicity studies. In the 4-week study, a decrease in currently formed corpora lutea at the 3,000 mg/kg. Prolonged length of estrous cyclicity.	Possible by both 2- and 4-week repeated toxicity study	Chugai Pharmaceutical Co., Ltd. Takai *et al.* (2009)
	Ethylene Glycol Monomethyl Ether (EGME)	30, 100 and 300 mg/kg/day for 2 and 4 weeks	PO	Crl:CD (SD)	Continuous diestrus at 100 mg/kg and higher regardless of the administration periods. Hypertrophy of corpora lutea with decreased cellular debris, and increased PCNA negative large atretic follicles and decrease of newly-formed basophilic corpora lutea at 100 mg/kg and higher after 2 or 4 weeks at histopathological examination	Possible by both 2- and 4-week repeated toxicity study	Eisai Co., Ltd. Dodo *et al.* (2009)
	Indomethacin (COX-1,2 inhibitor, NSAID)	0.4, 1.3 and 4 mg/kg/day for 2 and 4 weeks	PO	Crl:CD (SD)	Unruptured follicles or luteinized cysts at 4 mg/kg in both the 2-week and 4-week studies at histopathological examination. Follicular cysts at 4 mg/kg in the 4-week study. Estrous cyclicity was not disturbed both studies.	Possible by both 2- and 4-week repeated toxicity study	Astellas Pharma Inc. Tsubota *et al.* (2009)
	Compound X (PPAR α/γ dual agonist)	4, 20 and 100 mg/kg/day for 2 and 4 weeks	PO	Crl:CD (SD)	An increase in atresia of large follicles, a decrease in corpora lutea, granulosa cell exfoliation into antram of large follicles, corpora lutera with retained oocyte, and interstitial gland hyperplasia were observed in all treated groups in both the 2- and 4-week studies at histopathological examination of the ovaries. No disruption of estrous cyclicity.	Possible by both 2- and 4-week repeated toxicity study	Asahi Kasei Pharma Corporation Sato N. *et al.* (2009)

Table 1. (Continued)

Category	Chemical	Dose levels (mg/kg/day)	Route	Animal strain	Results	Detection of ovarian toxicity	Company and reference
Endocrine imbalance inducers	Atrazine (Herbicide, Endocrine modulator)	3, 30 and 300 mg/kg/day for 2 and 4 weeks	PO	Slc:SD	Prolongation of diestrus and loss of currently formed corpora lutea, decrease in the numbers of previously formed corpora lutea, increase in large-sized atretic follicles, and swelling of the previously formed luteal cells at 300 mg/kg in 2 week study at histopathological examination.	Possible by both 2- and 4-week repeated toxicity study	Nippon Shinyaku Co., Ltd. Shibayama *et al.* (2009)
	Bromocriptine (Dopamine agonist)	0.08, 0.4 and 2 mg/kg/day for the 2 and 4 weeks	SC	Crl:CD (SD)	Increases of ovarian weights were observed at 2 mg/kg and 0.4 mg/kg and higher in the 2-week and 4-week general studies, respectively. The number of corpora luteum was increased in the 0.4 mg/kg and higher in the 2- and 4-week general toxicity studies at the histopathological examination of the ovaries. Bromocriptine did not affect estrous cyclicity in both the general toxicity studies.	Possible by both 2- and 4-week repeated toxicity study	Sanwa Kagaku Kenkyusho Co., Ltd. Kumazawa *et al.* (2009)
	Chlorpromazine hydrochloride (D2, 5-HT2A H1, α1-antagonist)	3, 10, 30 and 300 mg/kg/day for 2 and 4 weeks	PO	Crl:CD (SD)	At histopathological examination, a decrease of ovarian weights at 10 mg/kg and higher in the 4-week study. An increase in large atretic follicles at 10 mg/kg and higher in the 2 and 4 weeks studies at histopathological examination. A decreased uterine weights and/or atrophic findings in the uterus and vagina at 30 mg/kg and 10 mg/kg and higher, mucification in the vaginal epithelium and alveolar hyperplasia in the mammary gland at 3 mg/kg and higher, and 10 mg/kg and higher in the 2 and 4 weeks studies, respectively. Irregular estrous cycles were seen at 3 mg/kg and higher, and 10 mg/kg and higher in the 2 and 4 weeks studies.	Possible by both 2- and 4-week repeated toxicity study	Takeda Pharmaceutical Co., Ltd. Izumi *et al.* (2009)
	Sulpiride (D2 Antagonist)	1, 10 and 100 mg/kg/day for 2 and 4 weeks	PO	Crl:CD (SD)	Increases in atretic follicle at 1 mg/kg or more and increases in follicular cysts at 10 mg/kg or higher in the 2-week study at histopathological examination. In the 4-week study, these findings at 1 mg/kg or higher, and a decrease in large follicles at 10 mg/kg or higher. Increased body weight gain at 10 mg/kg or higher in the 2- and 4-week studies. Development of mammary alveolus in these groups exhibited the.	Possible by both 2- and 4-week repeated toxicity study	Mitsubishi Tanabe Pharma Corporation Ishii *et al.* (2009)

PO, SC and IP mean treatment routes by gavage, subcutaneous and intraperitoneal treatments, respectively.

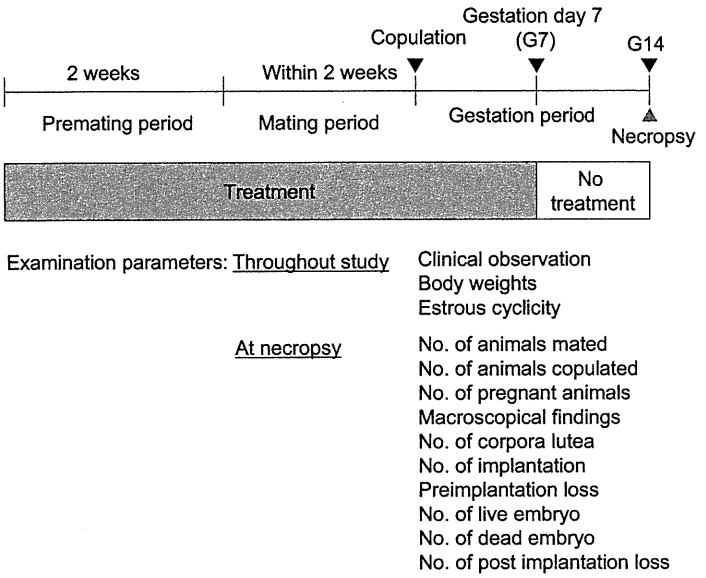

Fig. 1. Experimental design and examination parameters of female fertility study.

sion of Pathology, NIHS, who was responsible for the histopathological analysis of the validation study to confirm the standardization.

RESULTS

The summary results of the individual studies are shown in Tables 1 (Repeated toxicity study) and 2 (Female fertility study).

Hormone analogues

Medroxyprogesterone acetate (MPA)

Profiles of chemical: Synthetic progestagen

Company: ASKA Pharmaceutical Co., Ltd.

Studies: Repeated toxicity and fertility studies

Animals; Crl:CD(SD) rats

Doses, route and duration: Treatment by gavage at 0, 0.4, 2.0 and 10 mg/kg/day for 2- or 4-week in the repeated toxicity study, and the same doses prior to mating to Day 7 of gestation in the fertility study.

Results: In the general toxicity study, animals with irregular estrous cycle were increased in number and decreased ovary weight was observed at 2.0 mg/kg and greater in the 2- and 4-week-treated groups. On histopathological examination, atretic large follicles were increased and decreased new and old/large or old/small corpora lutea were observed at the same doses in the 2- and 4-week treated groups. In the fertility study, animals with irregular estrous cycle and elongation of mean estrous cycle increased in number at 0.4 mg/kg, which showed no effects on fertility. Decreased number of copulated animals and decreased gestation rate with low preimplantation loss were observed in the 2.0 mg/kg-treated group and no copulation was observed in the 10 mg/kg-treated group.

Conclusion: The changes in fertility induced by MPA were well correlated with histopathological changes of the ovaries after 2 and 4 weeks treatment, which suggests that 2-weeks administration period is sufficient to detect ovarian toxicity of MPA in the general toxicity study.

Reference: Ohtake *et al.* (2009)

Mifepristone

Profiles of chemical: A progesterone receptor antagonist,

Company: Kissei Pharmaceutical Co., Ltd.

Studies: 2 or 4 weeks general toxicity and the fertility studies.

Animals: Crl:CD(SD) rats

Doses, route and duration: Treatment by gavage at 0.8, 4, 20 and 100 mg/kg for 2- or 4- weeks in the repeated dose toxicity studies and at 0.8, 4 and 20 mg/kg from 2 weeks before copulation to postcoital day 7 for the female fertility study

Results: In the general toxicity studies, persistent estrus

Table 2. Summary of collaborative study -Female fertility study-

Category	Chemical	Dose levels	Route	Animal strain	Results	Evaluation	Company and reference
Hormone analogues	Medroxyprogesterone acetate (Progesterone)	0.4, 2.0 and 10 mg/kg/day	PO	Crl:CD (SD)	Irregular estrous cycle (prolonged) at 0.4 mg/kg and higher. Decreased fertility index at 2.0 mg/kg and higher. Increased body weight during pre-mating period at 10 mg/kg.	Reproductive toxicity was detected.	ASKA Pharmaceutical Co., Ltd. Ohtake *et al.* (2009)
	Mifepristone (Progesterone receptor antagonist)	0.8, 4 and 20 mg/kg/day	PO	Crl:CD (SD)	Irregular estrous cycle (persistent estrus) and decreased fertility index at 20 mg/kg. Shorter copulatory interval at 20 mg/kg. Increased post-implantation loss and decreased live embryo at 4 mg/kg.	Reproductive toxicity was detected.	Kissei Pharmaceutical Co., Ltd. Tamura *et al.* (2009)
	Tamoxifen (Estrogen receptor antagonist)	0.005, 0.03 and 0.2 mg/kg/day	PO	Wistar Hanover Crl:WI (HAN)	Irregular estrous cycle (anestrus) and increased pre-implantation loss at 0.005 mg/kg and higher. Decreased fertility index at 0.03 mg/kg and higher. Decreased live embryo at 0.005 mg/kg and higher. Decreased body weight gain during pre-mating period at 0.03 mg/kg and higher.	Reproductive toxicity was detected.	Banyu Pharmaceutical Co., Ltd. Tsujioka *et al.* (2009)
Primordial follicle damaging agents	4-vinylcyclohexene diepoxide (Group 2B carcinogen)	5, 20 and 80 mg/kg/day	IP	Crl:CD (SD)	Increased pre-implantation loss at 80 mg/kg. Decreased body weight during pregnancy period at 80 mg/kg.	Reproductive toxicity was detected.	Toa Eiyo Ltd., Ajinomoto Co., Inc. Ito *et al.* (2009)
	Busulfan (Antineoplastic Alkylating agent)	0.1, 0.5 and 1.5 mg/kg/day 2.5 mg/kg/day	PO IP	Crl:CD (SD)	Increased pre- and post-implantation loss at 1.5 mg/kg and higher. Decreased live embryo and increased corpora lutea and dead embryo at 1.5 mg/kg and higher. Decreased body weight gain during pregnancy period at 1.5 mg/kg. Decreased food consumption during pre-mating and pregnancy period at 2.5 mg/kg.	Reproductive toxicity was detected.	Kyorin Pharmaceutical Co., Ltd. Sakurada *et al.* (2009)
	Cisplatin (Antineoplastic)	0.25, 0.5 and 1.0 mg/kg/day	IP	Crl:CD (SD)	Increased pre-implantation loss at 1.0 mg/kg. Increased post-implantation loss, decreased live embryo and increased dead embryo at 0.5 mg/kg and higher. Decreased body weight during pre-mating at 1.0 mg/kg and pregnancy period at 0.5 mg/kg and higher.	Reproductive toxicity was detected.	Toyama Chemical Co., Ltd. Nozaki *et al.* (2009)
	Cyclophosphamide (Antineoplastic Alkylating agent)	5, 10 and 20 mg/kg/day	PO	Crl:CD (SD)	Increased pre- and post-implantation loss at 5 mg/kg and higher. Decreased live embryo and increased dead embryo at 10 mg/kg and higher. Decreased body weight and food consumption during pre-mating and pregnancy period at 10 mg/kg and higher.	Reproductive toxicity was detected.	Otsuka Co., Ltd.

Table 2. (Continued)

Category	Chemical	Dose levels	Route	Animal strain	Results	Evaluation	Company and reference
Metabolism imbalance inducers	Anastrozole (Antineoplastic, Aromatase inhibitor)	0.01, 0.1 and 5 mg/kg/day	PO	Crl:CD (SD)	Irregular estrous cycle (prolonged) and increased pre-implantation loss at 0.1 mg/kg and higher. Decreased fertility index at 5 mg/kg. Decreased live embryo at 0.1 mg/kg and higher, and decreased corpora lutea at 5 mg/kg. Increased body weight during pre-mating period at 5 mg/kg.	Reproductive toxicity was detected.	Daiichi-Sankyo Co., Ltd. Shirai *et al.* (2009)
	Di(2-ethylhexyl) adipate (DEHA)	200, 1,000 and 2,000 mg/kg/day	PO	Crl:CD (SD)	Irregular estrous cycle (prolonged) and increased pre-implantation at 2,000 mg/kg and post-implantation loss at 1,000 mg/kg and higher. Decreased live embryo and decreased body weight at 2,000 mg/kg. Staining around perineum at 1,000 mg/kg and higher.	Reproductive toxicity was detected.	Kowa Co., Ltd. Wato *et al.* (2009)
	Di(2-ethylhexyl) phthalate (DEHP)	300, 1,000 and 3,000 mg/kg/day	PO	Crl:CD (SD)	Irregular estrous cycle (prolonged) and decreased fertility index at 3,000 mg/kg. Decreased body weight during pre-mating period at 3,000 mg/kg and pregnancy period at 1,000 mg/kg and higher. Enlargement of liver at 300 mg/kg and higher. Loose feces, mucous feces and salivation at 1,000 mg/kg and higher.	Reproductive toxicity was detected.	Chugai Pharmaceutical Co., Ltd. Takai *et al.* (2009)
	Ethylene Glycol Monomethyl Ether (EGME)	30, 100 and 300 mg/kg/day	PO	Crl:CD (SD)	Irregular estrous cycle (prolonged) at 30 mg/kg and higher. Decreased fertility index at 300 mg/kg. Decreased corpora lutea, implantation and live embryo and increased post-implantation loss at 100 mg/kg and higher. Decreased body weight and food consumption during pre-mating and/or pregnancy period at 300 mg/kg.	Reproductive toxicity was detected.	Eisai Co., Ltd. Dodo *et al.* (2009)
	Indomethacin (COX-1,2 inhibitor, NSAID)	0.4, 1.3 and 4.0 mg/kg/day	PO	Crl:CD (SD)	Death of dams (8/10) at 4.0 mg/kg. Reproductive toxicity could not be evaluated at 4.0 mg/kg because of death of dams. Decreased body weight and food consumption during pre-mating period at 4.0 mg/kg. No adverse effect at 0.4 and 1.3 mg/kg.	Reproductive toxicity was not detected enough.	Astellas Pharma Inc. Tsubota *et al.* (2009)
	Compound X (PPAR α/γ dual agonist)	4, 20 and 100 mg/kg/day	PO	Crl:CD (SD)	Decreased fertility index at 100 mg/kg. Decreased corpora lutea, implantations and live embryo at 20 mg/kg and higher. Increased body weight at 20 mg/kg and higher. (estrous cycle was not evaluated)	Reproductive toxicity was detected.	Asahi Kasei Pharma Corporation Sato N. *et al.* (2009)

Table 2. (Continued)

Category	Chemical	Dose levels	Route	Animal strain	Results	Evaluation	Company and reference
Endocrine imbalance inducers	Atrazine (Herbicide, Endocrine modulator)	3, 30 and 100 mg/kg/day	PO	Slc:SD	Irregular estrous cycle (prolonged) at 30 mg/kg and higher. Salivation at 30 mg/kg and higher.	Reproductive toxicity was detected.	Nippon Shinyaku Co., Ltd. Shibayama *et al.* (2009)
	Bromocriptine (Dopamine agonist)	0.08, 0.4 and 2 mg/kg/day	SC	Crl:CD (SD)	Decreased fertility index at 0.4 mg/kg and higher. Effects on the next generation in 0.4 and 2 mg/kg could not be evaluated.	Reproductive toxicity was detected.	Sanwa Kagaku Kenkyusho Co., Ltd. Kumazawa *et al.* (2009)
	Chlorpromazine hydrochloride (D2, 5-HT2A H1, α1-antagonist)	3, 10 and 30 mg/kg/day	PO	Crl:CD (SD)	Irregular estrous cycle (prolonged) at 10 mg/kg and higher. Longer copulatory interval at 30 mg/kg. Decreased fertility index at 30 mg/kg.	Reproductive toxicity was detected.	Takeda Pharmaceutical Co., Ltd. Izumi *et al.* (2009)
	Sulpiride (D2 Antagonist)	1, 10 and 100 mg/kg/day	PO	Crl:CD (SD)	Irregular estrous cycle (prolonged) at 10 mg/kg and higher. Increased corpora lutea at 100 mg/kg. Increased post-implantation loss and dead embryo at 10 mg/kg and higher. Increased body weight and food consumption during pre-mating period at 10 mg/kg and higher.	Reproductive toxicity was detected.	Mitsubishi Tanabe Pharma Corporation Ishii *et al.* (2009)

PO, SC and IP mean treatment routes by gavage, subcutaneous and intraperitoneal treatments, respectively.

as seen in the vaginal smears, multiple cysts in the ovaries at necropsy, increases in luteinized cysts and enlargement of previous formed corpora lutea were observed in histopathological examination of the ovaries in rats receiving 20 mg/kg or more for 2 or 4 weeks. In the female fertility studies, persistent vaginal cornification was also observed at 20 mg/kg and the precoital interval was significantly shortened. All of the animals were completely infertile when dosed with 20 mg/kg during the post-coital period. An increase in pre-implantation losses was observed in the animals treated with 20 mg/kg during the pre-coital phase, while treatment with 4 mg/kg mifepristone during the post-coital phase induced an increase in post-implantation losses.

Conclusion: A 2-week administration period would be sufficient to detect the ovarian toxicity of mifepristone in the repeated-dose toxicity study and the pathological findings in the ovaries would reflect the alterations in the female reproductive endpoints in the female fertility study.

Reference: Tamura *et al.* (2009)

Tamoxifen

Profiles of chemical: Selective estrogen receptor modulator (SERM)

Company: Banyu Pharmaceutical Co., Ltd.

Studies: Repeated toxicity and fertility studies

Animals: Hanover Crl:WI(HAN) rats

Doses, route and duration: Treatment by gavage at 0.005, 0.03, or 0.2 mg/kg/day for 2- or 4- weeks g/kg/day in the repeated toxicity study, and the same doses from prior to mating to Day 7 of gestation in the fertility study.

Results: In the general toxicity and fertility studies, findings at 0.03 and 0.2 mg/kg/day included decreases in body weight gains associated with decreases in the food consumption, in 2- and 4-week repeated dose toxicity studies and fertility study. In the repeated toxicity study, the ovarian histopathological findings included increases in the large atretic follicles, increases in the interstitial cells and absence of newly formed corpus lutea at 0.2 mg/kg/day in the 2-week study and at 0.03 and 0.2 mg/kg/day in the 4-week study. The treatment induced estrogenic and antiestrogenic reactions in the uterus, while mucinous degeneration was detected in the vagina. In female fertility study the effects consisted primary of disturbance of estrus cycle and decreases in numbers of pregnant rats which were considered to be related the ovarian histopathological changes.

Conclusion: Based on these findings, the ovarian histopathological evaluation in the repeated toxicity study could anticipate the effects of tamoxifen on female fertility via ovarian dysfunction at slightly toxic doses, and 2-week treatment of tamoxifen at appropriate dose could be sufficient to detect ovarian toxicity by microscopic examination.

Reference: Tsujioka *et al.* (2009)

Primordial follicle damaging agents

4-vinylcyclohexene diepoxide (VCD) -1

Profiles of chemical: Acceleration of the natural process of apoptosis in small follicles

Company: Toa Eiyo Ltd.

Studies: Repeated toxicity study

Animals: Sprague-Dawley rats (Slc:SD) rats

Doses, route and duration: Intraperitoneally treatment at 5, 20 and 80 mg/kg once a day for 2- or 4- weeks in the repeated toxicity study.

Results: In the 2 weeks study, a decrease of small follicles was detected at 80 mg/kg. In the 4 weeks study, the incidence of the decrease was increased at 20 and 80 mg/kg in histopathological examination with dose-dependent manner.

Conclusion: A 2-week administration period was sufficient to detect the ovarian toxicity of VCD in histopathological examination of repeated-dose toxicity study at appropriate dose.

Reference: Ito *et al.* (2009)

4-vinylcyclohexene diepoxide (VCD) -2

Profiles of chemical: Acceleration of the natural process of apoptosis in small follicles

Company: Ajinomoto Co., Inc.

Studies: Fertility study

Animals: Sprague-Dawley rats (Slc:SD) rats

Doses, route and duration: Intraperitoneal treatment at 5, 20 and 80 mg/kg once a day from prior to mating to Day 7 of gestation in the fertility study.

Results: The number and the rate of implantation decreased and the rate of preimplantation loss showed increasing tendency in the 80 mg/kg group. As for the organ weights, decreases in absolute and relative ovary weights were observed in the 80 mg/kg group. In histopathological examination of the ovaries, decrease in the number of small follicles was observed at 80 mg/kg.

Conclusion: Decrease in the number of small follicles was observed at 80 mg/kg in the fertility study.

Reference: Kodama *et al.* (2009)

Busulfan

Profiles of chemical: Antineoplastic alkylating agents,

Company: Kyorin Pharmaceutical Co., Ltd.

Animals: Crl: CD(SD) rats

Doses, route and duration: Treatment by gavage at 0.1, 0.5, or 1.5 mg/kg/day for 2- or 4- weeks in the repeated toxicity study and the same doses from 2 weeks before mating to day 7 of pregnancy in the fertile study.

Results: In the 2-week general toxicity study, all rats treated with Busulfan showed normal estrous cyclicity and no toxicological changes in the weight and histopathology of the ovaries. In the 4-week study, a decrease in small follicles was observed histopathologically in 1 rat even at 0.5 mg/kg and in 4 rats at 1.5 mg/kg. PCNA immunohistochemistry of the follicles confirmed the above decrease in the number of small follicles at 1.5 mg/kg. In the female fertility study, increases in dead embryos and post-implantation loss were found in rats at 1.5 mg/kg.

Conclusion: The present study indicates that a 4-week administration period and appropriate assessment, including careful histopathology, of stage-based follicles are needed to detect small follicle depletion in general toxicity study as the first-tier screening.

Reference: Sakurada *et al.* (2009)

Cisplatin

Profiles of chemical: A platinum-based chemotherapy drug clinically used as an antitumor agent

Company: Toyama Chemical Co., Ltd.

Studies: Repeated toxicity and fertility studies

Animals: Crl:CD(SD) rats

Doses, route and duration: Daily treatment intraperitoneally at dose levels of 0.25, 0.5, 1.0 and 2.0 mg/kg for 2 weeks, or those of 0.125, 0.25 and 0.5 mg/kg for 4 weeks in the repeated toxicity study, and at 0.25, 0.5 and 1.0 mg/kg from 14 days prior to mating to Day 7 of gestation in the female fertility study.

Results: In the general toxicity study, a decrease in large follicle, an increase in atresia of medium and large follicles, and/or a decrease in currently formed corpus luteum were observed in animals receiving 1.0 and 2.0 mg/kg for 2 weeks, and decreases in small and/or large follicles and an increase in atresia of large follicle were observed in animals receiving 0.25 and 0.5 mg/kg for 4 weeks on the histopathological examination of the ovaries. In the female fertility study, copulation and fertility indices in animals receiving 1.0 mg/kg tended to be lower than that in the control animals.

Conclusion: Histopathological changes attributable to cisplatin dosing were detected by detailed observation of the ovary in the 2-week study, Therefore, it is concluded that a 2-week administration period is sufficient for evaluation of ovarian toxicity of cisplatin.

Reference: Nozaki *et al.* (2009)

Cyclophosphamide

Profiles of chemical: An alkylating agent used in combination cancer chemotherapy regimens

Company: Otsuka Pharmaceutical Co., Ltd.

Studies: Repeated toxicity and fertility studies

Animals: Crl:CD(SD) rats

Doses, route and duration: Treatment orally to female rats at dose levels of 0, 5, 10 and 20 mg/kg for the repeated-dose toxicity study for 2- or 4- week, and those of 0, 5, 10, and 20 mg/kg for the female fertility study from 2 weeks prior to mating to Day 7 of pregnancy.

Results: In the repeated-dose toxicity study, increases in large-sized atretic follicles as well as atrophy of corpora lutea were observed in the 20 mg/kg group of 4 weeks study by the histopathological examination of the ovaries. There were no drug-related changes in the ovary of 2 weeks study. In the female fertility study, the number of implantation was slightly decreased and the corpora lutea of pregnancy were not observed in 20 mg/kg group. The dose-dependent increase in incidence of post-implantation loss was observed, and no abnormalities were observed in the estrus cycle and mating in the all treated group.

Conclusion: The histopathological changes in the ovary are important endpoints for evaluation of drugs inducing ovarian damage as well as caesarean section. A 4-week administration period is sufficient to detect the ovarian toxicity of CP in the repeated dose toxicity study.

Reference: Sato M. *et al.* (2009)

Metabolite imbalance inducers

Anastrozole

Profiles of chemical: Aromatase inhibitor

Company: Daiichi-Sankyo Co., Ltd.

Studies: Repeated toxicity and fertility studies

Animals: CrjDu:F344 female rats for the 2- and 4-week repeated toxicity studies and Crl:CD(SD) rats for the female fertility study.

Doses, route and duration: Gavage treatment at 0.01, 0.1, 1 and 50 mg/kg for 2 or 4 weeks in the repeated toxicity study and at levels of 0, 0.01, 0.1, and 5 mg/kg from 2 weeks prior to mating to Day 7 of pregnancy for the female fertility study.

Results: In the general toxicity study, large abnormal atretic follicles, follicular cysts, a decrease in the corpus luteum and depletion of currently formed corpus luteum were observed in the 1 and/or 50 mg/kg groups of both the 2-week and 4-week studies in a histopathological examination of the ovaries. In the female fertility study,

the copulation rate and pregnancy rate were decreased in the 5 mg/kg group. Irregular estrous cycles, such as an extended estrous cycle or no estrous cycle, were also observed in the 0.1 and 5 mg/kg groups. At necropsy, decreased numbers of implantations, corpora lutea and live fetuses were noted in the 0.1 and/or 5 mg/kg groups.

Conclusion: Histopathological changes in the ovary are important endpoints for the evaluation of drugs inducing ovarian damage. A 2-week administration period was sufficient to detect the ovarian toxicity of anastrozole in a repeated-dose toxicity study.

Reference: Shirai *et al.* (2009)

di(2-Ethylhexyl)adipate (DEHA)

Profiles of chemical: Peroxisome proliferator-activated receptors (PPARs)

Company: Kowa Company, Ltd.

Studies: 2 or 4 weeks general toxicity and fertility studies.

Animals: Crl:CD(SD) rats

Doses, route and duration: Treatment by gavage at 200, 1,000 and 2,000 mg/kg for 2- or 4-weeks in the repeated toxicity study and the same doses from 2 weeks before mating until Gestation Days 7 in the female fertility study.

Results: In the general toxicity studies, increase in atresia of large follicle, decrease in currently formed corpus luteum and follicular cyst were observed in the 1,000 mg/kg and above groups. In the fertility study, a significant increase in mean estrus cycle length and post-implantation loss rate were observed in the 1,000 mg/kg and above groups, and a significant decrease in implantation rate and number of live embryos and a significant increase in pre-implantation loss rate were observed in the 2,000 mg/kg group.

Conclusion: The histopathological changes of ovary observed in the repeated-dose toxicity studies were correlated with the result that DEHA affected the estrus cycle in the female fertility study. A 2-week administration period is sufficient for detection of the ovarian toxicities following treatment with DEHA by new histopathological examination of the ovaries.

Reference: Wato *et al.* (2009)

di-(2-Ethylhexyl) phthalate (DEHP)

Profiles of chemical: Peroxisome proliferator-activated receptors (PPARs)

Company: Chugai Pharmaceutical Co., Ltd.

Studies: 2 or 4 weeks general toxicity and the fertility studies.

Animals: Crl:CD(SD)

Doses, route and duration: Treatment by gavage at 300, 1,000 and 3,000 mg/kg in 2- or 4-week in the repeated toxicity study and the same doses from 2 weeks prior to mating to Day 7 of pregnancy in the fertility study.

Results: Histopathology of the ovaries in both repeated-dose toxicity studies showed vacuolation of stromal cells in the groups receiving 300 mg/kg or more and an increased of large atretic follicle in groups at 1,000 mg/kg or more. In the 4-week study, a decrease in currently formed corpora lutea was observed in the 3,000 mg/kg group. In the female fertility study, the 3,000 mg/kg group showed prolongation of the mean estrous cycle and irregular estrous cycles. Cesarean section revealed a decrease of pregnancy rate in the 3,000 mg/kg group. No effects on fertility or early embryonic development were found in groups at 1,000 mg/kg or less. Conclusion: Histopathological changes in the ovary are important endpoints for evaluation of drugs which induce ovarian damage. In conclusion, for a repeated-dose toxicity study, a 2-week administration period is sufficient to detect ovarian toxicity caused by DEHP.

Reference: Takai *et al.* (2009)

Ethylene glycol monomethyl ether (EGME)

Profiles of chemical: Direct stimulators for luteal progesterone secretion

Company: Eisai Co., Ltd.

Studies: 2 or 4 weeks general toxicity and the fertility studies.

Animals: Crl:CD(SD) rats

Doses, route and duration: Treatment by gavage at 30, 100 or 300 mg/kg for 2- or 4-week in the repeated toxicity study, and the same doses from 2 weeks prior to mating until Day 6 of pregnancy for the fertility study.

Results: In the general toxicity studies, continuous diestrus was seen at 100 mg/kg and higher regardless of the administration periods. The ovarian morphological changes were observed at 100 mg/kg and higher after 2 or 4 weeks of administration characterized by hypertrophy of corpora lutea with decreased cellular debris, which indicated apoptosis, and increased imunohistochemically PCNA negative large atretic follicles. The newly-formed basophilic corpora lutea was scarcely found. In the fertility study, irregular estrous cycles, prolonged mating periods, lower pregnancy rates, and decreased corpora lutea of pregnancy were seen at 100 mg/kg and higher. Irregular estrous cycles observed in some animals at 30 mg/kg were minimal.

Conclusion: The ovarian histopathological changes in the general toxicity studies were well correlated with impaired female fertility found in the fertility study. A

general toxicity study with a treatment period for 2 weeks or more is plausible for evaluation of ovarian toxicity induced by EGME.

Reference: Dodo *et al.* (2009)

Indomethacin

Profiles of chemical: Nonselective inhibitor of cyclooxygenase (COX)-1 and -2

Company: Astellas Pharma Inc.

Studies: 2 or 4 weeks general toxicity and the fertility studies.

Animals: Crl:CD(SD) rats

Doses, route and duration: Treatment by gavage at 0.4, 1.3, or 4 mg/kg for 2- or 4-week in the general toxicity study, and the same doses from 2 weeks before mating until Gestation Days 7 in female fertility study.

Results: In the general toxicity studies, unruptured follicles or luteinized cysts were observed histopathologically in the 4 mg/kg group in both the 2-week and 4-week studies. Follicular cysts were found in the 4 mg/kg group in the 4-week study. Estrous cyclicity was not disturbed in both studies. In the female fertility study, no toxic effects on female fertility parameters were detected in the 0.4 and 1.3 mg/kg group treated with indomethacin, but 8 of 10 rats in the 4 mg/kg group died or were sacrificed before completion of the dosing period.

Conclusion: Two weeks of indomethacin treatment is sufficient to detect the unruptured luteinized follicles or luteinized cyst in the ovary. In addition, 4 weeks of dosing may be required for induction of follicular cysts, although we could not clearly show that these histopathological changes would affect female fertility functions. These present studies suggest that a precise histopathological examination may be able to predict the effect of test articles on female reproductive functions.

Reference: Tsubota *et al.* (2009)

Compound X

Profiles of chemical: Peroxisome proliferator-activated receptor (PPAR) α/γ dual agonist.

Company: Asahi Kasei Pharma Corporation

Studies: 2 or 4 weeks general toxicity and the fertility studies.

Animals: Crl:CD(SD) rats

Doses, route and duration: Treatment by gavage at 4, 20, and 100 mg/kg/day for 2- or 4- week in the repeated toxicity study and the same doses from 2 weeks prior to mating to Day 7 of pregnancy in the fertility study.

Results: In the general toxicity study, an increase in atresia of large follicles, a decrease in corpora lutea, granulosa cell exfoliation into antram of large follicles, corpora lutera with retained oocyte, and interstitial gland hyperplasia were observed in all of the treated groups in both the 2- and 4-week studies on histopathological examination of the ovaries. In the female fertility study, the pregnancy rate tended to decrease in the 100 mg/kg/day group. At necropsy, decreases in the number of corpora lutea, the number of implantations, and the number of live embryos were noted in the 20 and 100 mg/kg/day group. No changes were observed in animals given 4 mg/kg/day.

Conclusion: These findings indicated that histopathological changes in the ovary are important endpoints for evaluation of drugs inducing ovarian damage. A 2-week administration period is sufficient to detect ovarian toxicity of this test compound in the general toxicity study.

Reference: Sato N. *et al.* (2009)

Endocrine imbalance inducers

Atrazine

Profiles of chemical: A potent herbicide with endocrine-disrupting activity

Company: Nippon Shinyaku Co., Ltd.

Studies: 2 or 4 weeks general toxicity and the fertility studies.

Animals: Crl:CD(SD) rats

Doses, route and duration: Treatment by gavage at 3, 30 or 300 mg/kg for 2- or 4-week in the repeated dose toxicity study, and at doses of 3, 30 or 100 mg/kg from two weeks before mating to Day 7 of gestation in the female fertility study.

Results: In the two-week repeated-dose toxicity study, prolongation of diestrus and histopathological findings such as loss of the currently formed corpora lutea, decrease in the numbers of previously formed corpora lutea, increase in large-sized atretic follicles, and swelling of the previously formed luteal cells were observed in the 300 mg/kg group, suggesting that atrazine had an anovulatory effect through suppression of the luteinizing hormone surge. In the female fertility study, copulation failure caused by prolongation of diestrus was observed in one animal in the 100 mg/kg group, which could be due to the anovulatory effect of atrazine.

Conclusion: The effect of atrazine on female fertility can be assessed by detailed histopathological examination of ovaries in a two-week repeated-dose toxicity study, provided the appropriate dose levels are selected.

Reference: Shibayama *et al.* (2009)

Bromocriptine

Profiles of chemical: Blockade of prolactin surges

Company: Sanwa Kagaku Kenkyusho Co., Ltd.

Studies: 2 or 4 weeks general toxicity and the fertility studies.

Animals: Crl:CD(SD)

Doses, route and duration: Treatment subcutaneously at 0.08, 0.4 and 2 mg/kg for the 2- or 4- week general toxicity study and the same doses from 2 weeks prior to mating to day 7 of gestation in the female fertility study.

Results: Increases of ovarian weights were observed at 2 mg/kg and 0.4 mg/kg and higher in the 2-week and 4-week general studies, respectively. The number of corpora luteum was increased in the 0.4 mg/kg and higher in the 2- and 4-week general toxicity studies by the histopathological examination of the ovaries. bromocriptine did not affect estrous cyclicity in both general toxicity studies. In the female fertility study, although animals in any groups mated successfully, no females in 0.4 and 2 mg/kg groups were pregnant. There were no adverse effects on the reproductive performance in the 0.08 mg/kg group.

Conclusion: The histopathological changes in the ovary are considered important parameters for evaluation of drugs including ovarian damage. A 2-week administration period is sufficient to detect the ovarian toxicity of bromocriptine in a general toxicity study.

Reference: Kumazawa *et al.* (2009)

Chlorpromazine hydrochloride (CPZ)

Profiles of chemical: A phenothiazine psychoactive drug with an α1-adrenoceptor and dopamine D2 receptor antagonistic activity

Company: Takeda Pharmaceutical Co., Ltd.

Studies: 2- or 4-week general toxicity and the fertility studies.

Animals: Crl:CD(SD) rats

Doses, route and duration: Treatment by gavage at dosage levels of 3, 10 and 30 mg/kg/day for 2 and 4 weeks in the repeated-dose toxicity study and the same doses from two weeks before mating to Day 7 of gestation in the female fertility study.

Results: In the general toxicity study, ovarian weights were decreased at 10 mg/kg and higher in the 4-week study and an increase in large atretic follicles was observed histopathologically at 10 mg/kg and higher in the 2 and 4 weeks studies. In addition, decreased uterine weights and/or atrophic findings in the uterus and vagina at 30 mg/kg and 10 mg/kg and higher, mucification in the vaginal epithelium and alveolar hyperplasia in the mammary gland at 3 mg/kg and higher, and 10 mg/kg and higher were seen in the 2 and 4 weeks studies, respectively. Irregular estrous cycles were seen at 3 mg/kg and higher, and 10 mg/kg and higher in the 2 and 4 weeks studies. The fertility study with dosing from 2 weeks before mating to Day 6 of gestation showed irregular estrous cycles at 10 mg/kg and higher and prolonged copulatory intervals and a reduced fertility index at 30 mg/kg.

Conclusion: Oral CPZ treatment induced ovarian toxicity with 2 weeks or longer treatment and changed the fertility parameters and therefore it was concluded that a 2 weeks administration period is adequate to detect the ovarian toxicity of CPZ.

Reference: Izumi *et al.* (2009)

Sulpiride

Profiles of chemical: A D2 antagonist

Company: Mitsubishi Tanabe Pharma Corporation

Studies: 2 or 4 weeks general toxicity and the fertility studies.

Animals: Crl:CD(SD)

Doses, route and duration: Treatment by gavage at 1, 10, and 100 mg/kg/day for 2- or 4-week in a general toxicity study, and the same doses from the pre-mating period until Days 0-7 of gestation in the fertility study.

Results: In the ovarian histology in the 2-week study, increases in atretic follicle were seen at 1 mg/kg or more and increases in follicular cysts at 10 mg/kg or more. In the 4-week study, these findings were seen at 1 mg/kg or more, and a decrease in large follicles was seen at 10 mg/kg or more. Increased body weight gain was observed at 10 mg/kg or more in the 2- and 4-week studies. The females in these groups exhibited the development of mammary alveolus. In the fertility study, sulpiride-treated females showing persistent diestrus resulted in successful mating, and almost females got pregnant. However, increased implantation loss was observed at 10 mg/kg or more, which was considered to be caused by the adverse effect of sulpiride on oocyte development.

Conclusion: Sulpiride-induced ovarian toxicity was seen at 1 mg/kg or more in the 2- and 4-week repeated-dose toxicity studies, and the observed ovarian changes were considered to be related to adverse effects on female fertility.

Reference: Ishi *et al.* (2009)

Comparison of the results among the fertility, and repeated toxicity studies

The comparison of endpoints of ovarian toxicities and their lowest toxicity doses among the fertility, 2-week or 4-week toxicity studies is shown in Table 3. All compounds induced ovarian toxicity, and they were detectable in both the general toxicity and fertility studies. In the general toxicity studies, only 2 compounds, busulfan and cyclophosphamide, had no noteworthy findings

Table 3. Comparison of NOAEL and ovarian toxicities observed in female fertility and general toxicity studies.

Chemicals		2-Week general toxicity study	4-Week general toxicity study	Reproductive study
Medroxyprogesterone acetate	Lowest doses for ovarian toxicity	2.0 mg/kg	2.0 mg/kg	0.4 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase in atretic large follicles/ decreased currently/previously corposa lutea	Increase in atretic large follicles/ decreased currently/previously corposa lutea	Prolonged mean estrous cycle before mating
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Mifepristone	Lowest doses for ovarian toxicity	20 mg/kg	20 mg/kg	4 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase in luteinized cyst, Increase in atresia of large follicle	Increase in luteinized cyst, Increase in atresia of large follicle	Persistent estrus, Shorter pre-coital interval, Completely infertile animals
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Tamoxifen	Lowest doses for ovarian toxicity	0.2 mg/kg	0.03 mg/kg	0.005 mg/kg
	Endpoints of ovarian or reproductive toxicity	Hyperplasia of the interstitial cell, Increase in atretic follicle, large sized, Absence of currently corpus luteum	Hyperplasia of the interstitial cell, Increase in atretic follicle, large sized, Absence of currently corpus luteum	Increase in anestrus females, preimplantation loss
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
4-Vinylcyclohexene diepoxide	Lowest doses for ovarian toxicity	80 mg/kg	20 mg/kg	80 mg/kg
	Endpoints of ovarian or reproductive toxicity	Decrease in small follicle	Decrease in small follicle	Increase in preimplantation loss
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Busulfan	Lowest doses for ovarian toxicity	-	1.5 mg/kg	1.5 mg/kg
	Endpoints of ovarian or reproductive toxicity	No noteworthy findings	Decrease of small follicles	Increase in preimplantation loss
	Ovarian toxicity or reprotoxicity was detected?	No	Yes	Yes
Cisplatin	Lowest doses for ovarian toxicity	1.0 mg/kg	0.25 mg/kg	0.5 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase in atresia of medium and large follicle, Decrease in large follicle and newly formed corpus luteum	Increase in atresia of medium and large follicle, Decrease in large follicle and newly formed corpus luteum	Increase in preimplantation loss
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes

Table 3. (Continued)

Chemicals		2-Week general toxicity study	4-Week general toxicity study	Reproductive study
Cyclophosphamide	Lowest doses for ovarian toxicity	-	20 mg/kg	5 mg/kg
	Endpoints of ovarian or reproductive toxicity	Not noteworthy findings	Atrophy of corpus luteum	Increased preimplatation loss
	Ovarian toxicity or reprotoxicity was detected?	No	Yes	Yes
Anastrozole	Lowest doses for ovarian toxicity	50 mg/kg	1.0 mg/kg	0.1 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase in large sized atretic follicle, cystic follicle	Increase in large sized atretic follicle	Irregular estrous cycle, Increased preimplatation loss
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Di(2-ethylhexyl) adipate	Lowest doses for ovarian toxicity	1,000 mg/kg	1,000 mg/kg	1,000 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase in large sized atretic follicle	Increase in large sized atretic follicle.	Increase of estrous cycle and pre-implantation loss
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Di(2-ethylhexyl) phthalate	Lowest doses for ovarian toxicity	3,000 mg/kg	1,000 mg/kg	3,000 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase in large sized atretic follicles	Increase in large sized atretic follicles. Decrease in newly formed corpora lutea	Prolonged estrous cycle
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Ethylene glycol monomethyl ether	Lowest doses for ovarian toxicity	100 mg/kg	100 mg/kg	30 mg/kg
	Endpoints of ovarian or reproductive toxicity	Hypertrophy of corpora lutea, Increased large atretic follicles	Hypertrophy of corpora lutea, Increased large atretic follicles	Irregular estrous cycle
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Indomethacin	Lowest doses for ovarian toxicity	4 mg/kg	1.3 mg/kg	-
	Endpoints of ovarian or reproductive toxicity	Unruptrued follicle	Unruptrued follicle Increase in atretic follicle	Not evaluated. 4.0 mg/kg: Death 8/10 1.3 mg/kg: No change
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	-

Table 3. (Continued)

Chemicals		2-Week general toxicity study	4-Week general toxicity study	Reproductive study
Compound X	Lowest doses for ovarian toxicity	4 mg/kg	4 mg/kg	20 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase in atresia of large follicle	Increase in atresia of large follicle	Decrease in corpora lutea, implantations and live embryo
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Atrazine	Lowest doses for ovarian toxicity	300 mg/kg	300 mg/kg	30 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase in large atretic follicle, Loss of currently formed corpus luteum, Decrease in previously formed , Swelling of previously	Increase in large sized atretic follicle swelling of previous formed corpus luteum.	Increase of irregular estrous cycle
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Bromocriptine	Lowest doses for ovarian toxicity	0.4 mg/kg	0.4 mg/kg	0.4 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase in corpora lutea	Increase in absolute ovary weight, Increase in corpora lutea	Decrease in pregnant animals
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Chlorpromazine hydrochloride	Lowest doses for ovarian toxicity	10 mg/kg	10 mg/kg	10 mg/kg
	Endpoints of ovarian or reproductive toxicity	Increase of large sized atretic follicle	Increase of large sized atretic follicle	Irregular estrous cycle
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes
Sulpiride	Lowest doses for ovarian toxicity	1 mg/kg	1 mg/kg	10 mg/kg
	Endpoints of ovarian or reproductive toxicity	Follicular cyst, Increase of large sized atretic follicle	Follicular cyst, Increase of large sized atretic follicle	Irregular estrous cycle
	Ovarian toxicity or reprotoxicity was detected?	Yes	Yes	Yes

in the ovary in 2-week toxicity study. They are alkylating agents, and categorized into small follicle damaging chemicals.

Their lowest toxicity doses of 6 compounds in the general toxicity studies were lower than those in their fertility studies. Their lowest toxicity doses of 7 compounds in the fertility study were lower than those in their general toxicity studies. Their lowest toxicity doses of the remaining 4 compounds in the general toxicity studies were the same as those in fertility studies.

Histopathological terms in the ovary observed in the present validation study

The histopathological findings in the ovary observed in the present collaborative work are shown in Table 4. The most popular finding was an increase of large sized atretic follicles, and appeared in all categorized studies. Follicular cyst was observed in several categories. Decreases or depletion of corpus lutem formed in the current or previous estrous cycle was the next popular findings. Follicular cysts also wee found in 2 categorized areas. Decreases of small follicles were only observed in the compounds damaging small follicles. Histopathological changes related with non-ovulated follicles including unruptured follicle, unruptured luteinized follicle, luteinized cyst/luteal cyst, expansion of cumulus oophorus were observed in the compounds categorized into hormonal analogues and metabolite imbalance inducers.

DISCUSSION

This is a summary of the collaborative work organized by NIHS and JPMA to evaluate whether ovarian toxicities are detected by repeated-dose general toxicity studies in rats. Eighteen pharmaceutical companies that participated in the collaborative study and examined whether a 2-week testing period is adequate to detect ovarian toxicity. In this study, 2- or 4-week repeated-dose toxicity studies with ovarian histopathological examinations were conducted using 17 compounds which have been reported to be or were expected to be ovarian toxicants. These compounds were categorized into hormone analogues, primordial follicle damaging agents, metabolism imbalance inducers, and endocrine imbalance inducers. In order to compare the results with those of the ovarian histopathological findings using these compounds, a female fertility study was also conducted. In the present validation study, a common study design was applied, and careful histopathological examination of the female reproductive tract based on ovarian physiology (Yoshida *et al.*, 2009), PCNA immunohistochemical staining for detection of small follicles (Muskhelishvili *et al.*, 2005) and continuous observations of estrous cyclicity were added to the general toxicity study. The highest dosages of the general toxicity and female fertility studies were selected from the doses that demonstrated ovarian toxicity in published or in-house data. In principle, the same dosage was selected in both studies. Four weeks are considered to be the appropriate period to detect any ovarian toxicity in rats because approximately 27 days is accepted to be a period of follicular development from medium follicles (type 3b follicle classified by Pedersen and Peters (1968)) to large follicles, type 8 follicles classified by Pedersen and Peters (1968)) (Greenwald and Roy, 1994).

The present validation study demonstrated that all the compounds investigated induced ovarian toxicity in both general toxicity and fertility studies except lack of reprotoxicity of indomethacin. This result suggests that any of the parameters for the ovary in the general toxicity study might reflect female fertility toxicity. Although fifteen compounds affected the ovary in both 2- and 4-week treatment studies, 2 alkylating agents categorized as small follicle damaging chemicals, busulfan and cyclophosphamide, did not induce noteworthy changes in the ovary. However, ovarian toxicities were detected from these 2 compounds in the 4-week treatment. Their dose selections were considered to be appropriate for a 2-week study because typical general toxicities were observed with them. The 2-week treatment with the other 2 compounds in the same category, VCD and cisplatin, was sufficient to detect damage to small follicles using the present study design. Since both busulfan and cyclophosphamide are cytotoxic agents, other data such as the pharmacological action of the compounds investigated might be more informative for the prediction of ovarian toxicity.

A comparison of lowest toxicity doses in the ovary between the general toxicity and female fertility studies did not indicate any significance involving the compound category based on MOA category in the present validation study. The doses for the ovarian toxicity in the general toxicity studies were the same or lower than those in the female fertility study in 10 out of 17 chemicals, suggesting that a general toxicity study might be less sensitive for the detection of the female reproductive toxicity. If any chemical had effects on the ovary in the general toxicity study, the female fertility might be affected at lower levels.

The histopathological changes observed in the present validation study demonstrated useful indicators for the identification of ovarian toxicity. The most common findings were increases in large-sized atretic follicles and decreases in the corpus luteum. They indicate that the

Table 4. Histopathological terms used for the ovarian changes

Histopathological terms	Origin of components	References			
		Hormone analogues	Small follicle damaging agents	Metabolite imbalance inducers	Endocrine imbalance inducers
Decrease of small follicles	Small follicle		Hoyer and Divine (2001) Ito *et al.* (2009) Nozaki *et al.* (2009) Sakurada *et al.* (2009)		
Deacrease of large follicles	Large follicle		Nozaki *et al.* (2009)		
Increase of medium sized atretic follicle	Medium follicle		Nozaki *et al.* (2009)	Shirai *et al.* (2009)	Ishi *et al.* (2009)
Increase of large sized atretic follicle	Large follicle	Ohtake *et al.* (2009) Tsujioka *et al.* (2009) Tamura *et al.* (2009)	Nozaki *et al.* (2009) Sato M. *et al.* (2009)	Dodo *et al.* (2009) Ishi *et al.* (2009) Sato N. *et al.* (2009) Shirai *et al.* (2009) Takai *et al.* (2009) Tsubota *et al.* (2009) Wato *et al.* (2009)	Ishi *et al.* (2009) Izumi *et al.* (2009) Shibayama *et al.* (2009)
Follicular cyst	Large follicle			Shirai *et al.* (2009) Tsubota *et al.* (2009) Wato *et al.* (2009)	Ishii *et al.* (2009)
Unruptured follicle	Unruptured follicle	Tamura *et al.* (2009)			
Unruptured luteinized follicle	Unruptured follicle			Sato N. *et al.* (2009) * Shirota *et al.* (1998)	
Expantion of cumulus oophorus	Unruptured follicle			Davis *et al.* (1999) Tsubota *et al.* (2009)	
Exfoliation of granulosa cell	Granulosa cell			Sato N. *et al.* (2009)	
Absence/depletion of currently formed corpus luteum	Currently formed corpus luteum	Tsujioka *et al.* (2009)		Shirai *et al.* (2009)	
Decrease of currently formed corpus luteum	Currently formed corpus luteum		Nozaki *et al.* (2009)	Takai *et al.* (2009) Wato *et al.* (2009)	
Decrease of previously formed corpus luteum	Previously formed corpus luteum		Ohtake *et al.* (2009)		
Decrease of corpus luteum	Corpus luteum			Sato N. *et al.* (2009)	
Increase of corpus luteum	Corpus luteum				Kumazawa *et al.* (2009)

Table 4. (Continued)

Histopathological terms	Origin of components	References			
		Hormone analogues	Small follicle damaging agents	Metabolite imbalance inducers	Endocrine imbalance inducers
Hypertrophy of corpus luteum	Corpus luteum			Davis *et al.* (1997) Dodo *et al.* (2009)	
Swelling of previously formed corpus luteum	Previously formed corpus luteum				Shibayama *et al.* (2009)
Large size of previously formed corpus luteum	Previously formed corpus luteum	Tamura *et al.* (2009)			
Atrophy of corpus luteum	Corpus luteum		Sato M. *et al.* (2009)		
Corpus luteum with cavity	Corpus luteum				
Luteinized corpus luteum/ luteal cyst	Unruptured follicle	Tamura *et al.* (2009)		Tsubota *et al.* (2009)	
Increase of interstitial gland	Interstitial cells	Tsujioka *et al.* (2009)		Takai *et al.* (2009)	

* Expressing as granulosa cell exfoliation into antram of large follicles.

treatment resulted in a disturbance of ovulation and large follicle development, although MOA was different among the chemicals. The lasting disturbances will lead to atrophy of the ovary. Follicular cysts reflect an abnormal situation involving large sized follicular atresia. Decreases in the small follicles were only observed with the compounds that damaged small follicles, VCD, cisplatin and busulfan. This result indicates that a qualitative histopathological examination using single transverse sections might be suitable for screening of detecting damage to small follicles. Our results also support a position paper published by The Society of Toxicologic Pathology (STP) ovary evaluation working group on histopathological approaches for the assessment of rodent reproductive toxicity. This paper recommended qualitative evaluation of the ovary as the first-tier assessment (Regan *et al.*, 2005). In addition, our work also confirms that PCNA immunohistochemical staining is a very useful tool for the identification of small follicles (Muskhelishvili *et al.*, 2005). If effects on the small follicles are predicted in the qualitative analysis, a further approach including MOA should be performed. Our validation study also provided very informative evidence that the chemicals related to non-ovulated follicles induced common histopathological changes such as unruptured follicles, unruptured luteinized follicles, luteinized/luteal cysts, expansion of the cumulus oophorus, and cystic corpus lutea (Sato *et al.*, 2009; Tamura *et al*, 2009; Tsubota *et al.*, 2009). When the estrous cycle is normal and no degenerated oocytes are found, distinguishing the corpus luteum with a cavity from a cystic corpus luteum or a luteinized cyst after normal ovulation, which reflects incomplete luteinization, is very difficult. More information on these lesions is required.

In the fertility study, all the compounds except for indomethacin reflected alterations in the female fertility parameters, such as an irregular estrous cycle and increased pre-implantation loss. As for the irregular estrous cycle, it might be correlated with the disruption of ovulation, increases in atresia of the follicles and decreases in newly formed corpus lutea and so on. The hormonal alterations induced by the hormone analogues may directly affect the estrous cycle. In the metabolic imbalance inducers, prolongation of the estrous cycle and decrease in pregnancy rate were observed. The preimplantation loss described by the primordial follicle damaging agents VCD, cisplatin, busulfan and cyclophosphamide, was considered to be a consequence of decrease in the number of small follicles. In the case of endocrine imbalance inducers, bromocriptine induced a blockade of prolactin surges and subsequent reduction in progesterone secretion resulting in persistent of the corpus lutea and disturbance of implantation. Another endocrine imbalance inducer, chlorpromazine, suppressed ovulation and induced atresia of the follicles, resulting in a decrease in the implantation rate. According to these findings, the morphological changes observed in the ovaries in the repeated-dose toxicity studies could reflect alterations in the female reproductive parameters.

In conclusion, ovarian toxicity could be detected by careful histopatholgical examination. A 2-week dosing period may be sufficient for the evaluation of ovarian toxicity, except for cytotoxic compounds such as alkylating agents. The pathological findings of ovarian toxicity reflected the female fertility parameters.

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