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Challenges and Best Practices in Conducting Multigenerational Studies: A CRO Perspective

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Introduction

Multigenerational studies play a crucial role in reproductive toxicology, offering essential insights into substance-related effects across generations. However, Contract Research Organizations (CROs) face significant challenges in conducting these studies while adhering to stringent regulatory guidelines both for chemicals (OECD 416, OECD 426, OECD 443, U.S. EPA OPPTS 870.3800) and pharmaceutical compounds (ICH S5(R2)).

Among these, OECD 443 (EOGRT) studies have become particularly relevant for regulatory authorities evaluating substance registration dossiers. Their complexity requires meticulous planning due to the large number of animals involved (especially if all cohorts are included), intricate necropsy scheduling, and the demanding nature of specific assessments. A key challenge in conducting these studies is the careful selection of appropriate dosages. This poster outlines key considerations for optimizing study design, balancing regulatory compliance with scientific robustness. It also addresses critical methodologies for evaluating neurotoxicity, immunotoxicity, and ovarian follicle enumeration.

These assessments require standardized and harmonized approaches to generate reliable data for regulatory submission. Drawing on our experience, we emphasize the importance of proactive study planning, interdisciplinary collaboration, and adaptability in navigating an evolving regulatory landscape.

Dose level selection and staggered start dates approach

Despite years of experience, selecting appropriate doses for an OECD 443, with the optimal spacing between dose levels can still present challenges.

A well-defined scenario is when the maximum dose of 1000 mg/kg/day is permissible, as both dose selection and spacing are relatively straightforward. In such cases, a typical dose range would include 100, 300, and 1000 mg/kg/day. In this case, the only potential difficulty might be unexpected toxicity in 21-day-old animals; however, this critical issue could be addressed through a preliminary study.

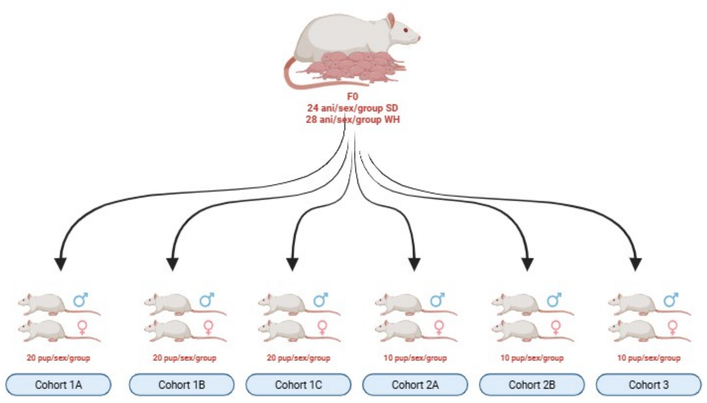
Identifying a dose that reliably induces approximately 20% toxicity, while remaining ethically acceptable and without compromising the ability to perform scheduled sacrifices is a significant challenge. The situation is further complicated in cases of sex-related toxicity, where it may become necessary to implement two distinct dosing regimens: one for males and one for females.

As a CRO, we have found it useful in some cases to split the study into two arrivals, spaced seven days apart. This approach is mentioned in Guidance Document No. 151, where it is proposed primarily to improve the planning of all study-related activities.

However, we have found it beneficial also for the following reasons:

1. In the event of unexpected toxicity, the dose levels in the first group can be reduced, and the new dosing regimen can be applied to the second batch of arriving animals.
2. Animal weight could be measured twice a week during the first week of treatment, as well as food consumption, and these parameters should also be evaluated for accurate dose selection.
3. The study can be divided into two separate rooms instead of a single large room. This is particularly advantageous considering the numerous activities concentrated around the time of birth, allowing the dam to benefit from a more controlled and less stressful environment.
4. When all cohorts are planned, it becomes easier to manage pup selection and dosing, address unexpected toxicity, perform tests such as the Auditory Startle Response, and carry out perfusion during necropsy.

Selection of cohorts (PND 21)



COHORT 1C

In cases where cohorts 2 and/or 3 are omitted, sufficient number of pups need to be maintained to investigate sexual maturation (3 per/sex/litter/dose).

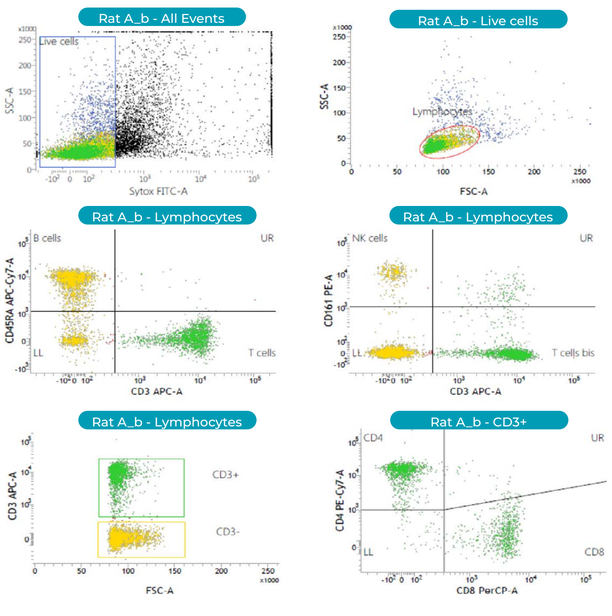
The animals could be grouped in an extra cohort labelled Cohort 1C, for example(OECD 443, 25 June 2025).

IMMUNOPHENOTYPING

Splenic lymphocyte subpopulation Cohort 1A

During the necropsy procedure the whole spleen is weighed. A portion of the spleen (approximately the half) is collected, weighed and transferred into appropriate culture medium (RPMI Dutch modified for lymphocytes). Splenocytes are mechanically separated and the main cell populations T (CD3+), T-helper (CD3+ CD4+), T-cytotoxic (CD3+ CD8+), B (CD45RA+) and NK (CD161+) are identified by means of specific antibodies and flow cytometric acquisition and analysis (BD FACS Verse).

The other part of the spleen is preserved in appropriate fixative for histopathological evaluation. The animals randomly selected for this assesment are the same randomly selected for blood collection. If the study is divided into two staggered start dates, 5 animals/sex/group will be randomly selected from the first arrival and 5 animals/sex/group will be selected form the second start.



Histopathology, Staging of the spermatogenic cycle and Ovarian follicles count Parental generation and Cohort 1A

Histopathological evaluation is performed in animals of the P generation and of Cohort 1A on tissues specified in the study plan from all males and all females in the control and high dose groups, from all animals in the low and medium dose groups killed or died during the treatment period, on all abnormalities detected during post mortem observation and in the reproductive organs of animals suspected of reduced fertility (e.g. that failed to mate or conceive). Histopathological examination plays a fundamental role in identifying a possible trigger and in adjusting the experimental design accordingly. Histopathology is key to identifying potential triggers and refining the study design. Therefore, completing the evaluation of the parental generation before the sacrifice of Cohorts 1A and 1B is advised.

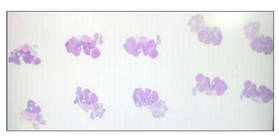
Staging of spermatogenic cycle – Parental generation and Cohort 1A

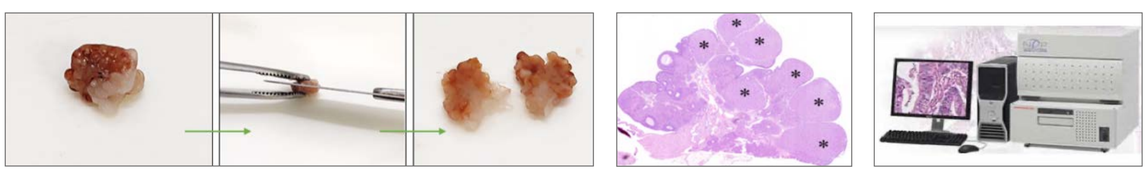
A detailed qualitative evaluation of the testes, taking into account the tubular stages of the spermatogenic cycle, is performed in all males of the control and high dose groups of the P generation and Cohorts 1A. The PAS-H-stained sections are used to identify the spermatogenic stages.

Ovarian follicles count - Cohort 1A

Ovarian follicle count is performed in 5 serial sections from one ovary in F1 females (Cohort 1A). The ovary is cut in half across its long axis and through the central suspending ligament. Both halves are then mounted in the same wax block to provide serial sections from the central plane of both halves of the ovary at the same time. From an even cut surface, the block is cut at sections of 5 microns (µm) thick, and every 20th section (or the section from every 100 µm interval) is retained from each half of the ovary, for a total of 5 double sections (or 10 single sections in total). This method is estimated to provide sections from a 1% sample of the ovary, the count should include the total number of primordial follicles, growing follicles and corpora lutea for the optimum statistical power of the sample. All serial sections are stained with haematoxylin and eosin (H&E). The criteria used for the quantitative ovarian follicle counts including tissue processing, selection of ovarian sections, follicle counting and relationship between follicle counting and functional outcome, is chosen on the basis of the main references used as guidelines and essentially their morphological differentiation is based on those proposed by Pedersen and Peters (1968) and adapted to rat species based on those proposed by Hirshfield and ReesMidgley (1978).

Number of Small follicles Levels					Number of Medium follicles Levels					Number of Large follicles Levels					Mean for animal			Number of Corpora lutea
1°	2°	3°	4°	5°	1°	2°	3°	4°	5°	1°	2°	3°	4°	5°	small	medium	large	
1	8	7	7	8	1	1	0	1	0	2	2	7	5	3	6	1	4	10
13	11	12	15	12	1	1	1	1	0	6	7	7	6	11	13	1	7	10
31	41	24	18	16	4	4	5	8	3	7	9	11	8	6	26	5	8	10
9	31	32	55	55	1	4	4	4	9	8	8	7	7	6	36	4	7	10
35	24	23	14	8	6	3	2	2	4	11	6	7	8	9	21	3	8	10
28	44	36	28	25	2	1	6	5	3	10	4	8	9	6	32	3	7	15
7	9	10	32	44	1	3	8	6	5	2	6	5	7	12	20	5	6	12
11	17	17	7	15	2	3	1	2	2	6	6	5	1	4	13	2	4	12
12	18	10	17	8	2	6	4	5	2	6	9	5	6	6	13	4	6	15
21	13	29	17	23	2	2	4	4	5	11	6	10	8	7	21	3	8	13
18	28	20	18	25	4	2	2	6	4	9	9	4	8	8	22	4	8	8
30	19	21	19	26	2	4	1	1	3	9	10	11	6	5	23	2	8	11
4	7	4	7	10	1	3	6	1	1	7	6	7	6	10	6	2	7	10
16	26	13	16	19	4	3	6	7	7	6	8	14	7	13	18	5	10	13
9	24	28	9	13	3	1	4	5	3	11	7	6	5	4	17	3	7	11
21	18	21	18	18	6	4	0	2	7	12	4	4	12	9	19	4	8	13
16	9	8	28	27	0	3	1	5	0	6	6	5	10	5	18	2	6	12
2	17	12	11	13	2	3	2	2	3	5	8	6	7	7	11	2	7	11
21	10	21	16	22	3	2	2	8	2	1	7	8	10	10	18	3	7	16
12	13	18	23	22	2	4	3	8	4	3	8	7	11	6	18	4	7	14
														Mean	19	3	7	12
														SD	7	1	1	2
														N	20	20	20	20

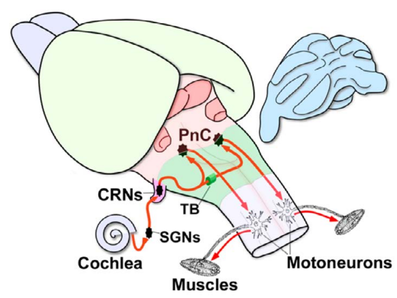




NEUROTOX ASSESSMENT

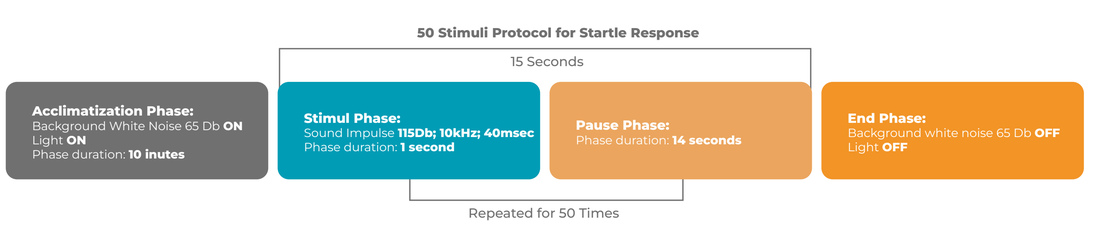
Startle Reflex (Acoustic Stimuli) – Cohort 2B

The evaluation of startle reflex response to acoustic stimuli is widely used for sensorimotor gating and hearing deficiency. The guideline states that the test should be conducted on postnatal (PND) interval 24–25. We have chosen to perform it on PND 24 before dosing.



Each animal is placed in the experimental chamber inside a device to restrict its movements. Initially a background white noise of 65 Db is set. The eight chambers (8 pups can be testes at the same time) are equipped with a loudspeaker to generate the stimuli (pure sound 115 Db 40 msec 10 KHz for 1 second) and loadcells to record animal movements. Each stimulus is followed by a 14-second pause. Each animal is subjected to 50 sound stimuli during which the amplitude related to each movement was recorded. In performing the auditory startle test, the mean response amplitude on each block of 10 trials (5 blocks of 10 trials) is determined.

The test requires approximately 15 minutes/animal. When the study is splitted between 2 staggered start date, there is the possibility to analyze the first set of data and follow-up observations.



Best Practise

- Use staggered start date to enhance scheduling, to manage the complex test such us Auditory Startle Response and whole-body perfusion at necropsy.
- Use of an additional Cohort (Cohort 1C, 20Pup/sex/litter) in case of that Cohorts 2 and 3 are omitted. Three pups/sex/litter are necessary to enhance statistical power for evaluation of sexual maturation (preputial separation and vaginal opening)
- Complete the histopathological evaluation of the parental generation, before the sacrifice of Cohorts 1A and 1B

References

- Hirshfield AN et al. Morphometric Analysis of Follicular Development In the Rat. Biology of reproduction 1978, 19: 597-605
- Pedersen T & Peters H Proposal for a classification of oocytes and follicles in the mouse ovary. Journal of Reproduction and Fertility 1968, 17: 555–557