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OECD guidelines and validated methods for *in vivo* testing of reproductive toxicity

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INTRODUCTION

p0010 The OECD Guidelines for Testing of Chemicals are a package of protocols and methods aimed at producing a toxicity test sufficiently well designed to enable it to be carried out in a similar manner in different countries and to produce results that will be fully acceptable to different regulatory bodies. Guidelines in this chapter are described for educational purposes only. Therefore, the official original and updated guidelines must be followed if intended for performing assays for regulatory purposes. The reproductive toxicity guidelines characterize the adverse effects against the reproductive system attributable to the exposure to chemicals for two purposes: (1) hazard characterization and (2) risk characterization. The reproduction/ developmental studies, included in the OECD Guidelines, are relevant in the risk characterization of chemicals. The endpoints evaluated in these protocols and the no-observed-adverse-effect levels (NOAELs) obtained are taken into consideration not only for the reproductive toxicity testing but also for the risk assessment of general systemic effects. For example, the NOAEL of systemic maternal toxicity in the teratogenicity study is frequently considered for risk characterization in short-term scenarios.

p0015 The results from these methods are recorded for the evaluation of multiple endpoints. Some of them describe the effects on the developmental process. The reproductive toxicity tests are relevant in the classification of substances. In the European Union, the substances classified as CMR (carcinogenic, mutagenic, reprotoxic) have strong restrictions in their use independently of the potential risk. The NOAEL obtained from the teratogenicity study is used in the classification and labeling of the substances. Also, the data of effects in the reproductive organs or systems obtained from studies of subchronic repeated dose are considered in the decisions for classification and labeling.

p0020 In general, the severity of effects on endpoints relevant to development or fertility should be evaluated relative to the systemic general toxicity effects. The teratogenicity studies relevant to humans are not interpretable for secondary effects of maternal toxicity just because they are observed at the same dose. However, in the fertility studies, the data are more relevant if there is no effect to parental toxicity (either the male or females in the F_0). If the development or fertility impairment is observed at the same dose causing significant systemic parental effects, it should be clarified that the effects are not secondary to the parental damage. Further mechanistic studies or additional ad hoc studies may be needed for better interpretation.

p0025 The criteria for interpreting the relationship between parental toxicity and effects on development or fertility are frequently a matter of discussion among experts in agencies making decisions for regulatory process. Table 10.1 summarizes the methods adopted by the OECD regarding reproductive toxicity. Method OECD 414 studies the effects of the exposure to substances during the gestation process for the evaluation of the effects in development. This test describes the prenatal effects in the implantation and organogenesis in test animals (rats/rabbits) and also the maternal effects produced during pregnancy.

p0030 In order to evaluate fertility in parental generation and prenatal and postnatal systemic effects in the first generation, method OECD 415 studies the toxicity to one generation in the rat or mouse. The exposure starts in the parental generation before mating to observe effects in spermatogenesis or the estrous cycle. After mating, only the pregnant female is exposed to the substance during gestation until the end of the nursing period.

p0035 A more complete method, OECD 416, not only describes the systemic effects on the first generation but also the fertility and functionality of their reproductive system to produce a second generation. The exposure to parental generation starts before mating so as to detect effects on the fertility and it lasts during mating, gestation and weaning of the F_1 generation. The F_1 offspring is administered the test substance during its growth, adulthood, mating and gestation of the F_2 generation until it is weaned.

p0040 Method OECD 421 is a screening test to detect postnatal effects due to prenatal exposure, usually in rats. It is also aimed to be used as a range-finding study for more detailed

Reproductive and Developmental Toxicology, Edited by Ramesh C. Gupta
ISBN: 978-0-12-382032-7

t0010 **TABLE 10.1** This table shows a summary of the reproductive toxicity protocols validated by the OECD and compares the different endpoints evaluated on each

OECD	Date (adopted)	EPA no.	EU	Species	Number and sex of animals	Dosage	Administration	Endpoints
414	2001	870.3700	B.31	rat/rabbit	20 females per concentration	3 concentration + control	Oral	– litters with implants – litters with live fetuses
415	1983		B.34	rat/mouse	20 females per concentration	3 concentration + control	Oral	– fertility – gestation – viability index – body weight – necropsy
416	2001	870.3800	B.35	rat	20 pregnant females per concentration	3 concentration + control – males during spermatogenesis – females during several estrus cycles	Oral	F_1 – growth – development – reproductive system F_2 – growth – development
421	1995	870.3550		rat	8 pregnant females per concentration	3 concentration + control 2 weeks prior to mating Males total of 28 days Females until day 3 postpartum	Oral	– gross lesions – identified target organs – infertility – clinical abnormalities – affected reproductive and litter performance – body weight changes – effects on mortality
422	1996	870.3650		rat	8 pregnant females per concentration	3 concentration + control 54 days approximately	Oral	– live births and postimplantation loss; – pups with abnormalities, runts; – time of death during the study or whether animals survived to termination; – implantations, corpora lutea, litter size and weights
426	2007	870.6300		rat	20 litters per concentration	3 concentration + control administered daily to mated females from the time of implantation (gestation day 6) throughout lactation (PND 21)	Oral	– body weight and clinical observations – brain weight – neuropathology – sexual maturation – other developmental landmarks (eye opening, incisor eruptions) – behavioral ontogeny – motor activity (including habituation) – motor and sensory function – learning and memory

tests. In this protocol, males are exposed during approximately 4 weeks (2 weeks before mating and 2 weeks postmating). Females are exposed 2 weeks before mating and then during pregnancy until weaning of the pups. The effects observed include fertility of the parental generation and systemic effects on the parental and first generation.

p0045 A combined method, using method OECD 421 and repeated dose toxicity studies, described in the protocol OECD 422 for simultaneous evaluation, entails the effects of a repeated exposure with a screening test of reproductive/developmental toxicity. As a screening test its results are of limited use; it is mainly used as a preliminary study to establish doses for further studies.

Method OECD 426 describes the neurotoxic effects of p0050
the test substance produced during the development of an organism. The females of the parental generation are dosed, from implantation (approximately day 6 after mating) to weaning of the first generation (day 21 after birth). This procedure allows the pups to be exposed during the neurological developmental process (pre- and postnatally) and

evaluate the effects produced by the test substance to the fetus and newborns.

p0055 Apart from these validated methods, there are some other methods (developmental immunotoxicity and one-generation extended reproduction toxicity study) that have not been validated yet by the regulatory agencies.

p0060 A method that has not been adopted yet by the OECD consists of the extension of the one-generation reproduction toxicity study (OECD 415) up to the sexual maturity of the first generation. This method does not imply the gestation of a second generation but a detailed study by histological and functional analysis of the first generation in order to detect any reasons for infertility and gestation of a new generation. The method for evaluating the developmental immunotoxicity has not been adapted by any international agency but it has been proposed by some committees and expert groups. It is based on the different immune response capacity by adult or developing organisms against chemicals. However, in the OECD there are no technical guidelines dedicated to immunotoxicity, either in developmental or in adult animals. Moreover, there are no legal requirements for the evaluation of developmental immunotoxicity, so it is improbable that this method will be adopted in the next few years.

p0065 Below, the methods adapted by the OECD and some other protocols are described in detail, including the general principles of the study, the main aspects of the procedure, the endpoints and the observations, data reporting and criteria for interpreting results, and summarizing the guidelines. This summary must not be used as an actual protocol to be performed for regulatory purposes. The original full OECD Guidelines must be used.

s0015 METHOD OECD 414 (PRENATAL DEVELOPMENTAL TOXICITY)

p0070 Among the methods for testing developmental toxicity, OECD guideline 414 (OECD, 2001a) provides general information concerning the effects of prenatal exposure on the pregnant test animal and on the developing organism. This protocol has its equivalents in the Environmental Protection Agency (EPA, number 870.3700) and in the European Union Test Method B.31. This includes the assessment of maternal effects as well as death, structural abnormalities or altered growth in the fetus. Although functional deficits are important parts of development, they are not included in these guidelines. Testing for these deficiencies and other postnatal effects are evaluated in the two-generation reproductive toxicity study (OECD, 2001b) and the developmental neurotoxicity study (OECD, 2007).

p0075 The preferred rodent species is the rat and the preferred non-rodent species is the rabbit. The test substance is administered to pregnant animals at least from implantation (day 5 after mating) to one day prior to the day of scheduled labor. The test examines the period of organogenesis (from day 5 to 15 in rats and 6 to 18 in rabbits) and also effects from preimplantation, through the entire period of gestation to the day before caesarean section. A tested substance or vehicle is usually administered orally by intubation. At least three dose levels and a concurrent control are used. Each test and control group contains a sufficient number of females to result in approximately 20 female animals (Table 10.1) with implantation sites at necropsy. Groups with fewer than 16 animals with implantation sites may be inappropriate. Dose levels should be selected taking into account any existing toxicity data. The highest dose should produce developmental and/or maternal toxicity (mild clinical signs or a decrease in body weight) but not death or severe suffering.

p0080 Clinical observations are made and recorded once a day, preferably at the same time(s) each day taking into consideration the peak period of anticipated effects after dosing. Animals are weighed on day 0, on the first day of dosing, every 3 days during the dosing period and on the day of scheduled labor. Food consumption is also recorded at 3-day intervals and should coincide with days of body weight determination. Females should be killed 1 day prior to the expected day of delivery. Females showing signs of abortion or premature delivery prior to scheduled kill should be sacrificed and subjected to a macroscopic examination. At the time of termination or death, the dam is examined macroscopically for structural abnormalities or pathological changes (caesarean section and subsequent fetal analyses).

Uterine contents s0020

p0085 Immediately after termination or as soon as possible after death, the uterus is removed and the pregnancy status of the animals is ascertained. Uteri that appear non-gravid are further examined. Gravid uteri including the cervix are weighed, except from animals found dead during the study. The number of corpora lutea (indication of implants) for pregnant animals is determined. The uterine contents are examined for numbers of embryonic or fetal deaths and viable fetuses.

Examination of fetuses s0025

p0090 The sex and body weight of each fetus are determined. Each fetus is examined for external skeletal and soft tissue alterations (e.g., variations and malformations or anomalies). For rats, half of each litter is prepared and examined for skeletal alterations. For rabbits, all fetuses are examined for soft tissue and skeletal alterations. The bodies of these fetuses are evaluated by dissection for soft tissue alterations, which include procedures for further evaluation of internal cardiac structure. The heads of one-half of the fetuses examined are removed and processed for evaluation of soft tissue alterations (including eyes, brain, nasal passages and tongue), using standard serial sectioning methods. The bodies of these fetuses and the remaining intact fetuses are processed and examined for skeletal alterations.

Data reporting s0030

p0095 Data are reported in tabular form, showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons, the time of any death or humane sacrifice, the number of pregnant females, the number of animals showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the types of fetal observations, and all relevant litter data.

s0035 ## Endpoints

p0100 Litters with implants are evaluated for developmental endpoints: number of corpora lutea as an indication of implantations, number and percent of live and dead fetuses and resorptions and number and percent of pre- and postimplantation losses. Litters with live fetuses are examined for the following developmental endpoints: number and percent of live offspring, sex ratio, fetal body weight, preferably by sex and combined; external, soft tissue and skeletal malformations and other relevant alterations, total number and percent of fetuses and litters with any external, soft tissue or skeletal alteration, and types and incidences of individual anomalies and other relevant alterations.

s0040 ## Interpretation of results

p0105 A prenatal developmental toxicity study provides information on the effects of repeated oral exposure to a substance during pregnancy. The results of the study should be interpreted in conjunction with the findings of subchronic, reproduction, toxicokinetic and other studies. Since emphasis is placed on general toxicity and developmental toxicity endpoints, the results of the study allow for the discrimination between developmental effects occurring in the absence of general toxicity and those which are only expressed at levels that are also toxic to the maternal animal.

s0045 # METHOD OECD 415 (ONE-GENERATION REPRODUCTION TOXICITY STUDY)

p0110 This protocol (OECD, 1983) has equivalence in the European Test Method B.34.

s0050 ## Principles of the test

p0115 This test guideline is designed to provide general information concerning the effects of the tested substance on male and female reproductive performance, such as gonadal function, estrous cycle, mating behavior, conception, parturition, lactation and weaning. The study also provides information about developmental effects, such as neonatal morbidity, mortality, behavior and teratogenesis.

p0120 This test is designed for use with rat or mouse. Males of parental generation (F_0) are dosed during growth and for at least one spermatogenic cycle (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effect on spermatogenesis by the tested substance. Females of the parental generation are dosed for at least two complete oestrous cycles (4 to 5 days) in order to elicit any adverse effects on estrus by the test substance. The animals are then mated. The test substance is administered to both sexes during the mating period and thereafter only to females during pregnancy and for the duration of the nursing period.

p0125 At least three treatment groups and a control group are used (Table 10.1). Each test and control group should contain a sufficient number of animals to yield about 20 pregnant females at or near term. Ideally, the highest dose level should induce toxicity but not mortality in the parental animals, the intermediate dose should induce minimal toxic effects attributable to the tested substance and the lowest dose should not induce any observable adverse effects on the parents or offspring.

p0130 The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the substance to affect fertility, pregnancy and maternal behavior in parental generation animals and suckling, growth and development of the F_1 offspring from conception to weaning. Daily dosing of the parental males begins when they are about 5 to 9 weeks old. In rats dosing is continued for 10 weeks prior to the mating period (for mice, 8 weeks). Males are killed and examined either at the end of the mating period or may be retained on test diet for the possible production of a second litter. For parental females, dosing begins after at least 5 days of acclimatization and continues for at least 2 weeks prior to mating. Daily dosing of the parental females continues throughout the 3-week mating period, pregnancy and up to the weaning of the F_1 offspring.

p0135 Animals dosed during the fertility study are allowed to litter normally and rear their progeny to the stage of weaning without standardization. If standardization is carried out, the following procedure is suggested: on day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by selection to yield, as nearly as possible, four males and four females per litter.

Endpoints s0055

p0140 Behavioral changes, such as signs of difficulty or prolonged parturition and all signs of toxicity, including mortality, are recorded. During pre-mating and mating periods and optionally during pregnancy, food consumption is measured weekly. After parturition, and during lactation, food consumption measurements are made on the same day as the litters are weighed. Parental generation is weighed on the first day of dosing and weekly thereafter. Each litter is examined as soon as possible after delivery to establish the number and sex of pups, stillbirths, live births and the presence of gross anomalies. Dead pups and pups killed at day 4 are preserved and studied for possible defects. Live pups are counted and litters weighed on the morning after birth and on days 4 and 7 and then weekly until termination of the study, when animals are weighed individually. Physical or behavioral abnormalities observed in the dams or offspring are recorded.

p0145 At the time of sacrifice or death the animals of the parental generation are examined macroscopically for structural abnormalities or pathological chances, with special attention to the organs of the reproductive system. The ovaries, uterus, cervix, vagina, testes, epididymides, seminal vesicles, prostate, coagulating gland, pituitary gland and target organs of all parental animals are preserved for examination. These organs are microscopically examined in all high dose and control animals and in animals which die during the study. Organs showing abnormalities in these animals are then examined in all other parental animals. In these instances microscopic examination is made of all tissues showing gross pathological changes. Reproductive organs of animals suspected of infertility may be subjected to microscopic examination.

s0060 ## Data reporting

p0150 Data are summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of fertile males, the number of pregnant females, the types of changes and the percentage of animals displaying each type of change (fertility, clinical abnormalities, body weight changes, effects on mortality and any other toxic effects).

s0065 ## Interpretation of the results

p0155 This reproductive toxicity study provides information on the effects of repeated oral exposure to a substance. The results of the study should be interpreted in conjunction with the findings of subchronic, teratogenic (see comments for OECD 414 guideline above) and other studies. Extrapolation of the results of the study to humans is valid to a limited degree, although it provides useful information on no-effect levels and permissible human exposure.

s0070 # METHOD OECD 416 (TWO-GENERATION REPRODUCTION TOXICITY STUDY)

p0160 The protocol for studying the effects of a chemical substance in a two-generation study has been adopted by different agencies. The OECD adopted it in 2001 as method 416 (OECD, 2001b), while the EPA named it 870.3800 and the European Union as B.35. This test is designed to provide general information concerning the effects of a tested substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the estrus cycle, mating behavior, conception, gestation, parturition, lactation and weaning, and the growth and development of the offspring. The study also provides information about the effects on the first generation (F_1) including neonatal morbidity, mortality and preliminary data on prenatal and postnatal developmental toxicity. In addition to studying growth and development of the F_1 generation (Table 10.1), the test is also intended to assess the integrity and performance of the male and female reproductive systems as well as growth and development of the second generation (F_2).

p0165 The tested substance is administered in graduated doses (at least three doses and a control) to groups of males and females. The rat is the preferred species for testing. Males of the parental generation are dosed during growth and for at least one complete spermatogenic cycle (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effects on spermatogenesis. Effects on sperm are determined by some sperm parameters (e.g., sperm morphology and motility) and in tissue preparation and detailed histopathology. Females of the parental generation are dosed during growth and for several complete estrus cycles in order to detect adverse effects on estrus cycle normality produced by the substance. The tested substance is administered to parental animals during their mating, during the resulting pregnancies and through the weaning of their F_1 offspring. At weaning the administration of the substance is continued to F_1 offspring during their growth into adulthood, mating, gestation, until the F_2 generation is weaned.

p0170 Each test and control group should contain a sufficient number of animals to yield preferably not less than 20 pregnant females at or near parturition. The objective is to produce enough pregnancies to assure a meaningful evaluation of the potential of the substance to affect fertility, pregnancy and maternal behavior and suckling, growth and development of the F_1 offspring from conception to maturity, and the development of their offspring (F_2) to weaning.

Dosing s0075

p0175 Daily dosing of the parental males and females begins when they are 5 to 9 weeks old. Daily dosing of the F_1 males and females begins at weaning. During the lactation period, direct exposure of the F_1 pups to the test substance may already occur. For both sexes (F_0 and F_1), dosing continues for at least 10 weeks before the mating period. Dosing is continued in both sexes during the 2-week mating period. Males are humanely killed and examined when they are no longer needed for assessment of reproductive effects. For parental females, dosing continues throughout pregnancy and up to weaning of the F_1 offspring. Treatment of F_0 and F_1 males and females continues until termination.

Observations s0080

p0180 This method studies different endpoints related to sperm parameters, observations of the offspring, physical development, functional investigations and pathological changes with special attention to the organs of the reproductive system. For the sperm parameters, the effects on spermatogenesis are evaluated by histopathological examination of testis and epididymis. Sperm evaluation for abnormalities is conducted in control and high dose F_0 and F_1 males; or in all males in each dose group when there are evidences from other studies of possible effects on spermatogenesis. If the sperm evaluation parameters have already been examined as part of a systemic toxicity study of at least 90 days (in subchronic or chronic repeated dose testing), they need not necessarily be repeated in the two-generation study.

p0185 For observations of the offspring, physical development of the offspring is recorded mainly by body weight gain. Other physical parameters give supplementary information, but these data are evaluated in the context of data on sexual maturation (age and body weight at vaginal opening or balano-preputial separation), and functional investigations (motor activity, sensory function, reflex ontogeny) of the F_1 offspring. The age of vaginal opening and preputial separation should be determined for F_1 weanlings selected for mating. Ano-genital distance should be measured at postnatal day 0 in F_2 pups if triggered by alterations in F_1 sex ratio or timing of sexual maturation.

p0190 All parental animals (F_0 and F_1), all pups with external abnormalities or clinical signs, and at least one randomly selected pup/sex/litter from both the F_1 and F_2 generation, are examined macroscopically for any structural abnormalities or pathological changes with special attention to the organs of the reproductive system. The uteri of all primiparous females are examined, in a manner which does not compromise histopathological evaluation, for the presence and number of implantation sites.

p0195 At the time of termination, body weight and the weight of organs of all F_0 and F_1 parental animals are determined (uterus, ovaries; testes, epididymis (total and caudal); prostate; seminal vesicles with coagulating glands and their fluids; brain, liver, kidneys, spleen, pituitary, thyroid and adrenal glands and known target organs). Terminal body weights for F_1 and F_2 pups that are selected for necropsy are determined and the organs (brain, spleen and thymus) from one selected pup/sex/litter are weighed.

p0200 Parameters observed in a prenatal developmental toxicity study (OECD, 2001a) about implantation and development of the fetus are also evaluated and described in the OECD TG 416 for two-generation reproduction toxicity.

s0085 Reporting of data

p0205 The evaluation of the date provided in this test includes the relationship, or lack, between the dose of the tested substance and the presence or absence, incidence and severity of abnormalities, including gross lesions, identified target organs, including necropsy and microscopic findings, affected fertility, clinical abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality and any other toxic effects. A two-generation reproduction toxicity study provides information on the effects of repeated exposure to a substance during all phases of the reproductive cycle. In particular, the study provides information on the reproductive parameters, and on development, growth and survival of offspring. The results of the study should be interpreted in conjunction with the findings of subchronic, prenatal developmental and toxicokinetic and other available studies.

s0090 METHOD OECD 421 (REPRODUCTION/ DEVELOPMENTAL TOXICITY SCREENING TEST)

p0210 The test is used as part of a set of initial screening tests for existing chemicals for which little or no toxicological information is available, as a dose range finding study for more extensive reproduction/developmental studies. The OECD Guideline 421 (OECD, 1995) was adopted by the EPA as Method 870.3550. This test does not provide complete information on all aspects of reproduction and development. It offers only limited means of detecting postnatal manifestations of prenatal exposure, or effects that may be induced during postnatal exposure. Due to the relatively small numbers of animals, the selectivity of the endpoints and the short duration of the study, this method does not provide evidence for definite claims of no effects. Of course, negative data do not indicate absolute safety with respect to reproduction and development. This information may provide some reassurance if actual exposures were clearly less than the dose related to the NOAEL of repeated dose subchronic or chronic dose studies.

Principle of the test s0095

p0215 Males should be dosed for a minimum of 4 weeks, including the day before scheduled labor. This includes a minimum of 2 weeks prior to mating, during the mating period and, approximately, 2 weeks post-mating. In view of the limited pre-mating dosing period in males, fertility may not be a particular sensitive indicator of testicular toxicity. Therefore, a detailed histological examination of the testes is essential. The combination of a pre-mating dosing period of 2 weeks and subsequent mating/fertility observations with an overall dosing period of at least 4 weeks, followed by detailed histopathology of the male gonads, is considered sufficient to enable detection of the majority of effects on male fertility and spermatogenesis.

p0220 This guideline is designed for performing the test with rats. Females should be dosed throughout the study. This includes 2 weeks prior to mating (with the objective of covering at least two complete estrous cycles), the variable time to conception, the duration of pregnancy and at least 4 days after delivery, up to and including the day before scheduled labor. Duration of study, following acclimatization, is approximately 54 days (at least 14 days pre-mating, (up to) 14 days mating, 22 days gestation, 4 days of lactation), as indicated in Figure 10.1, but it depends on female performance.

p0225 The guideline recommends the test substance to be administered orally, daily for 7 days a week, and each group should start with at least 10 animals of each sex. Except in the case of marked toxic effects, it is expected that this will provide at least eight pregnant females per group, which is the minimum acceptable number per group. At least three test groups and a control group are used. Two- to four-fold intervals are frequently optimal for setting the descending dose levels.

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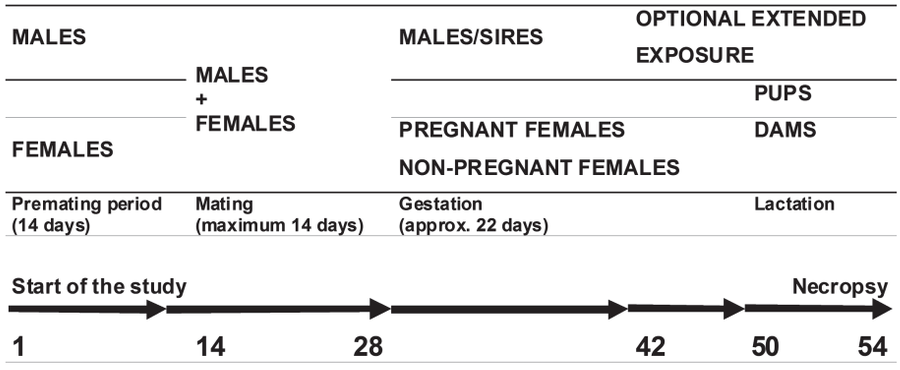

FIGURE 10.1 Temporal schedule for the Method OECD 421, indicating the period (days) of exposure.

p0230 Dosing of both sexes begins at least 2 weeks prior to mating and mating begins soon after the animals have attained full sexual maturity (10–12 weeks of age). Dosing is continued in both sexes during the mating period. Males should further be dosed after the mating period at least until the minimum total dosing period of 28 days (Table 10.1) has been completed. Parental female dosing should continue throughout pregnancy and at least up to, and including, day 3 postpartum. For studies where the test substance is administered by inhalation or by the dermal route, dosing is continued at least up to, and including, day 19 of gestation.

s0100 Observations

p0235 Food consumption and body weight are evaluated in males and females the first day of dosing, at least weekly during the test and at termination. Adult animals are examined macroscopically for abnormalities or pathological changes. Special attention is paid to the organs of the reproductive system. The number of implantation sites is recorded. The counting of corpora lutea is strongly recommended for estimating successful implantations over total implantations in order to deduce the number of implantation losses. The testes and epididymides of all male adult animals are weighed. Dead pups and pups killed at day 4 postpartum are carefully examined externally for gross abnormalities. The ovaries, testes, epididymides, accessory sex organs and all organs showing macroscopic lesions of all adult animals are preserved.

p0240 Detailed histological examination is performed on the ovaries, testes and epididymides (with detailed study on stages of spermatogenesis and histopathology of interstitial testicular cell structure) of the animals of the highest dose group and the control group. Examinations are extended to the animals of other dosage groups when changes are seen in the highest dose group.

s0105 Data reporting

p0245 The evaluation includes the relationship between the dose of the test substance and the presence or absence, incidence and severity of abnormalities, including gross lesions, identified target organs, infertility, clinical abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality and any other toxic effects. Because of the short period of treatment of the male, the histopathology of the testis and epididymis should be considered along with the fertility data, when assessing male reproductive effects.

p0250 The results that are reported from this test include body weight and food consumption changes; toxic response data by sex and dose, including fertility, gestation and other signs of toxicity; gestation length, toxic or other effects on reproduction, offspring, postnatal growth, etc.; nature, severity and duration of clinical observations; number of live births and postimplantation loss; number of pups with grossly visible abnormalities, number of runts; time of death; number of implantations, corpora lutea, litter size and litter weights; organ weight data for the parental animals; necropsy findings; microscopic findings of the male genital tract and in other tissues and absorption data.

METHOD OECD 422 (COMBINED REPEATED DOSE TOXICITY STUDY WITH THE REPRODUCTION/ DEVELOPMENTAL TOXICITY SCREENING TEST) s0110

p0255 This protocol (OECD, 1996) has also been adopted by the Environmental Protection Agency as 870.3650. This test is intended for identification of possible health hazards likely to arise from repeated exposure over a relatively limited period of time. The method comprises a reproduction/developmental toxicity screening test and, therefore, is also used to provide initial information on possible effects on male and female reproductive performance such as gonadal function, mating behavior, conception, development of the conceptus and parturition. This test does not provide complete information on all aspects of reproduction and development. It only offers limited means of detecting postnatal manifestations of prenatal exposure, or effects that may have been induced during postnatal exposure. It can also be used as a dose range finding study for more extensive reproduction/developmental studies. In this test, the dosing period is longer than in a conventional 28-day repeated dose study. However, it uses fewer animals of each sex per group.

Principle of the test s0115

p0260 This study is designed for use with rats. The tested substance is administered orally to at least three test groups of males and females and a control. Males are dosed for a minimum of 4 weeks (2 weeks before mating, during the mating period and approximately 2 weeks post-mating). In view of the limited pre-mating dosing period in males, fertility may not be a particularly sensitive indicator of testicular toxicity. Therefore, a detailed histological examination of the testes is essential. The combination of a pre-mating dosing period of 2 weeks and subsequent mating/fertility observations with an overall dosing period of at least 4 weeks, followed by detailed histopathology of the male gonads, is considered sufficient to enable detection of the majority of effects on male fertility and spermatogenesis.

p0265 Females are dosed throughout the study. This includes 2 weeks prior to mating (to cover at least two complete estrous cycles), the time to conception, the duration of pregnancy and at least 4 days after delivery, up to and including the day before scheduled labor.

Procedure s0120

p0270 The guideline recommends each group to start with at least 10 animals of each sex. Except in the case of marked toxic effects, it is expected that this will provide at least eight pregnant females per group which normally is the minimum acceptable number of pregnant females per group. The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the substance to affect fertility, pregnancy, maternal and suckling behavior, and growth and development of the F_1 offspring from conception to day 4 postpartum. Two- to four-fold intervals are frequently optimum for the dosing.

The animals are dosed with the test substance daily for 7 days a week.

p0275 After animals of both sexes have been acclimatized for at least 5 days, 2 weeks prior to mating, dosing begins. Mating begins soon after the animals have attained full sexual maturity (10–12 weeks of age). Males are dosed after the mating period at least until a minimum total dosing period of 28 days (Table 10.1). Daily dosing of the parental females continues throughout pregnancy and at least up to, and including, day 3 postpartum or the day before sacrifice.

s0125 Observations

p0280 General clinical observations are made daily. Once before the first exposure and at least once a week thereafter, detailed clinical observations are made in all animals. Changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, sensory reactivity to stimuli and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern) is evaluated. The duration of gestation should be recorded and is calculated from day 0 of pregnancy. Each litter is examined as soon as possible and live pups are counted and sexed, and litters are weighed within 24 hours of parturition. Males and females are weighed on the first day of dosing, weekly thereafter and at termination. Food consumption is measured weekly.

p0285 Hematological examinations are made in five males and five females randomly selected from each group and the following parameters are measured: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential. Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, are performed on blood samples obtained from the selected five males and five females of each group. Other determinations should be carried out if the known properties of the test substance may, or are suspected to, affect the metabolic profiles.

p0290 All adult animals in the study are subjected to a full, detailed, gross necropsy which includes careful examination of the external surface of the body, all orifices and the cranial, thoracic and abdominal cavities and their contents. Special attention is paid to the organs of the reproductive system. The testes and epididymides of all adult males are weighed and the ovaries, testes, epididymides, accessory sex organs and all organs showing macroscopic lesions of all adult animals are preserved. For five adult males and females, randomly selected from each group, the liver, kidneys, adrenals, thymus, spleen, brain and heart are trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection. Of the selected males and females, the following tissues are also preserved: all gross lesions, brain (representative regions including cerebrum, cerebellum and pons), spinal cord, stomach, small and large intestines, liver, kidneys, adrenals, spleen, heart, thymus, thyroid, trachea and lungs, uterus, urinary bladder, lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, and a section of bone marrow (or, alternatively, a fresh mounted marrow aspirate). These examinations should be extended to animals of other dosage groups, if treatment-related changes are observed in the highest dose group.

Data reporting s0130

p0295 Due to the limited dimensions of the study, statistical analysis in the form of tests for "significance" is of limited value for many endpoints, especially reproductive endpoints. The findings of this toxicity study are evaluated in terms of the observed effects, necropsy and microscopic findings. The evaluation includes the relationship between the dose of the tested substance and the presence or absence, incidence and severity of abnormalities, including gross lesions, identified target organs, infertility, clinical abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality and any other toxic effects. Because of the short period of treatment of the male, the histopathology of the testis and epididymis must be considered along with the fertility data, when assessing male reproduction effects.

p0300 The test report must include body weight changes, food consumption, toxic response data by sex and dose, including fertility, gestation and any other signs of toxicity, gestation length, toxic or other effects on reproduction, offspring, postnatal growth, nature, severity and duration of clinical observations, sensory activity, grip strength and motor activity assessments, hematological and clinical biochemistry tests, number of live births and postimplantation loss, number of pups with grossly visible abnormalities, number of runts, time of death during the study or whether animals survived to termination, number of implantations, corpora lutea (recommended), litter size and litter weights at the time of recording, body and organ weight data for the parental animals; necropsy findings, histopathological findings, absorption data and statistical treatment of results.

p0305 The study provides an evaluation of reproduction/developmental toxicity associated with administration of repeated doses. In particular, since emphasis is placed on general toxicity and reproduction/developmental toxicity endpoints, the results of the study allow for the discrimination between reproductive/developmental effects occurring in the absence of general toxicity and those which are only expressed at levels that are also toxic to parent animals. It could provide an indication of the need to conduct further investigations and could provide guidance in the design of subsequent studies and for justification of waiving full studies.

p0310 The experimental schedule for the study duration of this test is similar to that shown in method 421 (see Chapter 11), with a maximum duration of the study of 54 days.

METHOD OECD 426 (DEVELOPMENTAL NEUROTOXICITY STUDY) s0135

p0315 Developmental neurotoxicity studies are designed to provide data, including dose–response characterization, on the potential functional and morphological effects on the developing nervous system of the offspring that may arise from exposure in uterus and during early life. A developmental neurotoxicity study can be conducted as a separate

study, incorporated into a reproductive toxicity and/or adult neurotoxicity study, or added onto a prenatal developmental toxicity study.

p0320 This method was adopted by the OECD as Guideline 426 (OECD, 2007) and by the EPA as Method 870.6300.

s0140 Procedure

p0325 The preferred test species is the rat while other species can be used when appropriate. Each test and control group should contain a sufficient number of pregnant females to be exposed to the test substance to ensure that an adequate number of offspring (20 litters are recommended per dose level) is produced for neurotoxicity evaluation. On or before postnatal day (PND) 4, the size of each litter should be adjusted by eliminating extra pups by random selection to yield a uniform litter size for all litters. The litter size should not exceed the average litter size for the strain of rodents used (8–12).

p0330 At least three dose levels and a concurrent control should be used. The highest dose level should be chosen with the aim of inducing some maternal toxicity and may be limited to 1,000 mg/kg body weight. The tested substance (or vehicle) should be administered by the most relevant route to potential human exposure, and based on available metabolism and distribution information in the test animals. The tested substance (or vehicle) should, at least, be administered daily to mated females from the time of implantation (gestation day 6) throughout lactation (PND 21), so that the pups are exposed to the test substance during pre- and postnatal neurological development (Table 10.2).

t0015 **TABLE 10.2** Timing of the assessment of physical and developmental landmarks, and functional/behavioral endpoints (number of times when measurements are performed) for OECD Guideline 426

	Age periods		
	Pre-weaning	**Adolescence**	**Young adults**
Endpoints			
Physical and developmental landmarks			
Body weight and clinical observations	weekly	at least every two weeks	at least every two weeks
Brain weight	PND 22		at termination
Neuropathology	PND 22		at termination
Sexual maturation	–	as appropriate	–
Other developmental landmarks	as appropriate	–	–
Functional/behavioral endpoints			
Behavioral ontogeny		at least two measures	
Motor activity (including habituation)	1–3 times	–	once
Motor and sensory function	–	once	once
Learning and memory	–	once	once

Observations s0145

During the treatment and observation periods, detailed clinical observations are conducted periodically (a minimum of twice during the gestational and the lactational dosing period) using at least 10 dams per dose level. Clinical observations include changes in skin, fur, eyes, mucous membranes, occurrence of secretions and autonomic activity (lacrimation, piloerection, pupil size, unusual respiratory pattern and/or mouth breathing and any unusual signs of urination or defecation). Unusual responses with respect to body position, activity level (decreased or increased exploration of the standard area), coordination, posture, reactivity to environmental stimuli, presence of clonic or tonic movements, convulsions, tremors, stereotypies, bizarre behavior (biting or excessive licking, self-mutilation, walking backwards, vocalization) or aggression should be recorded. p0335

Signs of toxicity should be recorded, including the day of onset, time of day, degree and duration. Animals are weighed at the time of dosing at least once a week throughout the study, on or near the day of delivery, and on PND 21. For gavage studies dams should be weighed at least twice weekly. Food consumption is measured weekly at a minimum during gestation and lactation. p0340

For the offspring (at least one pup/sex/litter), during the treatment and observation periods, detailed clinical observations are conducted. The observations should be the same as for the dams. Changes in pre-weaning landmarks of development (pinna unfolding, eye opening, incisor eruption) are highly correlated with body weight, so this may be the best indicator of physical development. p0345

Neuropathological evaluation of the offspring is conducted using tissues from animals. For offspring killed through PND 22, brain tissues should be evaluated; for animals killed at termination (PND 70), both central nervous system (CNS) tissues and peripheral nervous system (PNS) tissues are evaluated. All gross abnormalities appearing at the time of necropsy should be noted. Tissue samples taken should represent all major regions of the nervous system. The purposes of the qualitative examination are to identify regions within the nervous system exhibiting evidence of neuropathological alterations, types of neuropathological alterations resulting from exposure to the test substance and to determine the range of severity of the neuropathological alterations. Morphometric (quantitative) evaluation should be performed as these data may assist in the detection of a treatment-related effect and are valuable in the interpretation of treatment-related differences in brain weight or morphology. p0350

Neuropathological evaluation should include an examination for indications of developmental damage to the nervous system, in addition to the cellular alterations. Some significant changes are alterations in the gross size or shape of the olfactory bulbs, cerebrum or cerebellum; alterations in the relative size of various brain regions, including changes in the size of regions resulting from the loss or persistence of normally transient populations of cells or axonal projections; alterations in proliferation, migration and differentiation, as indicated by areas of excessive apoptosis or necrosis, clusters or dispersed populations of ectopic, disoriented or malformed neurons or alterations in the relative size of various layers of cortical structures; alterations in patterns of myelination, including an overall size reduction or altered staining of myelinated structures; evidence of hydrocephalus, in p0355

particular enlargement of the ventricles, stenosis of the cerebral aqueduct and thinning of the cerebral hemispheres. For each type of lesion, the characteristics used to define each severity grade should be described, indicating the features used to differentiate each grade. The frequency of each type of lesion and its severity grade should be recorded and a statistical analysis should be performed to evaluate the nature of a dose–response relationships.

s0150 Data and reporting

p0360 A developmental neurotoxicity study provides information on the effects of repeated exposure to a substance during prenatal and early postnatal development. Since emphasis is placed on both general toxicity and developmental neurotoxicity endpoints, the results of the study allow for the discrimination between neurodevelopmental effects occurring in the absence of general maternal toxicity, and those which are only expressed at levels that are also toxic to the maternal animal.

p0365 The test report for this test should include the following results (Table 10.2): number of animals at the start and at the end of the study; number of animals and litters used for each test method; identification number of each animal; litter size and mean weight at birth by sex; body weight and body weight change data, including terminal body weight for dams and offspring; food consumption data, and water consumption data if appropriate; toxic response data by sex and dose level, including signs of toxicity or mortality, including time and cause of death; detailed description of clinical observations; score on each developmental landmark (weight, sexual maturation and behavioral ontogeny); detailed description of all behavioral, functional, neuropathological, neurochemical, electrophysiological findings by sex; necropsy findings; brain weights; diagnoses derived from neurological signs and lesions, including naturally occurring diseases or conditions; images of exemplar findings; low power images to assess homology of sections used for morphometry; absorption and metabolism data; statistical treatment of results and list of study personnel.

p0370 The discussion of results in the report must contain dose–response information, by sex and group; relationship of any other toxic effects to a conclusion about the neurotoxic potential of the test chemical, by sex and group, impact of toxicokinetic information on the conclusions, similarities of effects to any known neurotoxicants, data supporting the reliability and sensitivity of the test method, relationships between neuropathological and functional effects and NOAEL or benchmark dose for dams and offspring, by sex and group.

s0155 OTHER METHODS NOT INCLUDED IN THE OECD

s0160 Developmental immunotoxicity

p0375 The Agricultural Chemical Safety Assessment (ACSA) Technical Committee developed in 1999 a database for developmental immunotoxicity. The developing immune system can be significantly more sensitive than the adult immune system to xenobiotic-induced insult (Dietert and Holsapple, 2007). There are distinct differences between the immune system surrounding birth and that in the mature adult as well as differences in the nature of immunotoxic changes based on age. Immunosuppression is not the only concern. Immunotoxic changes that increase the risk for allergic or autoimmune responses should also be considered. Therefore, researchers should not assume that immunotoxicity assays validated for adult exposure assessment are inherently the most predictive for developmental immunotoxicology (DIT) evaluation. Many of those adult-based protocols were developed solely to detect immunosuppression, whereas DIT concerns include shifts in immune balance. However, majority of participants at the April 2008 ECETOC-ECVAM expert workshop (ECETOC-ECVAM, 2008) believe it is premature to include a DIT module in the enhanced one-generation technical guideline for the following reasons: no regulatory data requirement for DIT exists anywhere in the world, and only a handful of ad hoc "special studies" have been requested to date. Only recently one pesticide regulator has codified a requirement for an adult immunotoxicity study, so no contribution can be claimed to further international harmonization of hazard and risk assessment approaches. No standardized or validated methodology or OECD technical guidelines currently exist for the evaluation of immunotoxicity in either adult or developing animals, although discussions among experts have taken place (Holsapple *et al.*, 2005). Although an enhanced one-generation study would be suited for general use, DIT data are of dubious relevance outside the pesticides sector (Vogel *et al.*, 2010).

One-generation extended reproduction toxicity study s0165

p0380 This method has been proposed by different groups (Reuter *et al.*, 2003; Vogel *et al.*, 2010) in order to produce a more efficient protocol for testing the reproductive/developmental effects of chemical substances using one generation. This proposal uses a smaller number of animals, with benefits in economic effort and also ethically.

p0385 Previous evaluations of multi-generational tests (Reuter *et al.*, 2003; Cooper *et al.*, 2006; Myers *et al.*, 2008) show that in nearly all cases, the effects produced by the chemical had already been shown in the first generation and only in a minimal number of cases had the effect been visible in the second generation.

p0390 This method consists of the extension of the one-generation study (OECD 415) with detailed evaluation of different endpoints in F_1 generation. The F_1 generation is examined for functional, histological and pathological changes in its system and most exhaustively in the reproductive organs. Although this proposed method has not been approved by official organisms, it has already been used by industry. The benefits from using this method have been stated before and are related to the reduction in the number of animals and ethical reasons. Some of the arguments stated by industry for avoiding the use of this method are the expensive costs of detailed and exhaustive examinations of histopathology and functional changes in the animals.

CONCLUDING REMARKS AND FUTURE DIRECTIONS s0170

p0395 Different organizations at international levels (OECD, EPA, EU) have developed *in vivo* protocols for testing the reproductive toxicity of chemicals. These protocols are used all over the world and have been previously validated, so they are

considered reliable. The endpoints evaluated in those methods cover toxicity in the fertility in the parental generation to effects in the developmental process and include the birth of new generations of animals. The evaluation of the adverse effects produced on these tests provides useful information for the characterization of the toxic potential of a chemical. New protocols are being developed in order to reduce the number of animals used in the assessment and to improve the predictability for identification of human health hazards.

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