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An alternative method for preparation of tissue sections from the rat mammary gland

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ABSTRACT

In toxicopathological studies of the rat mammary glands, the guidelines of the Registry of Industrial Toxicology and Animal Data (RITA) recommend transverse sections of the inguinal mammary gland. However, occasionally limited amounts of mammary gland tissue are found in transverse sections. We compared transverse sectioning with an alternative method comprising horizontal sections of the rat mammary glands. Normal cycling female Sprague Dawley rats were sacrificed in proestrous, estrous, metestrous and diestrous, and samples from all mammary glands were collected. Transverse sections were prepared from the left-sided glands, and horizontal sections were cut from the right-sided glands. Sections were stained with HE, and epithelial and myoepithelial cells were visualized by immunohistochemical staining of cytokeratin 18 and alpha smooth muscle actin, respectively. Area of the mammary fat pad, mammary epithelium and connective tissue were measured in randomly sampled vision fields from each section. Horizontal sections contained a significantly larger area of mammary fat pad as well as glandular and connective tissue. No differences in tissue densities of epithelial or myoepithelial cells or connective tissue were observed between transverse sections and horizontal sections. Interestingly, densities of epithelium and connective tissue varied between cranial and caudal glands as well as the phases of the estrous cycle. In conclusion, horizontal histological sections of the rat mammary gland provided significantly larger samples of mammary gland tissue with no difference in tissue composition compared to transverse sections, which are recommended in the RITA guidelines.

Introduction

Female rats have six pairs of mammary glands (Fig. 1), which are localized in the mammary fat pad (MFP). From the nipple, the secretory component of each mammary gland comprises continuous branching ducts and ductules, terminating in alveolar lobules, alveolar buds and terminal end buds (TEB). Final development of the mammary glands occurs gradually with each estrous cycle from the onset of puberty, as stem cells in TEB proliferate and differentiate into ducts and alveolar buds and lobules. Alveolar lobules and ducts are lined by one to two layers of mammary epithelial cells (MEC), which are surrounded by a layer of contractile myoepithelial cells. The glandular epithelium is supported by connective tissue, which forms a relatively thicker sheath of fibrous tissue around the ducts compared to the alveolar lobules (reviewed by Masso-Welch et al., 2000; Russo and Russo, 1996) (Fig. 2).

In toxicopathology, histopathological examination of the mammary gland is indicated in, for example, studies of carcinogenicity (Bregman et al., 2003; EMEA/CPMP, 2002) and endocrine disruption (Lucas et al., 2007). Histopathological examination of the mammary gland can be focused on the occurrence of hyperplastic and neoplastic lesions (carcinogenicity studies) (EMEA/CPMP, 2002) or morphological changes such as feminization, masculinization or atrophy in studies of endocrine disruption (Lucas et al., 2007; Rudmann et al., 2005). Optimal tissue sectioning is critical for a valid result of a histopathological examination. Specifically,

Abbreviations: α-SMA, alpha smooth muscle actin; BrdU, 5-bromo-2-deoxyuridine; CK18, cytokeratin 18; CPMP, Committee for Proprietary Medicinal Products; DAB, diamno-benzidine tetra-hydrochloride; EGF, epidermal growth factor; EMEA, European Medicines Agency; HE, hematoxylin and eosin; HRP, horseradish peroxidase; IGF-1, insulin-like growth factor; IgG, immunoglobulin G; IHC, immunohistochemical; MCT, mammary gland connective tissue; MEC, mammary epithelial cells; MFP, mammary fat pad; PBS, phosphate buffered saline; RITA, Registry of Industrial Toxicology- and Animal-data; RT, room temperature; SEM, standard error of mean; TBS, tris-buffered saline; TEB, terminal end buds; TGF-β, transforming growth factor β; VIS, Visiopharm integrator system

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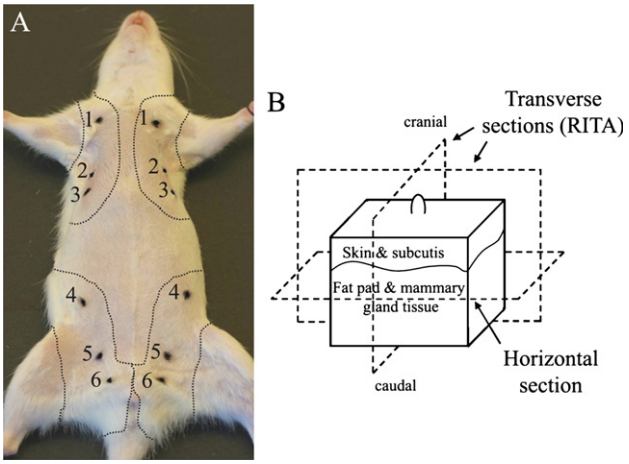

Fig. 1. Sampling of the rat mammary glands. (A) The picture shows the ventral thoracic and abdominal wall on an anaesthetized female Sprague Dawley rat, 10 weeks old. The rat has been clipped and the nipples marked with ink. The outline of all mammary glands as described by Popesko et al. (1992) has been drawn on the picture (dotted line). From the right side of the animal tissue samples measuring 1.5×1.5 cm were collected from all six mammary glands, and horizontal histological sections were cut through the mammary fat pad parallel to the skin surface. From the left side, tissue samples measuring 2.0×0.5 cm were collected from mammary glands 1, 2, 4 and 6. Transverse (as opposed to transverse longitudinal) histological sections through the nipple were cut from these tissue samples, as described in the RITA guidelines. Transverse sections through the nipple were also cut from mammary glands 3 and 5, however, not all of these transverse sections were exactly perpendicular to the length of the animal; instead, these transverse sections had random orientation. These random-orientation transverse sections would also represent the transverse longitudinal sections recommended in the RITA guidelines. (B) A schematic presentation of sectioning planes in the rat mammary gland. The RITA guideline recommends transverse sections of the rat mammary gland, oriented either perpendicular to the length of the animal, or parallel with the length of the animal. In this study, horizontal sections were compared to the transverse sectioning method.

changes relating to carcinogenicity as well as endocrine disruption are expected to affect primarily the mammary gland epithelium, and therefore, sectioning methods that provide as large as possible samples of mammary epithelium can advantageously be applied. The RITA guidelines suggest to prepare a transverse section, a longitudinal transverse section or both types of sections from the inguinal mammary gland (Ruehl-Fehlert et al., 2003). However, occasionally only limited amounts of mammary gland tissue are found in transverse sections. This can complicate histopathological examination, as large and representative samples of mammary gland tissue are fundamental for a valid histopathological evaluation.

The aim of the present study was to compare transverse sections of the rat mammary glands, with an alternative method comprising horizontal sections of the mammary glands, i.e., sections cut through the mammary gland tissue parallel to the skin surface. The main endpoints were areas of MFP, MEC and mammary gland connective tissue (MCT) in sections obtained by the two methods. To investigate if the alternative sectioning method introduced bias in tissue composition compared with the standard transverse section, the following endpoints were compared between the two sectioning methods: The density of epithelial and connective tissue, cytokeratin 18 (CK18) staining (epithelial marker) and α-smooth muscle actin (α-SMA) staining (myoepithelial marker) (Bocker et al., 1992). The study was performed with tissue from young sexually mature female Sprague Dawley rats exhibiting a normal estrous cycle. Thus, our results validate the alternative method comprising horizontal sections for use in preclinical safety studies in rats.

Materials and methods

Housing of animals, collection of vaginal smears and allocation to estrous cycle groups

Twenty-nine female and five male Sprague Dawley outbred rats (NTac:SD) were purchased from Taconic Europe, Denmark. The animals were approximately six weeks old at arrival. The animals were acclimatized for 17 days prior to study start, and were approximately 10 weeks old at study end and euthanasia. The animal experiment was performed in accordance with the national guidelines (Ministry of Justice, 2003). Female and male rats were housed separately in transparent type VI macrolone cages (floor area 1800 cm^2) with four or five animals per cage. The animal room environment was maintained at 18–24 °C, relative humidity at 30–70%, air change 8–15 times/h, light/dark cycle 12/12 h changing at approximately 0600 and 1800 h. The animals had free access to a complete pelleted rodent diet (Altromin 1320, Brogården, Hørsholm, Denmark) and drinking water (non-chlorinated, non-acidified) from an automated watering system. The male animals were housed in a cage next to the females, to stimulate the estrous cycle. To ensure that the females were sexually mature and normally cycling, and to ensure an approximately equal number of animals in each phase of the estrous cycle, vaginal smears were collected through experimental days 1–8 (after acclimatization) as described previously (Marcondes et al., 2002). The collected vaginal secretions were placed on glass slides, air-dried, stained with Hemacolor (Merck KGaA, Darmstadt, Germany) and mounted with Pertex (HistoLab Products Ab, Göteburg, Sweden). Confirmation of estrous cycle stage was based on histological examination of the internal reproductive organs as described recently (Westwood, 2008).

Tissue sampling and processing for histology

On experimental days 9–12 the female animals were anaesthetized with isofluran and euthanized by exsanguination through incision of the abdominal aorta. Following shaving of thoracic and abdominal skin, the nipples were marked with ink (Chartpak Ad Marker – Superblack P100, Chartpak, Leeds, MA, USA). The thoracic and abdominal skin and the underlying mammary glands (six glands) on each side of the animal were bluntly dissected from the muscles forming the thoracic and abdominal wall. Mammary glands and the overlying skin were placed on a piece of cardboard and fixed in 4% neutral buffered formaldehyde (VWR International AB, Stockholm, Sweden) for 24–48 h.

Trimming of the mammary glands was performed as described in Fig. 1. The tissue was dehydrated through graded concentrations of ethanol (Kemetyl A/S, Køge, Denmark) and finally transferred to xylene (Applichem, GmbH, Darmstadt, Germany) and embedded in paraffin. Of glands from the animals' right side horizontal sections of 2–3 μm thickness were cut through the mammary gland tissue parallel to the skin surface. Transverse sections through the nipple were cut from the mammary glands from the left side, as described in the RITA guidelines (Ruehl-Fehlert et al., 2003). Samples of duodenum (2 cm caudal to the pyloric sphincter) and colon (midpart), vagina, uterus and ovaries were fixed in 4% neutral buffered formaldehyde for 24–48 h and trimmed according to RITA guidelines (Ruehl-Fehlert et al., 2003).

Histochemistry and immunohistochemistry

Tissue sections were stained with hematoxylin and eosin (HE) (Sigma Aldrich, St. Louis, MO, USA) and Masson trichrome (Sigma