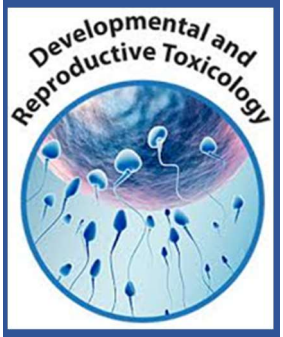


TOXGURUKUL FOUNDATION

TOXICOLOGY NEWSLETTERS

"It is not birth, marriage, or death, but gastrulation which is the most important time in your life."

— Louis Wolpert

February, 2020

Important Announcement

ToxGurukul Foundation Presents

Toxicology Webinar's Series on

'TOXICOLOGY AND RISK ASSESSMENT'

Commencing from April 2020

First speaker: **Dr. A. Wallace Hayes**

(Distinguished Fellow: American College of Toxicology; University of South Florida College of Public Health)

Topic: Basic Principles of Toxicology: Parts 1

Date and Time: **April 11, Saturday, 9.00 p.m.** Indian Standard Time (IST). Duration: 90 minutes

Course Moderator**: Dr. Varun Ahuja,** M.V.Sc., DABT (Lupin)

Please Note: We are also looking for **speakers for future webinars.** Speakers can be from anywhere from either in India or abroad. **Link for registration will be updated soon**.

Request for donations: Any sponsorship(s) to cover the cost of webinar conferencing**, please contact the undersigned. (**Sponsorship can be as little as a one-time payment of **Rs. 30,000/- ($400)** drawn towards **Toxgurukul Foundation).**

~ Dr. K.S. Rao
toxrao@gmail.com
+91-733-783-0074

Editor
Sapna Gupta
toxgurukul.india@gmail.com
editor.toxgurukul@gmail.com

REPRODUCTIVE TOXICITY ASSESSMENT

K.S. Rao[1], M.V.Sc., Ph.D., DABT; Kalpesh Patani[2], M.V.Sc.; Shekar Chelur[3], M.V.Sc., DABT, DIBTP; Mukesh Poshiya[4], M.V.Sc.
[1]Eurofins Advinus Limited, Bangalore; [2]Zydus-Cadila Healthcare Ltd., Ahmedabad; [3]Aurigene Discovery Technologies Ltd., Bangalore; [4]Jai Research Foundation, Vapi.

A. INTRODUCTION

Xenobiotic induced effects on the reproductive system can be pivotal in development of drugs and chemicals as human fertility is considered too fragile to be compromised. Due to no or very limited human data over reproductive toxicity, predicting it by animal studies making the reproductive toxicity assessment very useful to mankind.

Reproductive toxicity is evaluated under two broad categories of toxicity as Developmental & Reproductive Toxicity (DART) evaluation. Reproductive toxicity describes adverse effects on sexual function and fertility in adult males and/or females. the reproductive toxicity is categorized in three classes of toxicity: toxic effects on fertility, parturition, and lactation. Developmental toxicity includes adverse effects on the developing organism that may result from exposure prior to conception, during prenatal development, or postnatally until sexual maturation. In the developmental toxicity category, four classes of toxicity are evaluated: mortality, dysmorphogenesis (structural alterations), alterations to growth, and functional toxicities. The distinction between developmental and reproductive toxicity (DART) is somewhat arbitrary in that developmental exposures can result in effects on reproduction, and vice versa.

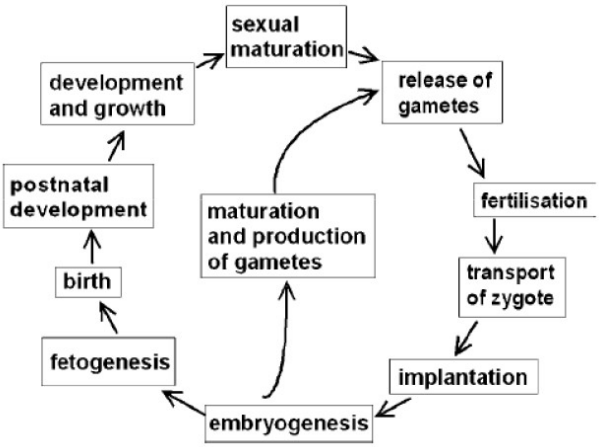


Figure 1: Mammals Reproductive cycle

Reproductive toxicity assessment as part of general toxicity studies involves several evaluations of reproductive and endocrine tissues in both males and females including

1. weights of testes, epididymis, and accessory glands; ovaries and uterus,
2. estimation of hormones and assessment of its impact on fertility,
3. histopathology evaluation of reproductive organs and endocrine organs is essential for safety assessment and for elucidation of mechanism of action.

Good understanding of background pathology, knowledge of reproductive endocrinology, development, and comparative biology and species differences are also important for evaluation. Wolffian ducts are progenitors of the upper male genital tract and give rise to the epididymis, vasa deferentia, and seminal vesicles. The müllerian ducts are the progenitors of the upper female genital tract and develop into the fallopian tubes at their cranial ends and fuse to form the uterus and upper part of the vagina at their caudal ends. The lower portions of the genital tract, which include the prostate gland, the penis, and scrotum in males, and the lower portion of the vagina, clitoris, and labia in the female, arise from the urogenital sinus and genital tubercle present in both sexes.

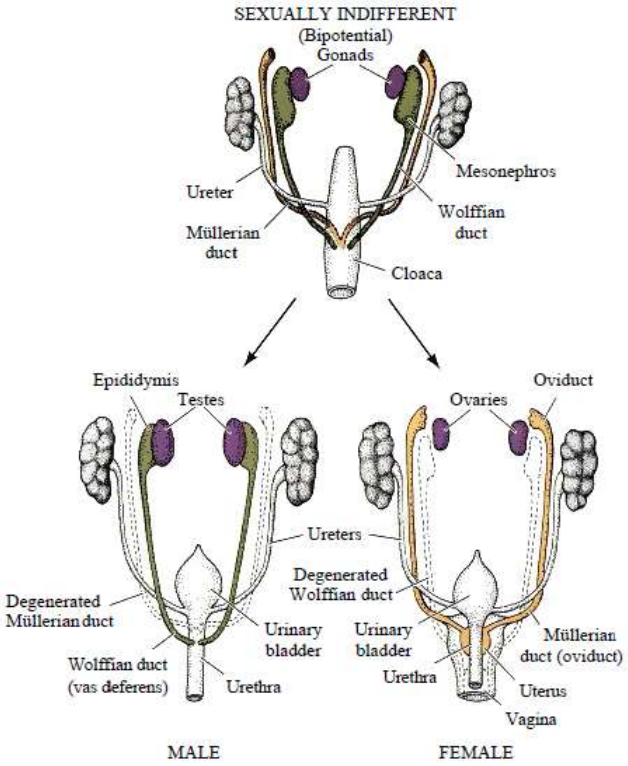


Figure 2: Development of the male and female internal genitalia

There is marked variability in the anatomy, biology, function, and number of the male accessory sex glands among preclinical species and humans. Major sites of toxicity in male reproductive system include impact on sperm production, sperm delivery, sperm quality and hormonal regulation (Table 1). The ovary is a complex organ with three major functions: production of

fertile oocytes, synthesis of steroid hormones, and synthesis of regulatory proteins. Some species differences occur in the histological appearance of the ovary (Table 2).

Table 1: Male reproductive organs and functions

Organ		Functions
Main organ	Testis	Sperm production Seminiferous fluid production by Sertoli cells Production of testosterone from Leydig cells
Transport	Rete Testis	Transport of sperms and fluid production
	Epididymis	Transport of sperms and resorption of fluid
	Vas deferens	Ejaculation
Accessary sex organs	Seminal vesicle	Nutrients
	Coagulation gland	Copulatory plug
	Prostate	Proteolytic enzymes
	Bulbourethral gland	Copulatory plug
	Preputial gland	Pheromones
Shared with urinary system	Urethra	Transport of semen
	Penis	Copulation

Table 2: Female reproductive organs and functions

Organ	Function
Ovaries	Production of eggs and sex hormones
Oviduct or fallopian tube	Transport of eggs
Uterus	Place of developing embryo and fetus
Vagina	Receives sperms from outside and passage of fetus
Cervix	Duct that connects uterus and vagina, mucus production

Estrous cycle duration in rodent strains ranges from 4 to 5 days. There are four phases- proestrus, estrus, metestrus and diestrus. Examination of vaginal smears (cytology) and/or vaginal histology are commonly used methods for identification of the stage of the rodent cycle and can be used as evidence of cyclicity.

Table 3: Vaginal smear cell types and numbers during the estrous cycle of the rodents

Stage of estrous cycle	Typical cell numbers				
	Leucocytes	Nucleated cells	Non nucleated cells	Cornified cells	Total cells
Estrus	-	-/+	+/-	+++	+++
Metestrus	+++	-	+	-/+	+++
Diestrus	++	+/-	+/-	+/-	+/++
Proestrus	-	++	-	-/+	+/++

- None or very few, + Low, ++ Moderate, +++ High

Histopathologic examination of the ovary, uterus and vagina should be conducted not only to determine the stage of the cycle, but also to assess cyclicity and identify the potential perturbations of the cycle. Evaluation of the mammary gland and pituitary gland can also aid in characterizing perturbations of the estrous cycle.

Follicle counting is not performed in general toxicity testing but is a useful tool in reproductive toxicity studies. Quantification of small follicles may provide additional information as a second-tier procedure to further characterize a suspected or demonstrated ovarian toxicant. Use of immunohistochemical methods, including human cytochrome P450 1B1 (CYP1B1) and proliferating cell nuclear antigen (PCNA), have been used to enhance the visibility of the primordial and primary follicles to facilitate manual counting with reduced variability and time.

Major mechanisms of toxicity on female reproductive system include direct toxicity to reproductive organs and indirect toxicity by hormonal imbalance. Regardless of mechanism, hormone imbalance will be disturbed in long term and will influence histopathological features of lesions in female reproductive organs. Background histological changes are more common in young male rodents than young female rodents but increase with age in both sexes.

Reproductive toxicants have a threshold of adversity. This contrasts with genotoxic carcinogens which are considered to have “no safe exposure level”. To this complexity of the threshold of adversity, reproductive toxicity adds the factor of the vulnerable window. The thalidomide tragedy triggered the development of more structured regulations and implementation of guidelines for (DART) testing.

Regulatory evolution of reproductive toxicity testing dates back to 1966, following the thalidomide tragedy in 1962, when US FDA recommended that outlined the three-segment protocol with which we are now familiar as Segment- I (Fertility Study), II (Teratology Study), III (Multi-Generation Study).

B. DOSE-RESPONSE ASSESSMENT

Dose setting, including levels, frequency, and interval of exposure, have a huge impact on dose response assessment. Current developmental toxicity study designs are conducted without attention to critical windows of development; therefore, the impact of exposure is considered to be uniform across the entirety of development. There are cases when suboptimal dose timing can miss a critical window and give a false negative result; for example, use of a single bolus dose of a chemical with rapid metabolism could preclude exposure over the critical window.

C. HYPOTHESIS-DRIVEN TESTING

Hypothesis-driven testing is the use of existing information about a chemical to generate hypotheses that could be tested using customized models and protocols. Modifications could be as straightforward as adjusting the dosing regimen so that the internal dosimetry of the chemical is more similar to human pharmacokinetics or choosing a model that is pharmacodynamically more similar to humans. Alternatively, modified testing could entail an entirely different approach that does not involve Segment II (embryofetal toxicity testing) like protocols.

In the drug discovery and development process, much is known about the activity of a compound. The developer usually knows the molecular target of the compound and possible secondary targets, either identified by high-throughput receptor binding/enzyme activity panels or inferred from *in vivo* safety pharmacology protocols or other toxicology protocols conducted prior to developmental testing. These data are generally not available for nonpharmaceutical chemicals, which (except for pesticides) are not designed to have specific biological activity, but there is still information available to shed light on the possible toxicity of a chemical including its relatedness to previously tested chemicals based on two or three-dimensional structure, physical chemical properties, or with relatively easily generated data on gene expression in a panel of cell types or high-throughput screening. This kind of information can be used to formulate and evaluate hypotheses about the toxicity of a new, related chemical.

Questions remain about the relationship between exposure level and response, particularly when the underlying information about a chemical is based on toxicity data using compounds other than the chemical of interest. Decisions about acceptable levels of exposure will need to incorporate an understanding about similarities and differences in toxicokinetics and disposition in target organs between the compounds, and uncertainty factors will continue to be necessary in decision-making about acceptable exposure levels. The selection of uncertainty factors may be influenced by the source of data, for example, in vivo, in vitro, in silico, or a combination of sources.

Hypothesis-based testing in the pharmaceutical industry considers the pharmacology, toxicology, and clinical use of the drug in designing a testing strategy. Considerations include both the effect of intended pharmacology and off-target effects on embryo-fetal and post-natal development.

Current guidelines for dose spacing typically recommend three doses plus an untreated control. The use of benchmark dose modeling is preferable to the NOAEL/LOAEL approach, but does not completely alleviate the dose spacing issue if dose levels are not near the point of departure (POD).

Considering enhancements as part of the evolution of the existing testing paradigm, dose setting should be designed based on the available data and hypothesis-testing rather than simply relying on the use of standard practices. In the ideal, dose setting should be based on the internal dose and not the administered dose and incorporate critical window information for the chemical or a mechanistically similar chemical. Dose-response methods should be used that allow for nonlinear curves.

D. CURRENT GUIDELINES

No.	Guidelines	When Needed	Approximate # of Animals
		A. Pharmaceuticals	
1.	ICH S5 (R2) Section 4.1.1 **(Rats)** Study of fertility and early embryonic development to implantation and Part II: toxicity to male fertility	Study should be completed before the initiation of large scale or long duration clinical trials (e.g. Phase III trials)	F_0-generation: 80 M & 80 F F_1-generation: 960 fetuses (GD-14)
2.	ICH S5 (R2) Section 4.1.3. Study for effects on embryo-fetal development in **rats**	Definitive developmental toxicity studies in two species are required for inclusion of women of childbearing potential (WOCBP) in clinical study. (e.g. Phase II trials)	F_0-generation: 88 F F_1-generation: 1100 fetuses (GD-20)
3.	ICH S5 (R2) Section 4.1.3. Study for effects on embryo-fetal development in **rabbits**		F_0-generation: 88 F F_1-generation: 790 fetuses (GD-29)
4.	ICH S5 (R2) Section 4.1.2. Study for effects on pre-and postnatal development, including maternal function	Phase III for drugs to be given to pregnant or nursing mothers for long periods or where there are indications of possible adverse effects on foetal development.	F_0-generation: 80 F F_1-generation: 960 pups F_2-generation: 960 fetuses (GD-14)
		B. Industrial Chemicals	
1.	OECD 421, Reproduction/Develop-mental toxicity screening test in **rats**	REACH Annex VIII (applicable for any registration of 10 tons or more per year)	F_0-generation: 48 F & 40 M F_1-generation: 500 pups
2.	OECD 422, Combined repeated dose toxicity study with the Repro. /Develop. toxicity screening test in **rats**	REACH Annex VIII	F_0-generation: 48 F & 40 M F_1-generation: 500 pups
3.	OECD 414, Prenatal developmental toxicity study in **rats**	REACH Annex IX (applicable for any registration of 100 tons or more per year)	F_0-generation: 88 F F_1-generation: 1100 fetuses
4.	OECD 414, Prenatal developmental toxicity study in **rabbits**	REACH Annex IX (applicable for any registration of 100 tons or more per year)	F_0-generation: 88 F F_1-generation: 800 fetuses
5.	OECD 443, Extended one-generation reproductive toxicity study in **Rats**	REACH Annex X	F_0-generation: 100 F & 100 M F_1-generation: 1200 Pups
		C. Agrochemicals	
1.	OPPTS 870.3700, Prenatal developmental toxicity study in **rats**	Before registration	F_0-generation: 88 F F_1-generation: 1100 fetuses
2.	OPPTS 870.3700, Prenatal developmental toxicity study in **rabbits**	Before registration	F_0-generation: 88 F F_1-generation: 800 fetuses

3.	OPPTS 870.3800, Reproduction and fertility effects in **rats**	Before registration	F_0-generation: 96 F & 96 M F_1-generation: 1200 Pups F_2-generation: 1200 Pups

E. REPRODUCTIVE TOXICITY TESTING OF PHARMACEUTICALS

The reproductive toxicity testing of all pharmaceuticals intended for human use is now performed according to the guidelines issued by the International Conference on Harmonisation (ICH). The original FDA guidelines defined a three-segment strategy for the reproductive toxicity of drugs. Segment I concerned the detection of effects on fertility following exposure of the adult. Segment II concerned the detection of teratogenicity or embryotoxicity. Segment III was the peri- and post-natal study designed to detect developmental effects following exposure during late pregnancy and lactation. Regulatory guidelines ICH S5 (R2) is the effective version to be followed for conducting studies, however ICH S5 (R3) is under draft version.

Dose Selection & Method (Segment II): A vehicle control and at least three dose levels of compounds are used, the highest of which produces some minimal adult toxicity and the lowest of which is a low order multiple of the anticipated human exposure level. Evaluation of pregnancy outcome often involves removal of fetuses about one day prior to delivery and evaluation of external, soft tissue, and skeletal alterations. In other protocols, males and females are dosed prior to mating and conceptuses evaluated after implantation, or pregnant animals are dosed and young are delivered and raised by their mothers or by foster mothers with testing of offspring viability and functional characteristics.

The guidelines define six phases of reproduction that need to be assessed: (A) adult fertility, (B) early embryonic development before implantation on the uterus, (C) embryonic organogenesis, (D) fetal development, (E) birth and pre-weaning development and (F) post-weaning development up to sexual maturity. The investigator is free to devise an experimental strategy best suited to the evaluation of the test compound in question, but the most probable option is the traditional three-segment design.

1. Fertility study

The fertility study is almost always performed in the rat and is intended to detect adverse effects on male and female gamete maturation, copulatory performance, fertilization, zygote development and implantation of the embryo. This covers ICH phases A and B. Groups of at least 20 males and females are treated for at least 2 weeks before and during mating. Treatment of the females is then continued at least through the implantation. The pregnant females are euthanized after mid gestation (GD 14) to allow inspection of their uterine contents. As per Indian regulatory

guidelines the females will be allowed to litter and offsprings growth and development will be evaluated till weaning. The males may be necropsied any time after pairing, but they are most often kept until completion of the female examinations, such that they are treated in total for about 8 weeks (i.e., the duration of an entire spermatic cycle). The reproductive organs are sampled from males and females for histopathology and testes/epididymides are screened for sperm analysis parameters like sperm count, morphology and motility. Hormone estimations, to be done based on requirement in males include luteinizing hormone, follicle-stimulating hormone, inhibin, testosterone, or prolactin. Hormone estimations in females include estradiol, progesterone, follicle-stimulating hormone, luteinizing hormone or prolactin Standard methods for serum hormone analysis include radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and multiplex immunoassay. Liquid chromatography/mass spectrometry (LC/MS)-based methods for measurement of testosterone and their metabolites.

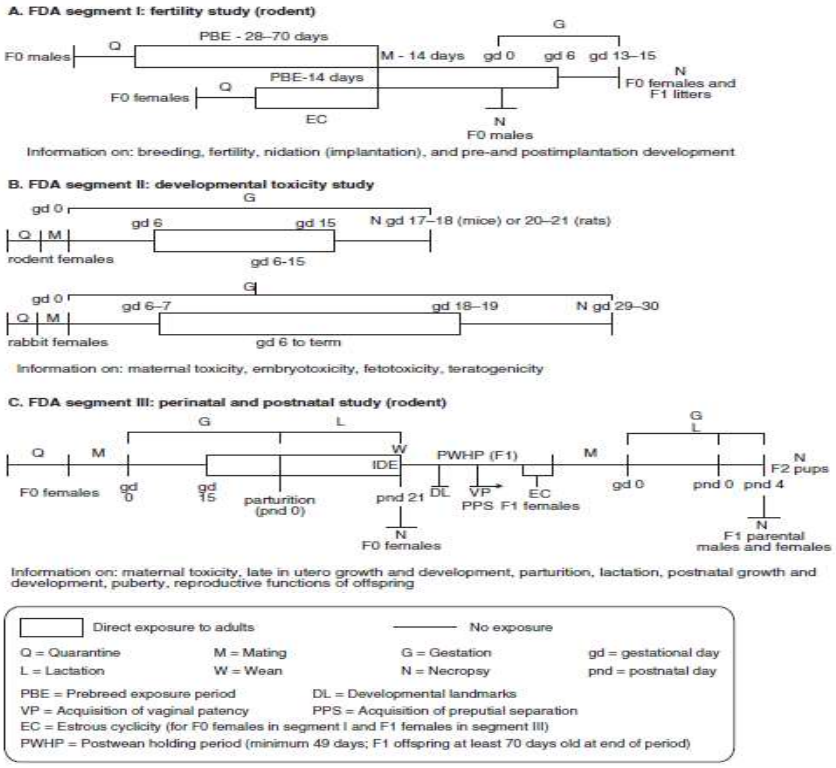
2. **Embryo-fetal developmental toxicity study (Segment II)**

This study is focused on ICH phase C, i.e., the period of organogenesis, embryonic cells are divided, differentiated, and migrated tremendously during this period therefore this is the principal period of sensitivity to classical teratogenic effects. Most types of structural birth defect can only be induced by exposure during this period. In view of the potentially catastrophic consequences of a teratogenic medicine reaching the market, this study is performed in two species, one rodent and one non-rodent, most often the rat and rabbit. Pregnant animals are treated during the period of organogenesis i.e. GD 6 to 17/19. Minimum of 20 litters for rodent studies or 16 litters for rabbit studies are used for meaningful biological evaluation. Treatment is stopped after closure of the palate in the embryo, after which the pregnant females continue to be monitored throughout the fetal period. They are then euthanized just before the anticipated day of parturition (GD 20 in rats and GD 29 in Rabbits). The uterine contents are examined and fetal examination like external, visceral and skeletal evaluations recorded and evaluated for any embryo-toxicity. Placenta also examined along with gross necropsy of dam to check any alteration.

3. **Pre- and post-natal study**

The design of the ICH pre-and post-natal study was based on the previous peri- and post-natal protocol devised the Japanese Ministry of Health and Welfare. The dosing period of the ICH study was extended to encompass the embryonic period in addition to the fetal and post-natal periods, hence the change of name. This protocol duplicates the exposure during the embryonic period already accomplished in the ICH embryotoxicity study, which is necessary in order to detect any functional defects induced by the

drug during organogenesis. Groups of about 20 mated female rats are dosed from day 6 of gestation throughout the embryonic and fetal periods, during parturition, and throughout lactation up to weaning. At weaning, one male and one female pup are selected from each litter to form the F1 generation. The dams and non-selected pups are necropsied. The selected F1-generation pups are not dosed, but their physical development is monitored before and after weaning, up to the age of sexual maturity. As per Indian regulatory guidelines F1 generation pups will be treated up to the maturity and later for gestation and lactation. Behavioral tests are performed with the aim of detecting any neurological defects of the pups. After attaining sexual maturity, the F1 pups are paired within each group to evaluate their fertility. The F1 females are euthanized two weeks after copulation for evaluating uterine parameters and fertility.



F. REPRODUCTIVE TOXICITY TESTING OF PESTICIDES

The U.S. Environmental Protection Agency (EPA) published teratogenicity testing requirements in 1978 under FIFRA. Essentially, it mandated how testing was to be conducted and reported, with exposure of pregnant dams to be initiated just before implantation and concluded the day before delivery. The EPA requires (1) a standard teratogenicity study with exposure during the main period of organogenesis and (2) a two-generation reproduction study.

The Office of Prevention, Pesticides, and Toxic Substances (OPPTS) revised the 1982 Health Effects Test Guidelines (EPA, 1998). The OPPTS-harmonized

guidelines have been developed for use in the testing of pesticides and toxic substances and the development of test data that must be submitted to the agency for review under federal regulations. The Organization of Economic Cooperation and Development (OECD) guidelines 414 and 416 and the OPPT guidelines under 40 CFR 798.4900 and 40 CFR 798.4700, OPP guidelines 83-3 and 83-4, provided the source material for developing these harmonized OPPTS test guidelines. Following is high level protocols for developmental and reproductive toxicity testing and notes the approximate dosing and breeding schedules.

Protocol for developmental toxicity testing

Rats	GD	20 females per dose group
	0	(Evidence of sperm in vaginal smear observation and/or plug in bedding) Begin exposure on gestation day 0/6 continue until day before parturition
	20	C-section and examine fetuses (on GD 20)
Rabbits		20 females per dose group
	0	Day of artificial insemination; natural mating can be used) Begin exposure on gestation day 0/7 continue until day before parturition
	29	C-section and examine fetuses (on GD 29)

At a minimum, the test substance should be administered daily from around the time of implantation to the day before Cesarean section on the day prior to the expected day of parturition. Alternatively, if preliminary studies do not indicate a high potential for preimplantation loss, treatment may be extended to include the entire period of gestation, from fertilization to approximately 1 day prior to the expected day of parturition. It is preferred that the dams are exposed from the time of mating i.e., GD 0.

Two-generation Study of Effects on the Reproduction Process in Rat

Age of animals	**F0/P1**
5-9 Weeks	Start of study. Exposure for 10 weeks in diet (or other route, based on the most likely human exposure scenario)
15-19 Weeks	1. Mating (conducted over 2 weeks or 3 estrous cycles; re-mating with proven male of same group can be considered) 2. Gestation (approximately 3 weeks) 3. Parturition to produce F1 litters
21-25 Weeks	1. Weaning (approximately 3 weeks) 2. Growth (approximately 15 weeks)
15-19 Weeks	1. Mating 2. Gestation (approximately 3 weeks) 3. Parturition to produce F2 litters 4. Weaning

In certain instances, such as poor reproductive performance in controls, or in the event of treatment-related alterations in litter size, the adults may be re-mated to produce F1a or F2a litter. If production of a second litter is deemed necessary in either generation the dams should be re-mated approximately 1-2 weeks following weaning of the last F1 or F2 litter.

VARIOUS REPRODUCTIVE INDICES MEASURED DURING DART STUDIES

$$Male\ Mating\ Index = \frac{Number\ of\ males\ mated}{Number\ of\ males\ cohabitated} X\ 100$$

$$Male\ Fertility\ Index = \frac{Number\ of\ males\ impregnating\ females}{Number\ of\ males\ mated} X\ 100$$

$$Female\ Mating\ Index = \frac{Number\ of\ females\ mated}{Number\ of\ females\ cohabitated} X\ 100$$

$$Female\ Fertility\ Index = \frac{Number\ of\ females\ pregnant}{Number\ of\ females\ mated} X\ 100$$

$$Implantation\ Index = \frac{Number\ of\ implants}{Number\ of\ corpora\ lutea\ (CL)} X\ 100$$

$$Preimplantation\ Loss = \frac{Number\ of\ CL - Number\ of\ implants}{Number\ of\ CL} X\ 100$$

$$Postimplantation\ Loss\ = \frac{Number\ of\ implants - Number\ of\ viable\ fetuses}{Number\ of\ implants} X\ 100$$

$$Gestation\ Index\ = \frac{Number\ of\ females\ with\ live\ born}{Number\ of\ females\ with\ evidence\ of\ pregnancy} X\ 100$$

$$Survival\ Index = \frac{Total\ number\ of\ live\ pups\ (at\ designate\ time\ point)}{Number\ of\ pups\ born} X\ 100$$

$$Live\ Birth\ Index = \frac{Number\ of\ pups\ born\ alive}{Total\ number\ of\ pups\ born} X\ 100$$

$$Fecundity\ index = \frac{Number\ of\ pregnant\ females}{Number\ of\ females\ with\ confirmed\ mating} X\ 100$$

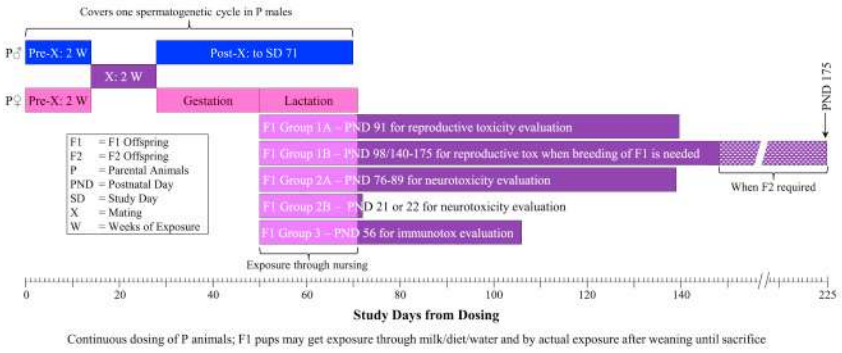
G. REPRODUCTIVE TOXICITY TESTING OF INDUSTRIAL CHEMICALS

For hazard assessment of chemicals in the regulatory domain, animal studies are being performed as default. For DART testing, the first harmonized guidelines were published in the 1980's for industrial chemicals (OECD 414 for developmental toxicity in 1981, OECD 415 and 416 for reproductive toxicity in 1983) [OECD 414, OECD 415, OECD 416] and in the 1990's for pharmaceuticals (ICH S5 for DART in 1993) [ICH S5]. OECD 415 (one generation reproduction toxicity study) has been withdrawn by OECD since 27th December, 2019. In addition to these definitive tests, a relatively quick screening method which can give initial clues about possible fertility and developmental effects of chemicals is the OECD 421 screening study adopted in 1995, and the combination with 28-day repeated dose, OECD 422, adopted in 1996 [OECD 421, OECD 422]. The most recent adopted guideline is OECD 443, the extended one-generation reproductive toxicity study (EOGRTS), which was finalized in 2011 [OECD 443] as per 3R principle and was developed as replacement for the two-generation reproduction toxicity study [OECD 416].OECD 421 and 422 revised in July 2016 and OECD 418 revised in June 2018.

Most of the above described individual guidelines evaluate toxicity of chemicals to only parts of the reproductive and developmental stages with the exception the two-generation reproductive toxicity study. Researchers now like to combine multiple reproductive and developmental toxicity studies into a single study and determine systemic exposure during dose range-finding or other general toxicity studies for the selection of appropriate doses. Although the two-generation toxicity study is considered "the gold standard" for the assessment of reproductive toxicity, it is complex in design, high in the utilization in animals (~2600 animals for study in rats) and with debatable value of the F2 generation. The two-generation toxicity study is also not designed to evaluate developmental neurotoxicity (DNT) or developmental immunotoxicity (DIT) endpoints, which require standalone studies using an additional 1280 animals.

H. EXTENDED ONE-GENERATION REPRODUCTIVE TOXICITY STUDY (EOGRTS)

This test assesses toxicity testing across life stages. The life stage toxicity was defined as the potential adverse effects of chemicals on preconception, development (embryo/fetal and newborn/pre-weaning life stages), adolescence, and adults of all ages for reproductive and developmental toxicity, any special sensitivity with respect to general toxicity and specific effects on the nervous, immunological, and endocrine systems at critical life stages. This is the latest test in the reproduction category and is also the most controversial.



The study starts with exposing a sufficient number of adult male and female rats (to achieve 20 litters/dose) to the test chemical for two weeks prior to mating through weaning. Both parents are then sacrificed on study day (SD) 71 and evaluated while pups are continuously dosed with the test chemical until their scheduled sacrifice after evaluation for possible toxicological effects as depicted below:

Groups of pups are evaluated for developmental neurotoxicity and at sexual maturity for reproductive, immuno, neuro, and general toxicity, and bred, when triggered, to produce F2 litters. The trigger to generate F2 animals in EOGRTS is based on developmental landmarks (e.g., anogenital distance, nipple retention, puberty onset) in F1 animals.

1. Outline of EOGRTS for parents including systemic dose determination.

Prior to and during cohabitation	
Housing prior to cohabitation	Group by sex
Housing during cohabitation	1:1 male:female
Clinical observations (CO)	≥ twice daily
PE	SD1 and weekly
BW	SD1 and weekly
FC	Weekly
Vaginal smear	Daily for 2 weeks
Blood	SD 14 or 28, test chemical concentration at SS (2 or 4 weeks)
Males following cohabitation	
Housing	Group
CO, PE, BW, FC	Same as above
Blood	At sacrifice, SD 71 or 85, test chemical concentration
Sperm analysis	At sacrifice, SD 71, using one testis/epididymis/vas deferens
Urine	Urinalysis
Blood	Hematology, Clinical Path, T4, TSH
Tissues	Histopathology
During gestation	
Housing	Individual for Dam
CO, PE, FC	Same as above
BW	Every 2 days
Blood	GD 14 or 17, test chemical concentration
At birth	
Blood	Dam for test chemical concentration
During lactation	
Housing	Dam and pups
CO, PE, FC	Same as above
BW	LD 4, 7, 14, 21
Blood	LD 4 and 14 for test chemical concentration
Milk	LD 4 and 14 for test chemical concentration
At termination	
Urine	Urinalysis
Blood	Hematology, Clinical Path, T4, TSH, test chemical concentration
Tissues	Histopathology
Uteri	All: presence and number of implantation site
Vaginal smears	All: stage of estrous cycle for correlation with histopathology

BW, body weight; CO, clinical observation; FC, food consumption; GD, gestational day; LD, lactational day; PE, physical examination; SD, study day; SS, steady-state; T4, thyroxin; TSH, thyroid stimulating hormone. Twenty-four hour or spot urine

samples may be collected on days of blood collection to determine test chemical concentration.

2. Outline of EOGRTS for F1 including systemic dose determination.

Blood samples	GD 21 or pups at birth, selected animals for test chemical concentration
At birth	Unique litter and group identification on PND 0 or 1
Culling	Reduce to 5 males and 5 females per litter on PND 4
Blood samples	From culled/litter/group for T4, TSH and test chemical concentration
Anogenital Distance	Males PND 4
Gross necropsy	All culled pups on PND 4
Housing until weaning	Litters with respective mothers
Nipple assessment	Male pups PND 12 or 13
Housing after weaning	Small groups of same sex and treatment
Clinical observations	≥ twice daily
Physical examination	Weekly at the time of weighing
BW before weaning	PND 4, 7, 14, 21 (at weaning)
BW after weaning	At weaning and weekly thereafter
Food consumption	Weekly following assigning to cohorts
Blood samples	PND 22 for T4, TSH and test chemical concentration
Sacrifice	Gross necropsy of pups not selected for cohort on PND 22
Tissues	Brain, spleen, thymus, mammary gland, target tissues on PND 22
Blood samples	PND 14, 56, between 76 and 89, 91, selected animals for test chemical concentration
Maturity assessment	Evaluated daily starting before the expected day in all selected animals

BW, body weight; PND, postnatal day; T4, thyroxin; TSH, thyroid stimulating hormones. Twenty-four hour or spot urine samples may be collected on days of blood collection to determine test chemical concentration.

3. Outline of EOGRTS for F1 cohort 1 with option for F2.

Cohort 1A: Reproductive systems and general toxicity assessment	
Number	20/sex/group (1 male and 1 female per litter per group)
CO, PE, BW, FC	See table outlining general considerations for F1 animals
Vaginal smears	Daily after patency until cornified smear is recorded
Estrous cycles	Period of two weeks from around PND 75
Termination PND 91	
Vaginal smears	All: stage of estrous cycle for correlation with histopathology
Ovary	Follicle and corpora lutea counts'
Blood	Hematology, Clinical Path, T4, TSH (10 randomly-selected animals)
Blood	PND 91 for test chemical concentration (4 animals/sex/dose group)
Urine	Urinalysis
Sperm analysis	Using one testis/epididymis (or vas deferens)
Tissues	Weights and histopathology
Immunotox (1 male or female per litter, all litters represented by at least 1 pup)	
Lymph nodes	Associated with and distant from the route of exposure
Spleen	½ for $CD4^+$ & $CD8^+$ T lymphocytes, B lymphocytes, NKC

Cohort 1B: Follow-up assessment of reproductive performance by mating F1 animals when needed	
Number	20/sex/group (1 male and 1 female per litter per group)
CO, PE, BW, FC	See table outlining general considerations for F1 animals
Vaginal smears	Pairing until evidence of mating
Cohabitation	After PND 90 and before PND 120 avoiding siblings
F2 Pups	Sac on PND 4
Termination PND 98 or at birth of F2 pups	
Vaginal smears	All: stage of estrous cycle for correlation with histopathology
Uteri	All: presence and number of implantation sites
Blood	Hematology, Clinical Path, T4, TSH
Urine	Urinalysis
Sperm analysis	Using one testis/epididymis (or vas deferens)
Tissues	Weights and histopathology
Immunotox (1 male or female per litter, all litters represented by at least 1 pup)	
Lymph nodes	Associated with and distant from the route of exposure
Spleen	½ for $CD4^+$ & $CD8^+$ T lymphocytes, B lymphocytes, NKC

BW, body weight; CD4 and CD8, T cells; CO, clinical observation; FC, food consumption; NKC, natural killer T cells; PE, physical examination; PND, postnatal day; T4, thyroxin; TSH, thyroid stimulating hormones. PND 4 blood samples are collected from culled pups, few pups are used to collect PND 14 blood sample. Twenty-four hour or spot urine samples may be collected on days of blood collection to determine test chemical concentration.

4. Outline of EOGRTS for F1 cohort 2 and 3.

Cohort 2A: neurobehavioral testing and neurohistopathology assessment as adults

Number	10/sex/group (1 male or 1 female per litter per group)
CO, PE, BW, FC	See table outlining general considerations for F1 animals
Auditory startle test	PND 24 ± 1
FOB	Between PND 63 and PND 75
Motor activity	Between PND 63 and PND 75
Termination after PND 75 and before PND 90	
Blood	For test chemical concentration
Tissues	Brain weight and full neurohistopathology - perfusion fixation
Brain (examination)	Multiple section from different regions of the brain

Cohort 2B: neurohistopathology assessment at weaning (PND 21 or PND 22)

Number	10/sex/group (1 male or 1 female per litter per group)
CO, PE, BW, FC	See table outlining general considerations for F1 animals
Termination PND 21 or PND 22	
Tissues	Brain weight and full neurohistopathology - perfusion fixation (optional)
Brain (examination)	Multiple section from different regions of the brain
Blood	For test chemical concentration

Cohort 3: developmental immunotoxicity assessment

Number	10/sex/group (1 male or 1 female per litter per group)
CO, PE, BW, FC	See table outlining general considerations for F1 animals

Termination PND 56 ± 3

Blood	For test chemical concentration
Assays	TDAR

BW, body weight; CO, clinical observation; FC, food consumption; FOB, functional observation battery; PE, physical examination; PND, postnatal day; TDAR, T-cell-dependent antibody response. Twenty-four hour or spot urine samples may be collected on days of blood collection.

I. SIGNIFICANCE OF EXPERIMENTAL DATA AND THEIR RELEVANCE TO HUMANS

Throughout many years, regulatory DART testing has proven its usefulness, as no major issues like those with DES and thalidomide (marketed before the implementation of modern hazard identifications) occurred. However, at the same time, inadequacies of animal safety testing have become evident. Uncertainties came up about the relevance of animal findings for the situation in man.

For example, aspirin is teratogenic in rodents but not in humans, and Thalidomide-induced phocomelia is seen after in utero exposure in humans and rabbits, but not in rodents. In addition, sulfoxaflor-induced fetal abnormalities and neonatal death in rats occur via a single MoA comprising sustained activation of the rat fetal-type muscle nicotinic acetyl-choline receptor resulting in a sustained muscle contracture. This MoA is considered not relevant for humans, given fundamental qualitative differences in sulfoxaflor agonism on the rat versus the human muscle nAChR.

Likewise, the temporal equivalence factors must be taken into account. There are 10- to 100-fold differences in time span for pre- and postnatal development up to sexual maturity, in rodents compared with humans. The period of major organ development in the rat covers a period of about 10 days, which compares with about 56 days in humans. Development of the limbs for example takes 3 days in the rat compared to 3 weeks in humans. This can be contrasted with the relatively small pharmacokinetic differences, perhaps of 2- or 3-fold in plasma levels of chemicals with the same administered dose in the rat and human. The consequence of these differences is that single episodes of high chemical exposure have greater opportunity to produce permanent effects on development in rodents compared with humans. The limited evidence from acute poisonings in pregnant women tends to support this hypothesis.

IMPORTANCE OF HISTORICAL CONTROL: Particular concern in reproductive toxicity testing especially embryo-fetal development is the maintenance of a historical control database. This database of information is useful in situations where the data are not otherwise obvious. Published databases can be useful for comparison, but they do not replace the significance of in-house historical control data.

J. IN VITRO AND IN SILICO APPROACHES FOR PREDICTIVE TOXICOLOGY

Predicting human developmental risk could in theory be based entirely on the testing of human cells or tissues or the *in-silico* manipulation of models of human development. Such a strategy was generally envisioned by the National Research Council in the 2007 report, Toxicity Testing in the 21st Century (National Research Council, 2007), and in the intervening decade, we have come much closer than expected to make such testing a practical reality.

The development of predictive models uses a large amount of data such as has been developed by the ToxCastTM and Tox21 efforts (US EPA, 2017a, 2017b). ToxCastTM has evaluated more than 1000 chemicals using multiple assays, and Tox21 includes data on many more chemicals using fewer assays.

K. CONCLUSION

The present whole-animal models used by the Food and Drug Administration include the 3 segment reproduction studies used for testing drug safety and the multigeneration studies used for food additives. The Environmental Protection Agency has adopted 2 similar versions of a 2-generation study for the Office of Pesticide Programs and the Office of Toxic Substances.

An important aspect of safety assessment of chemicals (industrial and agricultural chemicals and pharmaceuticals) is determining their potential reproductive and developmental toxicity. A number of guidelines have outlined a series of separate reproductive and developmental toxicity studies from fertilization through adulthood and in some cases to second generation. The Extended One-Generation Reproductive Toxicity Study (EOGRTS) is the most recent and comprehensive guideline in this series. EOGRTS design makes toxicity testing progressive, comprehensive, and efficient by assessing key endpoints across multiple life-stages at relevant doses using a minimum number of animals, combining studies/evaluations and proposing tiered-testing approaches based on outcomes. EOGRTS determines toxicity during preconception, development of embryo/fetus and newborn, adolescence, and adults, with specific emphasis on the nervous, immunological, and endocrine systems, EOGRTS also assesses maternal and paternal toxicity. However, EOGRTS guideline is complex, criteria for selecting doses is unclear, and monitoring systemic dose during the course of the study for better interpretation and human relevance is not clear.

Overall it is fair to say that the current reproduction/developmental screening studies are effective in providing unique data, especially

considering the limited number of animals used, but with some simple additions its value in risk assessment could be enriched even further.

L. BRIEF TERMINOLOGIES:

Developmental toxicology: the study of adverse effects on the developing organism that may result from exposure prior to conception, during prenatal development, or postnatally to the time of sexual maturation. The major manifestations of developmental toxicity include 1) death of the organism, 2) structural abnormality, 3) altered growth, and 4) functional deficiency.

Alterations (anomalies): structural alterations in development that include both malformations and variations:

Malformation/Major Abnormality: Structural change considered detrimental to the animal (may also be lethal) and is usually rare.

Variation/Minor Abnormality Structural change considered to have little or no detrimental effect on the animal; may be transient and may occur relatively frequently in the control population.

Conceptus: the sum of derivatives of a fertilised ovum at any stage of development from fertilisation until birth including the extra-embryonic membranes as well as the embryo or foetus.

Implantation (nidation): attachment of the blastocyst to the epithelial lining of the uterus, including its penetration through the uterine epithelium, and its embedding in the endometrium.

Embryo: the early or developing stage of any organism, especially the developing product of fertilisation of an egg after the long axis appears and until all major structures are present.

Foetus: the unborn offspring in the post-embryonic period.

Foetotoxicity: detrimental to the normal structure, development, growth, and/or viability of a foetus.

Resorption: a conceptus which, having implanted in the uterus, subsequently died and is being, or has been resorbed:

Early resorption: evidence of implantation without recognisable embryo/foetus.

Late resorption: dead embryo or foetus with external degenerative changes.

QUESTIONS

1. What do you understand by reproductive Toxicology?
2. Define Developmental toxicology.
3. What is DART?
4. What are the major guidelines used for reproductive toxicology?
5. Explain main developmental differences between male and female reprodtive organs.
6. Explain extended one-generation reproductive toxicity study (EOGRTS). What approach is used to determine EOGRTS?
7. What is the significance of experimental data (in vitro *v/s.* in vivo data) and their relevance to human?

---------End of the Document---------

ToxGurukul Foundation

ToxGurukul Foundation is a registered non-profit organization for professionals in the field of toxicology who are in search of a platform to learn and share the vast knowledge in this area. This syndicate belongs to independent professionals from different backgrounds of toxicology who share their knowledge to un-puzzle the Rubik's cube that each face in their daily work routine.

Website: ***www.toxgurukul.org***
Email: ***toxgurukul.india@gmail.com; editor.toxgurukul@gmail.com***

Important Links – ToxGurukul Foundation

Follow us

Join us

Subscribe

Library

Feedback

ToxGurukul Foundation (A Non - Profit Organization)

Regd. Office: Fl. No.10, New Ajanta Avenue, Building-4, Wing- A1, S. No. 135/136 Part, Kothrud, Pune-411038
Corporate Identity Number: U80904PN2019NPL182886
Email Id: toxgurukul.india@gmail.com