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Control data on endocrine sensitive endpoints for untreated Sprague-Dawley rats from the Ramazzini Institute colony

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Summary. *Background and aim:* Findings from laboratory animals as well as human studies suggest that Endocrine Disrupting Chemicals (EDCs) cause a number of reproductive health outcomes. Rats have been used extensively for developmental and reproductive physiology and endocrinology research and a number of endocrine sensitive endpoints have been well established in a variety of regulatory guidelines on rodent bioassays. We monitored the background data on some endocrine sensitive endpoints for untreated Sprague-Dawley rats from the Cesare Maltoni Cancer Research of the Ramazzini Institute colony (SD-CMCRC/RI). *Materials and methods:* General reproductive indices from dams and data for the entire litter were recorded. All the littermates were retained until the achievement of puberty and balanopreputial separation (BPS) was monitored in all the males; estrous cycle length and pattern were also evaluated in one female/litter. We compared our data with those provided by the Health and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI). *Results:* Overall, reproductive indices and pre-post weaning litter data of SD-CMCRC/RI rats were comparable with those reported by ILSI. *Conclusions:* Procedures for monitoring and physiological biological variations in our SD-CMCRC/RI rats fall within the range of values typically obtained for the selected endpoints. Further investigations are suggested in order to verify whether retaining all pups to sexual maturation can improve the sensitivity to discriminate between natural variation and treatment effects. A more comprehensive analysis of other relevant endocrine sensitive endpoints should be performed in order to provide a representation of the normal developmental landmarks and endocrine values at different ages.

Key words: endocrine endpoints, historical control data, Sprague-Dawley rats

Introduction

Findings from laboratory animals as well as human studies suggest that Endocrine Disrupting Chemicals (EDCs) cause a number of reproductive health outcomes, including abnormal puberty, irregular estrous cycle, reduced semen quality, testicular dysgenesis syndrome and other adverse effects involving disruption of the Hypothalamus-Pituitary-Gonadal (HPG) and/or Hypothalamus-Pituitary-Thyroid (HPT) axis (1).

The laboratory rat is widely used as the traditional animal model of choice for research on developmental and reproductive toxicity testing, conducted to support human health hazard identification and risk assessment. Considering the substantial conservation of reproductive process across rat and human, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended the laboratory rat as the species of choice for the endocrine screening and testing assays (2, 3).

A number of endocrine-related endpoints has been well established in a variety of regulatory guidelines on rodent bioassays, including areola/nipple retention and anogenital distance in pups at birth; balano-preputial separation (BPS) in males and day of vaginal opening (VO) in females as primary landmarks of sexual development. Information on the integrity and performance of both male and female reproductive systems, including gonadal function, estrous cycle, mating behavior, conception, gestation, lactation, and the growth and development of the offspring are also addressed by current test methods focusing on developmental and reproductive toxicity. All these endpoints, sensitive to endocrine disruption, are well recorded in many Organization for Economic Co-operation and Development Test Guidelines (OECD TGs) such as the Two-Generation Reproduction Study (OECD TG 416) (4), the Extended One-Generation Reproduction Study (OECD TG 443) (5) and the Developmental Neurotoxicity (DNT) study (OECD TG 426) (6). The National Toxicology Program (NTP) has also developed a range of techniques and testing regimes to evaluate the potential of environmental and occupational substances to affect development and damage reproductive systems. The NTP's Modified One-Generation Reproductive study design (MOG) provides information on the effects of substances on prenatal development, postnatal development, and reproduction (7). Recently, many OECD TGs have been revised placing additional emphasis on endocrine endpoints; the need for careful clinical observations of the animals, so that to obtain as much information as possible, is also stressed (8, 9).

The use of rodent models for research and testing on EDCs needs an awareness of a number of laboratory animal science issues in order to standardize methods of monitoring thus facilitating the reproducibility of results among laboratories (10).

We monitored untreated Sprague-Dawley (SD) rats belonging to the colony of the Cesare Maltoni Cancer Research Center of the Ramazzini Institute (CMCRC/RI) in order to provide background data on some endocrine sensitive endpoints for interpreting experimental results in developmental/reproductive studies.

General reproductive indices of the dams were recorded for subsequent interpretation of reproductive health effects of a tested substance. Indeed, changes in reproductive indices can be due to several factors, including alteration in hormone levels and fetal growth retardation (11).

Data for the entire litter, including litter size and sex ratio, were reported as an important endpoint in the overall evaluation of reproductive performance. A decreased litter size may indicate an adverse reproductive effect and can be used as a nonspecific indicator of reproductive toxicity. Altered sex ratios may be related to several factors, including selective loss of male or female offspring, sex-linked lethality (genetic germ cell abnormalities), abnormal production of X or Y chromosome-bearing sperm, or hormonal alterations that result in intersex conditions (masculinized females or feminized males) (11).

All the littermates were retained until the achievement of puberty without performing culling (reduction of litter size) a widely used procedure in reproductive toxicity studies (12). The BPS was monitored in males. Cleavage of the balano-preputial gland is an apical measure of the progression of puberty and it has been used as the primary endpoint of puberty onset in the male rat as it is an androgen dependent event (13, 14).

In one female per litter, the estrous cycle pattern was determined by observing changes in the vaginal smear cytology. Vaginal cytology is known to be dependent upon the hormonal balance and to respond rapidly to the administration of chemical possessing hormonal activity as, for example, oestrogenic agonist or antagonist activity. The inclusion of an assessment of estrous cyclicity, by examination of vaginal smears or washes, offers a quick and easy way to measure the sex hormone status within the female and is of value in interpreting other findings (for example, weight or pathological data for the female reproductive organs). Potentially this technique could also act as a simple initial marker of changing reproductive capacity with age in chronic studies (11).

A comparison with the laboratory's historical control data is an important aid to determine whether small increases or decreases (including not statistically significant ones) in an endpoint might constitute a treatment-related effect. As part of this evaluation, we compared our data with those provided by the Health

and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI) that provided a retrospective analysis of 43 multi-generation studies (16 in Wistar rats, 27 in Sprague-Dawley rats) conducted according to the United States Environmental Protection Agency (U.S. EPA) Reproduction and Fertility Effects Test Guideline (OPPTS 870.3800/OECD 416) (15).

Materials and methods

Male and female SD rats belonging to the colony used in the laboratory of the CMCRC/RI for over 40 years were used in the experiment. All the animals were kept in a single room at 23±3°C and at 40-60% relative humidity. The light/dark cycle was 12 hours. Rat feed (Dr. Piccioni Laboratory, Milan, Italy) and tap water were available *ad libitum*. Each lot of feed and tap water was periodically analyzed for biological (bacteria) and chemical (mycotoxins, pesticides, arsenic, lead, mercury, selenium) contaminants.

Eleven virgin female rats were cohabited with 11 breeder male rats of the same strain, one male per female, never brother and sister. Every day, the females were examined for presence of sperm by vaginal cytology. The day in which sperm was found in vaginal canal was defined as Day 0 of pregnancy (GD 0). The fertility index was defined as the number of animals inducing pregnancy or becoming pregnant divided by the number of mating sets. The gestation index was reported as the percentage of pairs with confirmed mating that have produced at least one pregnancy within a fixed period. Mean gestational length (duration of pregnancy) was the time from GD 0 to parturition. The day birth occurred was designated as post natal day 1 (PND). Each dam and delivered litter were housed in a common nesting box during the postpartum period. Newborns were housed with their mothers until weaning at PND 28. Sex was determined on PND 1 and sex ratio data was presented as percentage of males to total number of offspring. The mean litter size, including dead as well as live offspring, was calculated on PND 1. We totally evaluated 136 pups, 67 males and 69 females.

All the littermates were observed until the achievement of sexual maturity.

Starting on PND 35 until completion, all the males were examined daily (between 9:00 A.M. and 12:00 P.M.) for BPS. Each male rodent was removed from its cage and held in a supine position. Gentle digital pressure was applied to the sides of the prepuce, and the criterion was met when the prepuce completely retracts from the head of the penis. Each male rodent was examined daily until acquisition.

Starting from young adulthood (approximately PND 120) and for the duration of 3 weeks, daily vaginal lavage was performed on one female/litter. The female rat was removed from the cage and approximately 0.25 ml of physiological saline solution were drawn into a new clean dropping pipette. The tip of the pipette was gently inserted into the vaginal canal, the pipette bulb was firmly but gently depressed to expel the saline into the vagina and the saline was drawn back into the dropping pipette which was removed from the vaginal canal. A spray fixative (Cytofix™ Fixation Buffer, BD Biosciences, supplied by Di Giovanni srl, Bologna, Italy) was applied onto the slide prior to Papanicolaou stain. By using Papanicolaou staining, the maturity of nucleated epithelial cells can be distinguished with less mature cells stained turquoise and more mature cells pink- or orange-stained. Briefly, slides were successively submerged in alcohol 95%, 80%, 70% and water, then stained with Harris' Hematoxylin solution (Labochimicha srl, Padua, Italy). After a brief dipping in diluted hydrochloric acid and water to remove excess stain, the cells were dehydrated prior to immersion in the Orange G (Labochimicha srl,), an alcohol based cytoplasmic counterstain which stains keratin in brilliant orange. Slides were raised off in 95% alcohol and stained with the second counterstain, Eosin-Azure (E.A.) 50 (Labochimicha srl,), and rinsed off in 95% again. Finally, slides were immersed in absolute alcohol to dehydrate completely and in xylene. Slides were mounted with the Permount, then coverslipped and observed under a light microscope. The cytology of the vaginal smears allowed a classification in the following estrous stages: diestrus (D), predominance of leukocytes and a few scattered cornified epithelial cells; proestrus (P), predominance of round nucleated epithelial cells that may be dispersed or clumped; or estrus (E), all cornified cells (16). All the vaginal smear slides were evaluated by two pathologists in blind and

any discrepancy was solved by final consensus. For each female, measurement of estrous cycle length was performed by selecting the estrous stage and counting until the recurrence of the same stage. An analysis of estrous cycle pattern was also performed and reported as percentage of time in each stage.

Results

Results for dams and pre-weaning pups of SD-CMCRC/RI rats are reported in Table 1. The female's ability to achieve pregnancy, calculated as fertility index, turned out to be 91.6%. All the pregnant dams maintained pregnancy and delivered live pups (gestational index equal to 100%). The eleven dams displayed a similar gestational length (22.9±0.8 days). The mean litter size was 12.4±2.2 and sex ratio at birth (% males/total offspring) was 48.5±9.8.

Data on post-weaning endpoints are presented in Table 2. Balanopreputial separation, evaluated in all the littermates, was achieved at PND 45.0±1.9. The mean estrous cycle length, evaluated in one female/litter, was 4.9±0.3 days. Estrous cycle pattern, evaluated over a 3-week monitoring period, revealed a percentage of 51.4±9.2 days in diestrus; 24.8±6.3 in proestrus and 23.8±4.5 in estrus. The comparison of dams and pre-post weaning data of pups between SD-CMCRC/RI rats and inter-Laboratory control SD- derived rats data provided by ILSI is reported in Table 3.

Table 1. Dams and pre-weaning litter data from SD-CMCRC/RI rats.

Parameter	SD- CMCRC/RI
Fertility index (%)[a]	91.6
Gestational index (%)[b]	100 (11/11)
Mean gestational length (day) [c,d]	22.9±0.8
Total pups (n) delivered at PND 1 [e]	136
Litter size (n) [d,f]	12.4±2.2
Total male pups (n) at PND 1	67
Total female pups (n) at PND 1	69
Sex ratio at birth (%) [d,g]	48.5±9.8

[a:] Fertility index = (number of pregnant females/number of females cohabitated) x 100
[b:] Gestational index = (number of females with live born / number of females with evidence of pregnancy) x 100
[c:] Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition
[d:] Mean ± standard deviation
[e:] Live and stillborn pups are considered
[f:] Mean number of pups per litter at PND 1 (within 24 hours from delivery)
[g:] Sex ratio at birth = (no. of male offspring/no. of total offspring) x 100

Table 2. Post-weaning landmarks of pups from SD-CMCRC/RI rats.

Parameter	SD- CMCRC/RI
Age (PND) at balano-preputial separation (BPS) [a]	45.0±1.9
Estrous cycle length (days) [a]	4.9±0.3
Time in diestrus (%)[a]	51.4±9.2
Time in proestrus (%)[a]	24.8±6.3
Time in estrus (%)[a]	23.8±4.5

[a:] Mean standard deviation

Table 3. Comparison of dams and pre-post weaning data of pups between SD-CMCRC/RI rats and SD-derived rats*.

Parameter	SD- CMCRC/RI	SD-derived*
Fertility index (%)[a, b]	91.6	89.8±5.9
Gestational index (%)[b, c]	100	99.2±2.6
Mean gestational length (day) [b, d]	22.9±0.8	22.1±0.4
Litter size (n) [b, e]	12.4±2.2	13.7±0.9
Sex ratio at birth (%) [b, f]	48.5±9.8	52
Age (PND) at balano-preputial separation (BPS)[b]	45.0±1.9	45.3±2.1
Estrous cycle length (days) [b]	4.9±0.3	4.2±0.4

* Crl:CD®(SD)IGS BR, Crl:CD® (SD)IGS BR–VAF/Pluss, Crl:CD (SD), Crl:CD® (SD) BR, Crl:CD® BR, Crl:CD® BR–VAF/Pluss, CD®
[a]: Fertility index = (number of pregnant females / number of females cohabitated) x 100
[b:] Mean ± standard deviation
[c:] Gestational index = (number of females with live born / number of females with evidence of pregnancy) x 100
[d:] Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition
[e:] Mean number of pups per litter at PND 0 (within 24 hours from delivery)
[f]: Sex ratio at birth= (no. of male offspring/no. of total offspring) x 100. Standard deviation for control values is not reported by Marty MS et al. 2009

Discussion

Comprehensive historical control data are important in toxicity studies, as comparisons of data from study controls with historical ones may help to distinguish treatment-induced changes from spontaneously occurring background changes specific to species and strains (17).

Caution should be taken particularly when comparing certain endpoints, such as endocrine-related endpoints, with historical control databases from other laboratories, owing to possible inter-laboratory differences in procedures and classification schemes. Furthermore, subtle changes in species occur over time, owing to genetic alterations in strains or stocks of species and to change in environmental conditions, both in breeding colonies and in individual laboratories (17).

In our work, the reproductive indices and preweaning litter data of SD-CMCRC/RI rats were comparable with those reported by ILSI.

Interestingly, for SD-derived rats, data were separated by ILSI into litters that were standardized (i.e., culled) or not. In our work, pups were not culled, all the littermates were retained until the time of puberty. Culling is a procedure of artificial equalization of the number of offspring in litter used in rodent experiments to control litter size (18). The rationale for unculling litters is based on the possibility to explore the litter variability and to improve the sensitivity of the statistical analysis in detection of statistically significant and biologically important differences in maturational endpoints. Further statistical analysis on individual data from different laboratories could help to demonstrate whether culling evaluation of all the littermates *vs* one or two pups/sex/litter influences the outcome of data by reducing the probability of identifying a false negative result.

We also evaluated some endocrine relevant endpoints that are currently required by the OECD TGs, i.e BPS in males and estrous cyclicity in females.

The mean ages at BPS in SD-derived rat, reported by ILSI, ranged between 41.2 and 49.0 days, with a mean of 45.0±1.9 days. These values were remarkably closed to those obtained by the SD-CMCRC/RI male rats (45.3±2.1), indicating that the assessment was conducted in a consistent manner within and between studies. It is also noteworthy that BPS was monitored by the examination of all littermates. This procedure represents a new and interesting perspective, indeed, sexual maturation assessments are usually performed on only one weanling rat per sex after litter standardization or culling. For these reasons, the reported historical control values are usually based on observations for one weanling pup/sex/litter. Concern is expressed that culling could affect many health-related endpoints, including the onset of developmental landmarks and sexual maturation (12, 18).

In females, estrous cycle data are used to complement other data and do not typically indicate an adverse effect alone. Cooper and Goldman (19) reported that estrous cycle pattern is an important parameter in order to detect changes that might be masked when only examining estrous cycle length. Altered estrous cyclicity or complete cessation of vaginal cycling in response to toxicants should be considered an adverse female reproductive effect. In our work, data on estrous cycle length were within the expected range (e.g., 4-5 days). While estrous cycle length was reported in the ILSI review, estrous cycle pattern was not included due to the lack of the evaluation or of an agreed-upon method for correctly assessing cycle normality and duration (15). Consequently, a comparison for this endpoint was not possible.

Conclusions

Overall, the data for endocrine sensitive endpoints from our untreated SD-CMCRC/RI rats are comparable to the value reported in the scientific literature, suggesting that procedures for monitoring and physiological biological variations in our SD rats fall within the range of values typically obtained for the selected endpoints. In particular, BPS values were comparable for unculled SD-CMCRC/RI rats and other culled SD-derived rats. Further data on other relevant endocrine sensitive endpoints, such as anogenital distance, vaginal opening, first estrus and relative body weight at the time of acquisition, sperm analysis need to be investigated in our SD colony in the future, in order to provide a more comprehensive perspective

for interpreting data from treated animals, particularly with regard to reproductive and developmental toxicity bioassays.

Author contribution:
Fabiana Manservisi, Laura Falcioni, Luciano Bua, Ilaria Menghetti: concept and design of study, data collection, data interpretation and analysis, drafting, revision, approval of final manuscript; Daniele Mandrioli, Giovanna Galeati, Marcella Spinaci, Carlo Tamanini and Fiorella Belpoggi: data interpretation and analysis, critical revision of the entire text, approval of final manuscript.

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