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GUIDANCE DOCUMENT FOR HISTOLOGIC EVALUATION OF ENDOCRINE AND REPRODUCTIVE TESTS IN RODENTS

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No. 106

# GUIDANCE DOCUMENT FOR HISTOLOGIC EVALUATION OF ENDOCRINE AND REPRODUCTIVE TESTS IN RODENTS



INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among UNEP, ILO, FAO, WHO, UNIDO, UNITAR and OECD

**Environment Directorate** 

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#### FOREWORD

This document only includes Part 1 of the Guidance Document for Histologic Evaluation of Endocrine and Reproductive Tests in Rodents, and the annex to the Guidance Document. All other parts are posted directly on the public website due to the size of the document. The proposal for developing this Guidance Document as part of the project for updating TG 407 (Repeated Dode 28-Day Oral Toxicity Study in Rodents) was approved by the Working Group of National Coordinators of the Test Guidelines Programme (WNT) in 2007.

Guidance was recommended by the peer review panel following validation studies of the updated TG 407 study. The Validation Management Group (VMG) for mammalian testing and Endocrine Disrupter Testing and Assessment (EDTA) Task Force supported this recommendation during meetings in 2006. In January and March 2007, a draft Guidance Document, developed by a few consultants, was reviewed by the VMG-mammalian and the EDTA Task Force respectively; the WNT confirmed that such a draft should be developed. In 2008, the EDTA Task Force and WNT meetings were invited to comment on the draft Guidance Document and the WNT agreed that the document be presented at several workshops, in Europe and in the United States.

In May 2008, the WNT was invited to send written comments on the revised draft Guidance Document and the Secretariat provided the new EDTA Advisory Group (AG) and WNT with information on the workshops, and recommended that regulators attend the workshops. A few issues identified by the consultant were submitted (i) to the workshops with the request to provide scientific interpretation, and (ii) to the WNT and EDTA AG with the request to provide regulatory interpretation.

The guidance was presented to pathologists and the general scientific community at a series of meetings in June and September 2008 (the Society of Toxicologic Pathologists and the Teratology Society Meetings; and the European Teratology Society/European Society of Toxicologic Pathologists meeting). The presentation took the form of posters, workshops and handouts of the guidance. Feedback was taken from all the meetings. Comments on the guidance were also received from the VMG mammalian, EDTA AG and WNT. These have all been incorporated into the final Guidance Document.

A very short annex *Considerations on Endocrine data interpretation for the updated TG 407* was prepared on the basis of the comments received from the WNT and from the above meetings.

The WNT approved the draft Guidance Document, with a few changes to the introduction, at its meeting held on 30 March-2 April 2009. This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and the working Party on Chemicals, Pesticides and Biotechnology of the OECD.

This document is published on the responsibility of the Joint Meeting of the Chemicals Group and Management Committee of the Special Programme on the Control of Chemicals of the OECD.

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# ENDOCRINE DISRUPTION: GUIDANCE FOR HISTOPATHOLOGIC EVALUATION

## **General Introduction**

This Guidance Document has been prepared to provide the pathologist reading slides from endocrine and reproductive studies (eg the updated TG407 and longer studies) with sufficient information to be able to identify the often subtle histopathologic changes that are associated with endocrine disruption in target tissues.

The histopathology guidance was initially developed to assist pathologists involved in Test Guideline 407 studies. A need for guidance was suggested by the peer review panel following validation studies of the updated TG407 study and the VMG-mammalian and the EDTA TF endorsed this during meetings in 2006. The guidance focuses on areas that were inconsistently reported during validation studies ie male and female reproductive tissues, pituitary gland and mammary glands. It aims to provide a consistent approach to terminology and grading and advice on morphological changes associated with endocrine disruption pathways. After positive feedback from these groups it was proposed that the guidance could be used for other reproductive/endocrine studies in addition to the TG407.

The guidance was presented to pathologists and the general scientific community at a series of meetings through 2008. (The Society of Toxicologic Pathologists, The Teratology Society Meetings and The European Teratology Society/European Society of Toxicologic Pathologists). The presentation took the form of posters, workshops and handouts of the guidance. Feedback was taken from all the meetings. Comments on the guidance were also received from the VMGmammalian, EDTA and WNT. These have all been incorporated into the final guidance document

The peer review panel also proposed that guidance could be provided for data interpretation on the following points (from the TG407 studies):

- differences in the end-points that do not reach statistical significance
- distinction between findings that are consistent with endocrine disruption and non-specific findings that are not related to potential endocrine disruption
- weight differences for body-weight independent organs
- additivity of findings in a number of organs

The VMG-mammalian and EDTA also suggested that the guidance should be discussed by workshops within the pathology community so that a scientific consensus could be reached on histopathologic interpretation for these organs. The points about data interpretation were also discussed at these workshops. In addition the WNT was asked to provide comments on the points.

The discussions at the workshops and comments provided by countries via the WNT were not conclusive and did not provide clear guidance on the four points of interpretation raised by the peer review Panel. A summary of the comments provided is given in Annex 1. It should be stressed that interpretation from the results should consider all the endpoints. A decision on whether a chemical is causing endocrine disruption should be taken on the weight of evidence, based on the results from a number of tests and not solely on histopathology data.

It is also acknowledged that the study of endocrine effects is a rapidly evolving area and evidence accruing subsequent to the writing of this guidance should be taken into account in the interpretation of data. The OECD is committed to developing a framework for evaluating endocrine activity using standardized, validated and internationally harmonized test methods. It is important to ensure that these methods are conducted in a reliable manner to facilitate an unambiguous interpretation.

# **Organisation of the guidance**

Each section of the guidance has been structured to provide the following information on potential target tissues for endocrine disrupting chemicals:

- 1. Normal structure and function
- 2. Normal morphological variation of structure
- 3. Morphological patterns associated with hormone disruption
- 4. Recommended terminology and severity grading
- 5. Critical aspects of histopathologic evaluation
- 6. References

#### **Normal Structure and Function**

When evaluating an endocrine tissue for evidence of hormone imbalance it is essential to be familiar with the normal structure and function of the tissue. When dealing with a functional unit such as the male and female reproductive systems it is also necessary to consider the various parts (testis, epididymis, prostate and seminal vesicles for male and ovary, uterus, and vagina for female) as integrated units because there is considerable functional and endocrinologic interdependence of the tissues on one another. Section 1 of each tissue will provide a brief review of the normal physiology and morphology of the tissue that is deemed relevant for identifying changes in hormonal balance.

# Normal Morphological Variation of Structure

The results of hormone imbalance (endocrine disruption) can produce subtle histopathologic changes that can be challenging for the pathologist to recognize. Although it is important to identify these subtle indicators of endocrine disruption, it is also important that the pathologist does not mistakenly over diagnose changes that constitute normal morphological or physiological variation of structure within the tissues being examined. The second section provides examples of common variations that may be mistaken for drug induced changes.

#### **Morphological Patterns of Hormone Disruption**

There are many different ways of disrupting hormonal balance including altered steroidogenesis, altered androgen/estrogen metabolism, hormone receptor agonism/antagonism, increased clearance of hormones and administration of estrogenic/androgenic xenobiotics. In many cases, the pattern of changes in the various hormone dependant tissues provides an indication of the underlying cause of the hormone disruption. To aid interpretation of these underlying causes, some of the most common patterns of change associated with specific endocrine disruption mechanisms are described for each system.

# **Recommended Terminology and Severity Grading**

To try and ensure consistency of identification and interpretation of changes associated with endocrine disruption, it is critical that consistent terminology, consistent grading and consistent thresholds of

recording are used by those charged with the task of examining TG407 studies. The guidance document provides a list of recommended terminology with their diagnostic criteria and some suggested criteria for grading severity of findings.

# **Critical Aspects of Histopathologic Evaluation**

This guidance document has been prepared to aid the pathologist in detecting changes that are characteristic of endocrine disrupting chemicals. The endocrine system is, by its nature, a dynamic self regulating system that functions within a range of "normal variability". This normal physiologic range is reflected by a normal range of morphological features and it is important that the pathologist does not over interpret these normal variations in structure, especially since their evaluation of "normality" within the study is based on only 5 control animals/sex. The final section 5 provides guidance on the critical aspects of the histopathologic evaluation that will help the pathologist to identify those changes that are outside the "normal" expected range of variation. This attempts to provide practical guidance on things to be aware of so that you do not under or over diagnose changes.

#### References

Finally, a reference list is provided for each organ system that provides additional reading material when additional detail is needed.

# PART 1: GUIDANCE FOR DISSECTION AND TRIMMING OF ENDOCRINE TISSUES

A comprehensive set of Guides for Organ Sampling and Trimming in Rats and Mice has been devised and published online and in a series of manuscripts in Experimental Toxicologic Pathology by the Registry Nomenclature Information System (RENI). The guides can be readily accessed online from the following website: <u>http://www.item.fraunhofer.de/reni/trimming/trimm.php?lan=en</u>

Close adherance to these guides is recommended to obtain consistent and appropriate sampling of the various endocrine tissues. Critical aspects of sampling and sectioning are briefly summarized below.

# Male Reproductive System

Critical aspects of tissue dissection and handling of the testes and epididymides have been reviewed by (Foley, 2001). Additional guidance on sampling, fixation, and sectioning is also provided by (Lanning *et al.*, 2002; Creasy, 2003)

#### Testes

It is essential that the testes are fixed in an appropriate fixative such as Modified Davidsons or Bouins Fixative. They should NOT be fixed in formalin since this results in cellular shrinkage and precludes detection of the types of subtle morphological changes that are likely to occur with endocrine disruption. Modified Davidsons fixative (Latendresse *et al.*, 2002) is preferred to Davidsons fixative since periodic acid Schiffs (PAS) does not stain the spermatid acrosome (required for detailed staging of the spermatogenic cycle) when conventional Davidsons fluid is used. Bouins fixation results in differential tubular shrinkage, with tubules in the center of the testis showing greater shrinkage than more peripherally located tubules. The shrunken central tubules are often surrounded by interstitial proteinaceous fluid. This is an artifact of fixation and should not be mistaken as a real finding.

A transverse or longitudinal section of the testis may be used, or one of each may be employed. It is important to include the rete testis in the section since this can provide evidence of estrogen induced fluid disturbances in the rete and efferents ducts.

It is important to avoid trauma and squeezing of the testis during the necropsy dissection since this will result in sloughing of the germ cells into the tubular lumen, which may be mistaken for a test article related change.

## Epididymides

The epididymis can be preserved in the same fixative as the testis or in formalin. Formalin provides slightly better preservation of cellular detail. It is important to ensure that the epididymis does not dry out between dissection and fixation, to prevent drying artifact, particularly of the corpus.

A longitudinal section that incorporates the initial segment, head, body and tail of the epididymis is essential for a comprehensive evaluation of changes. Findings are often localized in specific regions of the epididymis. In addition, the location of sloughed testicular germ cells within the length of the epididymis provides a useful indication of how long ago the disturbance in testicular spermatogenesis began.

## **Prostate and Seminal Vesicles**

Prostate and seminal vesicles are best fixed in formalin.

The amount of secretory content of the accessory sex organs is very androgen dependant and represents a large proportion of the weight of the glands. Therefore it is critical that the organ weight of these tissues includes all the secretory content. This can best be achieved by dissecting out the accessory sex organs attached to the urinary bladder as a unit. The bladder should then be removed from the accessory sex gland unit over a weigh boat to ensure that any seminal fluid that leaks is caught and weighed with the gland. Care should be taken to prevent urine leaking into the weigh boat. Since the organ weight is generally more sensitive than the histopathological appearance of the organ, this procedure is critical. The guidelines recommend weighing the prostate, seminal vesicles and coagulating gland as a unit. Since the ventral prostate and the seminal vesicles are particularly sensitive to androgen status, further dissection and weighing of these individual tissues could be performed to gain additional organ weight data.

# Mammary gland



Sampling of the inguinal mammary gland is recommended. There is variation in the morphology and the response to endocrine disruption of mammary gland depending on their site along the mammary chain. Since glandular tissue is relatively abundant in the inguinal region and because it can be consistently sampled using the inguinal lymph node as a marker, sampling at this site is recommended to minimize variation.

A good longitudinal section of mammary gland is necessary to ensure a general overview of the entire gland rather than a peripheral sample that may only include the terminal branches. The best way of ensuring this for males and females is to include the subcutis and use the lateral iliac lymph node as a gross landmark for sampling. In females, both the nipple and the lymph node may be used as landmarks.

Whole mounts of mammary glands have been used very successfully to demonstrate changes in morphology and volume of the glandular tissue in response to endocrine disruption (Sourla et al., 1998; You et al., 2002). Although this methodology is not a specific recommendation for the TG407 study, it should be considered, to confirm or provide more detailed information on any changes seen in the mammary gland during this initial screen

#### Female Reproductive System

All female reproductive tract tissues are best fixed in formalin. Because the oestrous cycle-associated morphological changes observed in the reproductive tract do not occur uniformly along its length, sampling of tissues from specific areas of the vagina, uterus and ovaries is essential if an accurate histological evaluation of the system is to be performed and meaningful comparisons between animals made. Additional guidance on preparation of the uterus and vagina for sampling, fixation, and sectioning is also provided by the OECD Report of the Initial Work Towards the Validation of the Rodent Uterotrophic Assay - Phase 1(No. 65: http://www.oecd.org/document).

#### Vagina

A transverse section should be taken from the mid-vagina, avoiding the posterior (caudal) one-third of the organ as this is covered by a permanently keratinised stratified squamous epithelium. Care should be taken to avoid incorporating vulval or vaginal skin in this section. Weighing of the vagina is not required by the TG 407.

#### Uterus

The ovaries and vagina should be separated from the uterus, which is then weighed prior to fixation. The weight of the uterus will vary significantly depending on the stage of the estrous cycle and the volume of fluid within the lumen. This makes uterine weight a very variable parameter. After fixation, a transverse section should be taken midway along the length of each uterine horn. A longitudinal horizontal section should also be obtained from the uterine cervix/body and posterior (caudal half) of the attached horns.

#### Ovary

At necropsy, the ovaries should be separated from the oviducts and weighed before fixation. Once fixed, the ovaries should be halved longitudinally and one or more sections obtained from the middle of the organ. Suboptimal sampling (for example, from the periphery of the ovary) will result in sections that fail to include a representative selection of follicular and luteal structures.

# Pituitary

The small size of the pituitary, and its location within the sphenoid bone of the skull can make it difficult to remove without damage. Fixation in-situ is recommended, followed by careful dissection and post fixation weighing. This procedure also minimizes the possibility of the tissue drying out prior to fixation.

Sectioning of the pituitary should aim to include the maximum area of the pars distalis while still including the pars nervosa and pars intermedia. A consistent sampling is necessary, since different types of secreting cells are located in different areas within the pars distalis (see example in the RENI dissection guide for lactotroph distribution).

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## Annex : Considerations on endocrine data interpretation for the updated TG407.

The peer review panel recommended that guidance be provided for data interpretation from TG407 studies on the following points:

- 1. Differences in the end-points that do not reach statistical significance
- 2. Distinction between findings that are consistent with endocrine disruption and nonspecific findings that are not related to potential endocrine disruption
- 3. Weight differences for body-weight independent organs
- 4. Additivity of findings in a number of organs

#### 1) How to interpret differences in the end-points that do not reach statistical significance?

Statistical evaluation should have priority in principle because it may make translation of findings more clear. However, toxicological findings should be also interpreted on the basis of biological relevance and plausibility, regardless of statistical significance. This is especially important with the low animal number per group specified in OECD TG 407.

In the case of lesions/findings that are rather common (background lesion) and are known to have a high variability, clear statistical significance is important and in these cases it is important that the effects can be attributed to test substance. Aspects important for such assessment are amongst others: existence of a dose-effect relationship, increased severity etc.

If it concerns a rare effect, statistical significance is less important. However, it is recognized that a firm conclusion in the absence of statistical significance remains a problem, especially when there are no other indications that the tissue/organ is a target of the compound

As an example: findings induced by endocrine disruptors in the normal cycling rats are very complicated. The number of animals examined in OECD TG407 may not be enough to detect these effects, especially weak ones, on the female reproductive system. The slight changes might be overlooked if endpoints with statistical significance are only accepted as toxicity. Morphological deviations from normal estrous cycle stages should be considered and may indicate an adverse effect. It should be emphasized that the physiology of the female reproductive system and normal variation thereof should be understood in order to be able to identify end-points that may indicate endocrine disruption (regardless of statistical significance).

# 2) How to distinguish between findings that are consistent with endocrine disruption and non-specific findings that are not related to potential endocrine disruption?

Mechanistic information is very important. Without this it would be difficult to differentiate between specific and non-specific findings. Various endocrine mechanisms are involved in the regulation of body homeostasis and might specifically affect body weight gain and food consumption which are usually considered unspecific effects. In reproductive toxicity studies a dose-related reduction in female food consumption and body weight gain during the premating period will often be associated with a decrease in the number of corpora lutea, i.e. oocytes available for fertilisation. The effect probably relates to a reduced general fitness of the female which translates into a decrease in fecundity. Although frequently considered an unspecific effect, the underlying pathways are unresolved and an endocrine mechanism may well be

assumed. Nevertheless, for the purpose of determining a NOAEL for the study, a clear distinction is not required.

Detection of chemical specific findings can be difficult for endocrine disruptors. Receptor-mediated responses often show common findings. For example, histopathological changes in the ovary, uterus, vagina and mammary gland by progesterone treatment for 2 weeks are similar to those in sulpiride, a D2 blocker. Therefore, obtained findings must be translated taking into account individual cases. Some findings may clearly indicate endocrine disruption but the other may not. In the latter case, clear distinction would not be needed. Knowing patterns of histopathological changes but no specific findings induced by the treatment might be informative to detect the effects on the female reproductive system.

If a clear cut answer is needed concerning interpretation of a potential ED effect i.e. whether it's a direct, specific effect or an indirect mediated effect, a tier two study may need to be performed to investigate MOA.

3) What changes constitute 'positive' versus 'negative' effects (e.g. weight changes for body-weight independent organs)?

This question is not clear. Does this mean positive effects vs. negative effects (beneficial vs. detrimental) or positive vs. negative (effect vs. no effect)?

This question does not concern histopathologic evaluation of studies. It is questionable whether it is applicable to females in short-term studies as lower body weights are not likely to modulate ovarian toxicity in short-term studies. Lasting lower body weights might affect female reproductive organs in long-term studies.

# 4) How to interpret additivity of findings in a number of organs?

Question is not clear. If findings in multiple organs of individual animals aggregate into a pattern in a doserelated manner that can be associated with a specific mode/mechanism of action this should serve to strengthen the conclusion that the test substance is responsible. Findings in a number of organs (not necessarily the same) from different animals would not "add up" in the same way in the interpretation.

Histopathological examination in the female reproductive organs, i.e. ovary, uterus, vagina, mammary gland and pituitary, and major target organs of toxicities including the liver and kidney might be minimum requirement for the detection of any toxicity in the female reproductive system.

In addition, checking continuous estrous cycle might be informative for

- 1) Confirmation of presence of LH/FSH surge
- 2) Prediction of endocrine imbalance such as persistent diestrus or persistent estrus.