4. NORMAL BACKGROUND VARIATION OF STRUCTURE

Introduction

4.1 This section illustrates several examples of normal morphological changes in the female reproductive tract that can complicate the evaluation of reproductive tissues from TG 407 studies. These alterations may be developmental in nature or simply reflect the normal physiology of the oestrous cycle. Artefacts caused by suboptimal sampling and/or sectioning of the reproductive organs may also occur. Oblique sections through epithelia, for example, can mimic focal areas of epithelial hyperplasia or squamous metaplasia. Similarly, inadequate ovarian sections may not be representative of the organ in terms of the follicular and luteal stages present.

4.2 An awareness of these normal background and artefactual changes is important in order to avoid erroneously labelling them as treatment-related. The situation is complicated by the fact that the incidence of many common spontaneous variations may be influenced by substances with endocrine activity. In TG 407 studies the normal background variation for the test animal population is based on five control animals per sex. Given this small group size, it is important that small increases in the incidence of spontaneous lesions are not over-interpreted as treatment-related.

4.3 As TG 407 studies use young, sexually mature, non-mated adult rats, spontaneous age-related changes and alterations associated with pregnancy should not be encountered and are not discussed here.

Spontaneous changes

Figure 4.1 – Vagina: dioestrus (rat, H&E). Minimal neutrophil infiltration (N) of the stratum germinativum. This is a normal but inconsistent feature of dioestrus and should not be misdiagnosed as vaginitis.
Figure 4.2 – Uterus: late oestrus (rat, H&E). A marked neutrophil infiltration (N) of the endometrial glands may be observed during late oestrus/metoestrus, reflecting the dominance of oestrogen during these stages. This is a normal finding and should not be mistaken for endometritis.

Figure 4.3 – Uterus: early oestrus (rat, H&E). Early squamous metaplasia (SM) of the endocervix, affecting the luminal and/or glandular epithelium, may be occasionally observed in young control rats. This morphological alteration may also develop following treatment with oestrogenic compounds.
4.4 Ovarian luteal cysts (Figure 4.4) are occasional spontaneous findings in young rats that form when tertiary and Graafian follicles fail to ovulate but undergo lutenisation. Increased numbers of cystic corpora lutea may be observed following administration of compounds with gonadotrophic activity (Table 5.1).

4.5 Luteal cysts should be differentiated from follicular cysts; these develop from secondary follicles that fail to progress through folliculogenesis. Follicular cysts are fairly common spontaneous findings in aged rodents but are not typically observed in young rats. They may develop after dosing with antioestrogenic substances such as tamoxifen and CGS 18320B (Table 5.1). Cysts of the ovarian bursa or rete ovarii may also be occasionally encountered in young rodents as spontaneous developmental lesions.

![Figure 4.4 – Ovary: corpus luteum (rat, H&E). Cyst (C) formation within a corpus luteum (CL). Encircling the cyst are small, granulosa-like cells admixed with large, plump luteal cells – the latter differentiate this structure from a follicular cyst. The fluid-filled cyst cavity is lined by a simple squamous epithelium (not visible).](image)

![Figure 4.5 – Ovary: corpus luteum (rat, H&E). A patchy, often marked, mononuclear inflammatory cell infiltration (I) may be observed in degenerating corpora lutea. This is a normal feature of luteal regression. Consistent absence of this change in treated animals may reflect persistence of corpora lutea – an effect of some endocrine disrupting substances.](image)
Common artefacts

Figure 4.6 – Normal vulva (rat, H&E). This may be inadvertently sampled and included in histological sections of the vagina. The vulval epithelium is stratified squamous in type and covered by a thin, permanent stratum corneum (SC). Numerous small rete pegs (RP) project into the underlying dermis. Sweat glands and occasional hair follicles (not shown) may also be visible in the dermis.

Figure 4.7 – Uterus: dioestrus (rat, H&E). Oblique sections (Ob) through the normal endometrial columnar epithelium (CE) should not be confused with foci of squamous metaplasia, a potential treatment-related effect.
Figure 4.8 – Ovary: prooestrus (rat, H&E). Preovulatory (Graafian) follicles (GF); the absence of free-floating primary oocytes within the follicular lumina is a sectional artefact.
5. MORPHOLOGICAL PATTERNS OF ENDOCRINE DISRUPTION

Introduction

5.1 Endocrine disrupting substances can affect the hypothalamic-pituitary-ovarian (HPO) axis in several ways, producing alterations in the female reproductive tract that may be detected histologically. The updated TG 407 is concerned with detecting substances with oestrogenic, antioestrogenic, androgenic and antiandrogenic activity. Potent endocrine disruptors typically cause overt histological changes in the vagina, uterus and ovary that are relatively straightforward to identify. Ethinyl oestradiol (a potent oestrogenic compound), for example, induces ovarian atrophy in association with hyperplastic and hypertrophic changes in the uterus and vagina.

5.2 Detection of substances with weak endocrine activity is more problematic. Compounds such as genistein and nonylphenol do not usually cause profound histopathological changes in individual reproductive tract organs; they may, however, disrupt the synchrony of the normal oestrous cycle-associated morphological alterations in the reproductive tract. Thus, whilst individual tissues may be histologically normal, they fail to correlate in terms of oestrous cycle stage when collectively evaluated as a unit. The challenge of identifying asynchronous morphological changes in the female reproductive tract when assessing TG 407 studies is discussed further below.

Overview of morphological patterns of endocrine disruption

5.3 A useful classification of the morphological alterations that may be observed in the female rodent reproductive tract following xenobiotic administration has been described by Yuan (1998). This scheme comprises three types of morphological response, based on the combined histological appearance of the vagina, uterus and ovary and is summarised below:

- **Type I** atrophic vagina, uterus and ovary
- **Type II** atrophic ovary with hyperplastic/hypertrophic uterus and vagina
- **Type III** hyperplastic/hypertrophic ovary, uterus and vagina

5.4 Using this classification scheme, Table 5.1 provides an overview of the morphological alterations likely to be encountered in the female reproductive tract following administration of (anti)oestrogenic or androgenic substances. The expected histopathological changes and underlying mechanisms of endocrine disruption associated with each type of response are summarised. Although compounds with (anti)oestrogenic or androgenic activity typically induce type I and II responses, the type III response is included here for reference. Figures 5.1 to 5.13 illustrate examples of the morphological alterations that may be observed. This part of the guidance document concludes with a summary of recommended terminology for the recording of these changes.

5.5 Note that overt histopathological alterations associated with short-term antiandrogen administration are typically limited to the male reproductive tract (Kunimatsu et al, 2004), although ovarian interstitial stromal cell hyperplasia/hypertrophy has been described in rats following subacute oral dosing with flutamide, a potent androgen antagonist (Andrews et al, 2001).
**Table 5.1 – Overview of the morphological responses associated with endocrine disruption (modified after Yuan, 1998).**

<table>
<thead>
<tr>
<th>MORPHOLOGICAL PATTERN</th>
<th>EXPECTED MORPHOLOGICAL ALTERATIONS</th>
<th>EXAMPLE SUBSTANCE</th>
<th>MECHANISM OF ENDOCRINE DISRUPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I</strong></td>
<td><strong>VAGINA</strong> Thin, atrophic epithelium comprising 2-3 cell layers</td>
<td><strong>OVARY</strong> Atrophy with inactive interstitial glands; glandular cells are small and spindle-shaped</td>
<td><strong>CGS 18320B</strong> (non-steroidal aromatase inhibitor)</td>
</tr>
<tr>
<td><strong>(Atrophic vagina, uterus and ovary)</strong></td>
<td><strong>UTERUS</strong> Thin, atrophic endometrial and glandular epithelium consisting of low columnar cells</td>
<td><strong>UTERUS</strong> Sparse endometrial glands and atrophic myometrium</td>
<td><strong>Note</strong></td>
</tr>
<tr>
<td></td>
<td><strong>OVARY</strong> Cystic anovulatory follicles may be present</td>
<td><strong>OVARY</strong> Reduction in numbers of follicles and corpora lutea may not be observed in short-term studies</td>
<td><strong>Endogenous production of ovarian oestrogen is thus reduced, initiating widespread atrophic reproductive tract changes</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Note</strong></td>
<td></td>
<td><strong>Modulation of feedback control on the hypothalamus and pituitary by reduced endogenous oestrogen levels may suppress gonadotrophin secretion, resulting in the formation of follicular cysts from anovulatory follicles</strong></td>
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<td></td>
<td>• Atrophic changes are more readily detected in the vagina compared with the uterus and ovary</td>
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<td></td>
<td>• Ovarian atrophy may be particularly difficult to detect in short-term studies. Early (primordial to tertiary) follicular stages develop independently of hormonal stimulation and are thus frequently observed in the atrophic ovaries of non-cycling rats from subacute studies. Similarly, corpora lutea formed during previous cycles may also be present</td>
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<td></td>
<td>• Therefore, in short-term studies, the presence of normal follicular and luteal structures does necessarily imply unperturbed reproductive function</td>
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Table 5.1 (cont.) – Overview of the morphological responses associated with endocrine disruption (modified after Yuan, 1998).

<table>
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<tr>
<td></td>
<td>VAGINA</td>
<td>UTERUS</td>
<td>OVARY</td>
</tr>
<tr>
<td><strong>Type II</strong> (Hyperplastic/hypertrophic vagina and uterus with atrophic ovary)</td>
<td>Hyperplasia and hypertrophy of epithelium</td>
<td>Hyperplasia and hypertrophy of luminal and glandular epithelium</td>
<td>Atrophy with inactive interstitial glands</td>
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<td></td>
<td>Keratinisation of superficial epithelium; may be incomplete with retention of nuclei in cells of stratum corneum</td>
<td>Neutrophil infiltration of endometrium</td>
<td>Cystic follicles may be observed (tamoxifen)</td>
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<td></td>
<td>Areas of epithelial mucification may be noted; superficial epithelial cells are hypertrophied and contain large mucin-filled vacuoles.</td>
<td>Foci of squamous metaplasia present in luminal and glandular epithelium</td>
<td>Reduction in numbers of follicles and corpora lutea may not be observed in short-term studies</td>
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<td></td>
<td>Epithelial mucification typically predominates over keratinisation following androgen administration</td>
<td>Cystic endometrial glands may be observed</td>
<td>Interstitial stromal cell hypertrophy/hyperplasia may be noted (tamoxifen &amp; flutamide)</td>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VAGINA</td>
<td>UTERUS</td>
<td>OVARY</td>
</tr>
<tr>
<td><strong>Type III</strong></td>
<td>Hyperplasia and hypertrophy of epithelium</td>
<td>Hyperplasia and hypertrophy of luminal and glandular epithelium</td>
<td>Enlarged with increased numbers of corpora lutea and tertiary follicles. These changes are less pronounced with prolactin analogues</td>
</tr>
<tr>
<td></td>
<td>Keratinisation of superficial epithelium; may be incomplete with retention of nuclei in cells of stratum corneum</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Areas of epithelial mucification may be noted; superficial epithelial cells are hypertrophied and contain large mucin-filled vacuoles.</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Proliferative changes associated with prolactin analogues are typically limited to mild epithelial hypertrophy and mucification</td>
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</table>

Prolactin analogues (luteotrophs) prolong the lifespan of corpora lutea; this results in persistent progesterone secretion and development of a pseudopregnancy-like state.

Dopamine-depleting compounds (e.g. reserpine) impair the release of dopamine (a prolactin-inhibiting factor) from the hypothalamus. By increasing endogenous prolactin secretion, such substances have an indirect luteotrophic effect.
Selected examples of morphological alterations associated with endocrine disruption

A. Vagina

**Figure 5.1 – Vagina: atrophy (rat, H&E).** Detection of chemically induced atrophy in the vagina is straightforward. Note the marked attenuation of the vaginal epithelium which comprises 2-3 layers of small cells. Generalised atrophy of the female reproductive tract occurs normally in aged, reproductively senescent rats, but should not be encountered as a background finding in the young, sexually mature animals specified by the TG 407.

**Figure 5.2 – Vagina: hyperplasia/hypertrophy with mucification (rat, H&E).** Superficial epithelial cells are hypertrophied with large, intracytoplasmic, mucin-filled vacuoles (V). The stratum germinativum (SGerm) consists of the two most basal cell layers. Mucification of the vaginal epithelium is a potential alteration associated with oestrogenic substances, and is the expected morphological response of the vagina to administration of large doses of androgens. Luteotrophic compounds (e.g. prolactin analogues) typically cause mild epithelial hypertrophy and mucification.

**Figure 5.3 – Vagina: hyperplasia/hypertrophy with mucification associated with pregnancy (rat, H&E).** This figure clearly illustrates the effects of progesterone on the vaginal epithelium following oestrogen priming. Note the marked vacuolation (V) and hypertrophy of the superficial stratum germinativum cells.
B. Uterus

Figure 5.4 – Uterus: atrophy of luminal epithelium (rat, H&E). A low columnar epithelium lines the uterine lumen; endometrial glands (not shown) are reduced in number.

Figure 5.5 – Uterus: hypertrophy/hyperplasia of luminal epithelium (rat, H&E). Proliferative changes in the luminal and glandular epithelium are features of the type II (oestrogenic) and type III (gonadotrophic/luteotrophic) morphological responses.
Figures 5.6 and 5.7 – Uterus: squamous metaplasia (rat, H&E). Figure 5.6 (above) shows squamous metaplasia (SM) in the luminal epithelium of the endocervix – a spontaneous background finding in a control animal. Figure 5.7 (below) illustrates hyperplasia/hypertrophy of the luminal epithelium (HE) together with a focus of squamous metaplasia (SM) in a rat treated with the synthetic oestrogen diethylstilbestrol.
Figures 5.8 and 5.9 – Ovary: atrophy (rat, H&E). Figure 5.8 (above) shows an obvious reduction in the number of follicles and corpora lutea; this characterises ovarian atrophy as observed in long-term studies and aged female rats. In subacute studies, this alteration may not be observed, reducing the sensitivity of the ovary as an endpoint for the detection of (anti)oestrogenic effects. Changes in the ovarian stroma and interstitial glands, however, may be induced by the short-term administration of endocrine disrupting substances (Figures 5.10 and 5.11). Figure 5.9 (below) shows a normal ovary from a young, sexually mature control animal for comparison.
Hyperplasia and hypertrophy of stromal interstitial cells is a common feature of ovarian atrophy in ageing rodents, and has been reported following short-term dosing of young adult rats with tamoxifen and flutamide. Pale-staining, plump stromal cells are arranged in variably sized islands and clusters (IC). This ovarian section is from an aged control animal; note the orange-brown lipofuscin pigment present in some stromal cells, a normal finding in the ovaries of old, reproductively senescent rats.

High power view of a cluster of large, pale-staining stromal cells supported by a fine fibrovascular stroma. The ovarian surface epithelium (OSE) and tunica albuginea (TA) are also visible.

Early cyst (C) formation within a corpus luteum (CL). Encircling the cyst are small, granulosa-like cells admixed with large, plump luteal cells – the latter differentiate this structure from a follicular cyst (Figure 5.13). The fluid-filled cyst cavity is lined by a simple squamous epithelium (not visible).
Figure 5.13 – Ovary: follicular cyst (rat, H&E). Cyst (C) formation within a secondary follicle. The surrounding zona granulosa (ZG) is attenuated; luteinisation of the granulosa cells is not a feature of follicular cysts. The cyst cavity is lined by a simple squamous epithelium (SE, inset).

Reproductive tract asynchrony

5.6 As previously noted, weak endocrine disruptors are difficult to detect as they fail to significantly perturb the HPO axis of mature female rats and produce overt histopathological changes in the vagina, uterus and ovary. Such substances may, however, be associated with asynchrony of the oestrous cycle-associated morphological changes that normally occur in the reproductive tract.

5.7 Observation of potential reproductive tract asynchrony in short-term studies is a difficult and ambitious enterprise, even for an experienced toxicological histopathologist. A good working knowledge of the normal histological appearance of the reproductive tract at each stage of the oestrous cycle is essential. Careful evaluation of the female control rats (limited to 5 animals in TG 407 studies) should be performed in order to gain an appreciation of the range of physiological and background spontaneous changes that are normal for the strain and age of rat being utilised.

5.8 For asynchrony to be confidently diagnosed, a profound lack of correlation in the histological appearance of the reproductive tract tissues should be observed. As previously discussed, reproductive tract staging is initially based on the appearance of the vagina; the presence of markedly incompatible morphological alterations in the uterus and ovary is consistent with asynchrony.

5.9 Given the highly dynamic nature of the female reproductive system and small group sizes utilised in TG 407 studies, great care should be taken by the study pathologist to avoid erroneously interpreting minor normal variations in morphology as evidence of reproductive tract asynchrony.
SUMMARY OF RECOMMENDED TERMINOLOGY

A. VAGINA

- Epithelial atrophy
  - attenuated vaginal epithelium comprising 2-3 cell layers

- Epithelial hyperplasia/hypertrophy
  - thickened vaginal epithelium comprising increased number of cell layers/enlarged cells; may occur with:
    - keratinisation - superficial epithelial cells are variably keratinised
    - mucification - superficial epithelial cells are variably vacuolated
    - keratinisation and mucification - combination of changes is present

B. UTERUS

- Atrophy
  - attenuated, low columnar luminal and glandular epithelium
  - sparse endometrial glands and atrophic myometrium

- Epithelial hyperplasia/hypertrophy
  - thickened luminal and/or glandular epithelium comprising increased number of cell layers/enlarged cells

- Endometrial inflammation
  - inflammatory cell (neutrophil) infiltration of endometrial stroma and/or glands

- Cystic endometrial glands
  - dilation/cystic change in endometrial glands

- Squamous metaplasia
  - transformation of luminal/glandular columnar epithelium into a stratified squamous epithelium

C. OVARY

- Atrophy
  - inactive interstitial glands +/- reduced numbers of follicles/corpora lutea; may occur with
    - follicular cysts
    - interstitial stromal cell hypertrophy/hyperplasia

- Increased numbers of follicles/corpora lutea

- Luteal cysts
  - cystic change in corpus luteum; cyst cavity lined by a simple squamous epithelium and surrounded by lutenised and non-lutenised granulosa cells

- Incomplete lutenisation of corpora lutea
  - corpora lutea are composed of lutenised and non-lutenised granulosa cells

D. FEMALE REPRODUCTIVE TRACT

- Type I response
  - generalised reproductive tract atrophy

- Type II response
  - proliferative changes in uterus and vagina accompanied by ovarian atrophy

- Type III response
  - generalised reproductive tract proliferation

- Asynchrony
  - marked lack of correlation in the histological appearance of the uterus and/or ovary relative to the vagina
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