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Test Procedures to Evaluate Effects of Chemical Exposure on Fertility and Reproduction

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Various methods have been developed to assess the effects of chemical substances on reproduction. In some instances, the tests have been developed to define the effects of treatment on specific segments of the reproductive cycle. In other cases, studies are conducted to determine the cumulative effects of treatment during one or more generations. The structure, advantages, and disadvantages of three types of conventional reproduction studies are reviewed. An outline of the procedural sequences, observations, and record evaluation required in the three-period reproduction study, the three-generation reproduction study, and the multigeneration reproduction study are presented.

Introduction

Experiments are conducted to assess the effects of chemical substances on reproduction for a variety of reasons. These may include: screening programs to discern therapeutic materials to preserve pregnancy; test systems to identify materials that can be used for contraceptive uses; and finally, tests to identify unwanted chemical alterations on reproduction. It is evident that the induction of a similar result from several different substances could lead to different interpretations regarding the potential hazard. An example might involve a series of experiments in which the absolute prevention of implantation was produced if the substances were administered within 48 hr after copulation. This result would be viewed as highly favorable if the test system were designed to discover a postcoital contraceptive. The result would be extremely unfavorable if we were evaluating an over-the-counter analgesic; this observation would prevent further consideration of the substance as an over-the-counter drug. A similar result from an industrial chemical used in a completely closed system would not weigh heavily in the hazard evaluation of the material. Many factors must be considered when performing a hazard assessment for use in a risk-benefit analysis.

Reproduction is a cyclic process (Fig. 1) that can be modified at any one of several points. Classically, changes occurring during differentiation and development are considered teratogenic responses. Embryonic exposure to appropriate doses of mercury or thalidomide can result in abnormal morphological development. Changes produced during embryonic exposure might include somatic abnormalities that would subsequently prevent gametogenesis. Postnatal exposure to estrogenic or

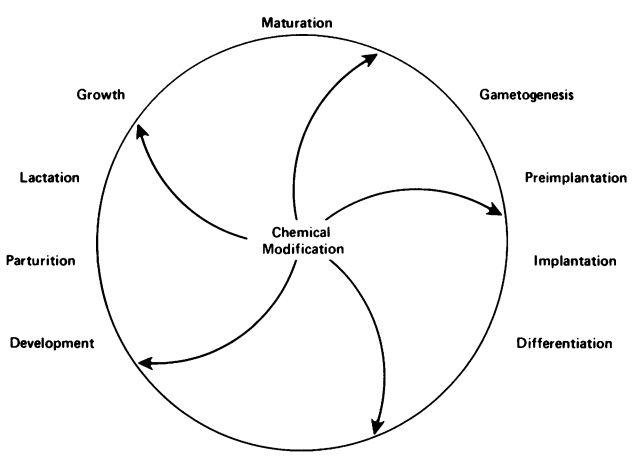

FIGURE 1. Cyclic events involved in reproductive and postnatal function.

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progestational compounds, ionizing irradiation, and nutritionally inadequate diets can also impair gametogenesis.

During the preimplantation stage, blastocyst survival can be reduced by exposure to metallic ions, particularly copper or mercury. Tubular or uterine infections can also reduce the blastocyst survival. An impairment in libido, reducing the incidence of copulation, could result in a direct reduction in the number of viable blastocytes. Materials producing a sedating effect will reduce mating behavior in experimental animals. Certain nutritional deficiencies and nonreproductive-system systemic toxic effects can reduce mating performance.

Impairment in reproductive efficiency can result from adverse effects on neonate survival at parturition or during lactation. Parturition initiation may be initiated prematurely or may be delayed. Uterine motility might be influenced in a manner that would impair parturition; maternal behavior changes might be induced that would cause rejection of the newborn. Maternal behavior effects commonly observed in rodent animals involve hyperactivity which may lead to cannibalism, or hypoactivity which may result in neglect of the young. Lactation may be adversely affected by delaying initiation or reducing the total volume or nutrient content of milk. It is also possible for chemical agents to be transferred into the milk and be subsequently consumed by the nursing young.

Test procedures used to evaluate the effects of chemical exposure on fertility and reproduction must be capable of detecting functional impairments in each of the sequential events shown in Figure 1.

Test Procedures

Studies designed to evaluate the effects of chemicals on the reproductive processes usually utilize rodent animals. These animals are selected because of their early sexual maturity, short gestation and lactation periods, and their comparative ease of maintenance (Table 1). Evaluation should not be limited to rodents if other organisms will provide information more directly applicable to human exposure or response.

Historically, two different types of tests have been recommended for evaluation of overall reproductive affects. One has been recommended by the Food and Drug Administration, Bureau of Drugs; the other by the Food and Drug Administration, Bureau of Foods and by the Environmental Protection Agency in their administration of the Federal Insecticide, Fungicide and Rodenticide Act for pesticide regulation. A third type of protocol has been proposed (*7*). This protocol retains the advantages of the three-generation reproduction study but requires only approximately one-half the calendar time to complete.

These test procedures reflect the anticipated differences in exposure of the consumer to drugs, food additives, foods, and other materials. The types of observations taken in each type of study are similar. Differences exist in length of exposure to the test material and frequency of observations. Routinely, separate studies are conducted to evaluate potential teratogenic or mutagenic responses.

Three-Period Reproduction Study

The three-period reproduction study is usually specified for assessing the undesired reproductive effects of drug agents. Drugs are normally taken intentionally; therefore, it is assumed that exposure during various periods of the reproductive cycle can be controlled.

Table 1. Relative timing of reproductive events of mammals[a]

Species	Age at sexual maturity, days[b]	Estrus cycle recurrence, days	Gestation time, days			Length of gestation, days
			Implentation	Primitive streak	Organogenesis	
Hamster	42.0–54.0	4.0	4.5–5.0	6.0	7.0–14.0	16.0–17.0
Mouse	28.0	4.0–5.0	4.5–5.0	7.0	7.5–16.0	20.0–21.0
Rat	46.0–53.0	4.0–5.0	5.5–6.0	8.5	9.0–17.0	21.0–22.0
Rabbit	120.0–240.0	[c]	7.0	6.5	7.0–20.0	31.0–32.0
Guinea pig	84.0	13.0–20.0	6.0	10.0	11.0–25.0	65.0–68.0
Pig	200.0–210.0	19.0–23.0	10.0–12.0	11.0	12.0–34.0	110.0–116.0
Sheep	150.0–300.0	16.5	10.0	13.0	14.0–35.0	142.0–150.0
Cat	210.0–245.0	14.0	13.0–14.0	13.0	14.0–26.0	58.0–71.0
Dog	270.0–425.0	182.5	13.0–14.0	13.0	14.0–30.0	57.0–66.0
Rhesus monkey	1,642.0	24.0–38.0	9.0	18.0	20.0–45.0	164.0–168.0

[a] Compiled from data of Boyer (*1*), Christie (*2*), Gruneberg (*3*), Nicholas (*4*), UFAW (*5*), and Witschi (*6*). Day on which evidence of mating is observed is defined as day 0 of gestation; values represent days.

[b] Ranges depend on species, nutrition, and other factors; values are days from birth.

[c] No estrus cycle.

The three periods of exposure are outlined in Table 2. Period II restricts chemical exposure to the period of organogenesis and is, thus, a conventional theratology study. Procedures for the evaluation of a teratogenic response have been presented (*7*).

Table 2. Three-period reproduction study.

		Treatment period	Parameters evaluated
Period I	Males		
	Rat	60 day premate, mate	Fertility
	Females		
	Rat	14 day premate, mate, gestation, lactation	Fertility Lactation Behavior Perinatal survival Development
Period II	Females		
	Rat	6–15 day gestation	Fetal development
	Rabbit	6–18 day gestation	Survival
Period III	Females	15–21 day gestation, lactation	Perinatal survival Development

In period I, the males and females are dosed with the test material on a daily basis for a period equal to the gametogenic period for that sex prior to mating. Treatment of the males and females continues during the mating period. Females are treated continuously during gestation and lactation. The parameters that are evaluated include fertility in both the male and female, lactation and behavioral effects in the female, and neonatal survival and development in the offspring. The experiment is terminated when the litters are weaned.

Period III permits treatment only during the last fourth of gestation and during the subsequent lactation period. The male receives no treatment. Lactation and behavioral responses are evaluated in the female; survival and development are evaluated in the neonates.

An advantage of this test sequence is the partial separation of exposure during specific segments of the reproduction period. Another advantage is that only four months of calendar time are required to complete this test sequence. A serious disadvantage is that there are no subsequent studies conducted to evaluate the reproductive performance of the F_1 offspring.

Three-Generation Reproduction Study

The conventional three-generation reproduction study is usually used to evaluate direct or indirect food additives and pesticide residues on food crops. The F_0 parental animals are exposed to treatment for approximately 60 days prior to mating, (Fig. 2). Treatment then continues, uninterrupted, until the final F_{3b} litters have been weaned. Each parental generation produces two litters. The F_{1b} litters provide the F_1 parental generation producing the F_{2a} and F_{2b} litters. The F_{2b} litters provide the F_2 parental generation. This test procedure offers the advantage of providing subsequent reproductive performance evaluations on animals in both the F_1 and F_2 parental animals following *in utero* exposure. Two disadvantages are associated with the three-generation reproduction study; there is no sequence separation and the study requires 20 months to complete.

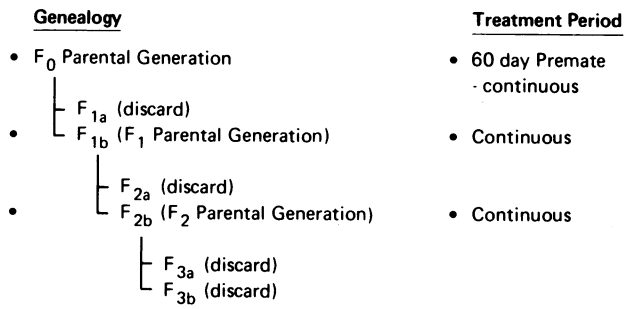

FIGURE 2. Three-generation reproduction study sequence of events.

Multigeneration Reproduction Study

A multigeneration reproduction study protocol has been developed (*7*); treatment of the F_0 parental females is initiated prior to the implantation of the F_{1a} litters (Fig. 3). Treatment continues uninterrupted until the final F_{2b} litters have been weaned. This sequences provides *in utero* and continuous postnatal exposure for the F_{1a} litters which provide the F_1 parental generation. Initiation of treatment post-mating but preimplantation of the F_{1a} litters provides *in utero* exposure while still providing the maximum logistical flexibility in obtaining F_{1a} offspring with comparable parental age and date of birth. The procedures employed and observations

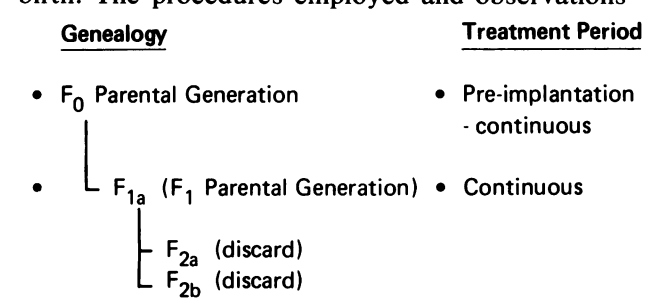

FIGURE 3. Multigeneration reproduction study sequence of events.

taken in this protocol are similar to those taken in the conventional three-generation study. This study does not provide sequence separation. It has the advantages of providing *in utero* exposure and subsequent reproductive performance evaluation of the F_1 parental generation and a required calendar time of the ten months to complete. This reduction in required calendar time, when compared to a three-generation study, offers the opportunity of increasing the number of materials evaluated and defining the affected sequence if effects are observed.

Materials and Methods

Similar animal models and methods are utilized in each of the general reproduction studies described. Normally an experiment consists of a control group and three treatment groups. F_0 parental animals are obtained from a stock colony at an age to provide young sexually mature animals after the required pre- or post-mating treatment period. The procedures that are customarily used require approximately 10 male and 20 female animals in each treatment group.

Mating Procedures and Records

When the parental animals reach sexual maturity (Table 1), each male is paired with two females from the same group. Successful mating is determined by the presence of a copulation plug, sperm, or blood in the vagina. If a female does not exhibit additional evidence of copulation at the end of a subsequent estrous cycle, she is returned to her original cage. At the end of two estrous cycles, all males within the same group are rotated and exposed to different females in the same group. No more than three males should be paired with any female during a given breeding cycle. The number of observed copulations, the number of estrous cycles required to obtain a mating, and the number of resulting pregnancies are recorded. These data are used to calculate the mating and fertility indices (Table 3). The offspring are weaned 21 days post partum. If a second litter is to be produced, the females are mated approximately 28 days after the first litter is weaned.

Table 3. Parental reproduction efficiency indices.[a]

Index	
Mating	$= \frac{\text{Number of copulations}}{\text{Number of estrus cycles}} \times 100$
Fecundity	$= \frac{\text{Number of pregnancies}}{\text{Number of copulations}} \times 100$
Male fertility	$= \frac{\text{Number of males impregnating females}}{\text{Number of males exposed to fertile nonpregnant females}} \times 100$
Female fertility	$= \frac{\text{Number of females conceiving}}{\text{Number of females exposed to fertile males}} \times 100$
Parturition	$= \frac{\text{Number of parturitions}}{\text{Number of pregnancies}} \times 100$

[a] Only one copulation counted per estrus cycle.

Table 4. Progeny survival indices.

Index	
Live birth	$= \frac{\text{Number of viable pups born}}{\text{Total number of pups born}} \times 100$
24-hr survival	$= \frac{\text{Number of pups viable at lactation day 1}}{\text{Number of viable pups born}} \times 100$
4-day survival	$= \frac{\text{Number of pups viable at lactation day 4}}{\text{Number of viable pups born}} \times 100$
12-day survival	$= \frac{\text{Number of pups viable at lactation day 12}}{\text{Number of pups retained at lactation day 4}} \times 100$
21-day survival	$= \frac{\text{Number of pups viable at lactation day 21}}{\text{Number of pups retained at lactation day 4}} \times 100$

Progeny Procedures and Records

All pups are examined for physical abnormalities at birth. The numbers of viable, stillborn and cannibalized members of each littler are recorded. Observations for clinical signs are made daily. The numbers of survivors on days 1, 4, 12, and 21 post parturition are recorded. On the fourth day of lactation, litters with more than ten pups may be reduced to that number by sacrificing randomly selected individuals. A final examination for physical abnormalities is made. Individual body weights are recorded at weaning on lactation day 21. These data are used to calculate the progeny survival indices shown in Table 4.

Body Weight Evaluations

Body weights and weight gains should be recorded to determine if the treatment is having an adverse effect on food consumption or general animal well being. The following sequence will provide adequate information: parental females, weekly from selection and days 1, 4, 12, 21, and 28 following parturition; parental males, weekly from selection and at time of paring for mating; progeny, as litters on days 1, 4, and 12 following birth and individually at weaning on day 21.

Results Evaluation

Data obtained from treated groups should be compared by appropriate statistical methods to the concurrent control results. Applicable methods should be used for parameters which yield discontinuous or nonparametric results. Parental body weight gains and weight of progeny may be compared by F-test and students' *t*-test (*8*). Anomalies may be compared by either the chi-square or binominal expansion method (*8*). Reproductive and survival indices may be compared by a nonparametric rank order method (*9*). Other statistical methods may be substituted where appropriate.

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