

A Multi-Laboratory Round Robin Comparison of Mammary Gland Whole Mounts Contributes to Protocol Standardization

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Abstract

The development of the mammary gland (MG) – particularly, the timing and extent of epithelial growth and ductal branching – can be affected by prenatal and early postnatal exposure to certain endocrine disrupting chemicals, including pharmaceuticals and dietary constituents. These developmental effects have been observed in MG whole mounts using varied preparation and evaluation techniques. Thus, current studies of mammary gland development and risk assessment in part because it is not a standardized endpoint for chemical toxicity testing. In order to improve reliability and standardization of MG whole mount assessments, in Nov. 2009, experts from 7 laboratories in Canada, U.S., and Argentina participated in a round robin evaluation of MG whole mounts from mice and rats treated *in utero* with vehicle and two different chemicals. MGs from female offspring at postnatal days (PND) 4, 21, and 45 were evaluated by each laboratory using unspecified techniques. Glands were evaluated for alterations in development including the timing and extent of growth and branching. All 7 laboratories reported similar findings of significantly altered morphology at day 45 for both treatments in mice (rats not evaluated). For PND 21 Mice, 5/7 and 6/7 labs reported altered morphology in both rats and mice. There was no consensus on the effects in either species on PND 4. At the conclusion of this exercise, the laboratories reached consensus on many aspects of study design and whole mount assessment methods and these details contribute to standardizing protocols for MG whole mount preparation and evaluation. With further validation, the assay could be added to existing chemical testing protocols or those under development at EPA and OECD. Research needs were also identified by the participating experts. Disclaimer: The views expressed are those of the authors and do not necessarily reflect the views or policies of NIHES.

Introduction

The development of the mammary gland (MG) – particularly, the timing and extent of epithelial growth and ductal branching – can be affected by prenatal and early postnatal exposure to certain endocrine disrupting chemicals, including pharmaceuticals and dietary constituents. These developmental effects have been observed in MG whole mounts using varied preparation and evaluation techniques. Thus, current studies of mammary gland development are not always comparable and data from whole mounts are often not considered in regulatory risk assessments, in part because it is not a standardized endpoint for chemical toxicity testing. In order to improve reliability and standardization of MG whole mount assessments, in Nov. 2009, experts from 7 laboratories in Canada, U.S., and Argentina participated in a round robin evaluation of MG whole mounts from mice and rats treated *in utero* with vehicle and two different chemicals.

A meeting of the participating laboratories was conducted in conjunction with an interdisciplinary workshop: Mammary Gland Evaluation and Risk Assessment, November 16-17, 2009, in Oakland, CA (see also SOT #2040).

Methods

A. Animals

- 25 time-pregnant CD-1 mice (Charles River)
- 25 time-pregnant Long Evans hooded rats (Charles River)
- All litters (mice and rats) equalized to 10 pups at birth, after pooling by treatment – 5 to 7 females per litter
- All animals had NIH-31 chow and water *ad libitum*, 12 hr light cycle, autoclaved white paper bedding and nesting materials

B. Treatments

- MICE: Vehicle, Treatment A and B gavage dosed starting on GD9
- RATS: Vehicle, Treatment A and B by gavage dosed starting on GD13
- Both species, 8-9 dams/treatment groups
- Collected body weight, trunk blood, and mammary glands (4 and 5 from both sides of the animal) from females on PNDs 4, 21, and 45
- No VO or smears, but serum is stored at -80°C
- Mammary glands were mounted onto charged slides, covered with Parafilm, a second slide and an inverted 50 ml conical tube filled with water. Sat for 45 min to 1.5 hr.
- Mounted slides fixed in Carnoy's (ethanol, chloroform, and glacial acetic acid; 6:3:1), rinsed in water, Carnex's stain for 24-48 hr, and increasing alcohols. Cleared in xylene and permanently mounted under Permount. Dried for 2-4 weeks before transit.
- Seven laboratories reviewed the slides blind to treatment status. No uniform methodology was established prior to the exercise.

EXPERT PANEL/ROUND ROBIN LABORATORIES

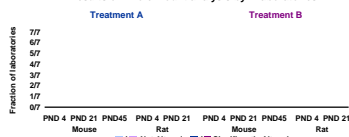
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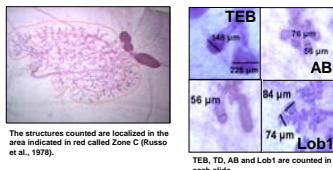
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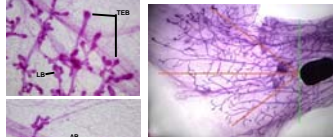
Results of whole mount analysis by 7 laboratories



All seven laboratories reported statistically significant altered mammary gland morphology from two different treatments at PND 45 in mice (rats not evaluated at PND 45). The majority of the laboratories also reported abnormal development in rats and mice from both treatments at PND 21. Findings at PND 4 were generally consistent with no effect for Treatment B and mixed for Treatment A in both rats and mice.



The structure outlined are localized in the area indicated in red called Zone C (Russo et al., 1978).



Budding and Developmental Pace:
• Lateral (LB) and alveolar (AB) budding important in the rat
• Budding is a precursor to branching and indication of stage of differentiation
• Visually assess slides over time to compare the developmental pace of growth and differentiation.

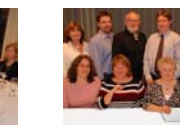
Results

Protocol – Areas of agreement

- Sample size - single randomly selected pups from ~10-12 litters will produce the data needed to detect differences
- Litter size must be equalized at birth or soon after, keeping both male and female pups in the litter.
- Body weight of the animal should be measured just prior to the time of necropsy.
- PND 45 was identified as the optimal time for whole mount assessment in this evaluation. Other useful time points include PND 4, 21, and 90, and age at VO.
- The 4th and 5th mammary glands of the mouse and rat are the most easily accessible and reliable indicators of mammary developmental toxicants
- Histopathology is suggested in frontal plane orientation so the gland profile is comparable to the mammary whole mount.
- Timing of VO should be assessed, as well as estrous status at necropsy.
- Use of digital images is recommended
- The definition of terminal end buds in rats is teardrop-shaped ductal end structures that are 100 mm or greater in diameter between birth and about 100 days after birth. In mice, the structure would measure 0.03 mm² or larger.
- The following parameters/end points should be measured (quantitatively) in both rat and mouse mammary gland whole mounts:
 - Ratio of terminal end buds to other end types present.
 - Distance of epithelial outgrowth from the nipple toward the lymph node.
 - Distance from the lymph node to the end of longest extended duct.
- For mouse only:
 - Visually assess the relative amount of side branching, presence of lobules/alveoli, and duct thickness.
 - Digitally assess branching density on both sides of the lymph node because duct branching density is asymmetric and known to be greater closer to the nipple.
- For rat only:
 - Visually assess budding, duct width, and differentiation from the lymph node to the outer edges.
 - Digitally assess the density in Area C if possible (outer 5 mm margin of the gland)

Protocol – Areas for continued discussion

- Use of inbred vs. outbred strains
- Assessment of phytoestrogen content of chow and laboratory contamination from test compound
- Measurement of internal dose
- Methods for removing mammary glands and for fixing, staining, and clearing



Measurement of epithelial outgrowth:
• Average distance to several end points
• Draw an imaginary perpendicular line at the growing edge of the MG and measure distance to the lymph node
• At the time of VO, measure distance between glands #4 and #5 to assess stage-specific growth. Grown together in rat by PND 45

Conclusions

There was good agreement between the 7 laboratories on whether MG development had been altered from the two treatments. All 7 laboratories reported similar findings of significantly altered MG morphology at PND 45 for both treatments in mice (rats not evaluated), and a majority of labs (5/7 and 6/7) reported altered morphology in rats and mice at PND 21. At the conclusion of the exercise, laboratories reached consensus on many aspects of study design and whole mount assessment methods. They also unanimously agreed:

- Mammary gland whole mount preparations should be routinely incorporated into test protocols that include *in utero* or early life exposures in order to determine potential effects of chemicals on early life mammary gland development. They further noted that histopathology alone was not suitable for detecting altered mammary gland morphology, but could be useful in conjunction with whole mounts.
- Mammary gland alterations they had observed were developmentally abnormal and are of concern. These changes could suggest:
 - An increased potential to develop intraductal hyperplasia, ductal bridging, or other preneoplastic conditions often present by 90 of age.
 - Altered potential for spontaneous tumor development.
 - Altered susceptibility to chemical carcinogens or another exogenous insult (a second "hit").
 - Potential for functional abnormality (inadequate lactation).
 - Indication that endocrine disruption has occurred (other tissues should be evaluated for effects).

Discussion

With further validation, mammary gland whole mount assessment could be added to existing chemical testing protocols or those under development at EPA and OECD, including:
• OECD extended one generation developmental reproductive study
• EPA Endocrine Disruptor Screening Protocol male and female pubertal
• NTP Reproductive Assessment by Continuous Breeding
A standardized protocol is being developed at NTP/NCTR and will be published shortly. Recent experimental work suggests male MG development is sensitive to estrogens and so both male and female MG should be examined.

Another round robin exercise is being considered

Acknowledgements

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References

Russo and Russo, 1978, J Natl Cancer Inst 61:1451-1459