Experimental and Toxicologic Pathology 66 (2014) 469–475

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

Efficiency of different decalcification protocols for nasal osseous structures in a rat experimental model of allergic rhinitis, and their effects on epithelial histology: An attempt at standardization

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ARTICLE INFO

Article history:
Received 25 July 2014
Accepted 3 September 2014

Keywords:
Decalcification
Allergic rhinitis
Experimental models
Nasal structures
Respiratory epithelium
Histology

ABSTRACT

Introduction: Decalcification of osseous specimens is required for histological analysis; this however may cause tissue damage. In rodent models of allergic rhinitis (AR), epithelial histologic assessment necessitates prior decalcification of the nasal osseous structures. However, respiratory epithelium is highly susceptible to damage, and rat nasal architecture is elaborate and its sectioning is challenging. Nevertheless, decalcification is not standardized in experimental AR. We therefore undertook this task, in order to reduce experimental bias.
Methods: Six-to-eight week-old Wistar rats underwent an AR protocol. Subsequently, nasal structures were decalcified in the following mediums: (i) formic acid 10% for 5 and 20 days; (ii) formic acid 15% for 5 and 15 days; (iii) Morse Solution for 5 and 20 days and (iv) EDTA for 20 and 40 days. Decalcification efficiency/speed was evaluated via radiographic analysis. Furthermore, specimens were stained with hematoxylin and eosin and assessed for preservation of epithelial features.
Results: Specimens were appropriately decalcified in 5 days in the formic acid-based mediums and in 20 days in EDTA with minimal epithelial damage. EDTA for 40 days had no unacceptable adverse effects; conversely, 15 and/or 20 days in acid-based agents provided no extra benefit for decalcification and were detrimental to the epithelium.
Conclusions: EDTA treatment for 20 days is appropriate for decalcification of nasal structures in rat models of allergic rhinitis; further incubation preserves epithelial integrity but is not required. When urgency is a factor, formic-acid-based decalcification for 5 days yields acceptable results.

1. Introduction

Bone-containing specimens require treatment with calcium-reacting agents, that is, acids which turn insoluble calcium salts into soluble, and/or chelators which take up calcium ions (Alers et al., 1999; Mattuella et al., 2007). Acids decalcify rapidly, but deterioration of tissue staining can occur after long incubation (Alers et al., 1999; Begum et al., 2010). Conversely, chelators have little effect on non-osseous tissues, but decalcification is slow (Yamamoto-Fukud et al., 2000).

In experimental rodent protocols of allergic rhinitis (AR), decalcification of the nasal bone is required. Nevertheless, decalcification techniques have not been standardized in this setting: as they are routine laboratory practices, they are not usually questioned in regard to modifications that could lead to higher tissue quality (Fernandes et al., 2007). However, tissue quality is especially important in models of AR, as the subtlety of pathologic alterations may cause their obscuring by decalcification-induced epithelial damage. Alongside these *disease*-specific issues, there are also *anatomy*-related ones: rat nose has numerous and elaborate turbinates lined by a plethora of epithelial types that occupy distinct areas (Harkema et al., 2006). For instance, *olfactory* epithelium covers approximately 50% of the nasal cavity, whereas *transitional* epithelium is

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http://dx.doi.org/10.1016/j.etp.2014.09.001
0940-2993/

situated caudal or posterior to *squamous* epithelium (SE) (which, in turn, is located at the vestibule (Mery et al., 1994)). Nevertheless, neither of these is suitable for AR investigations (Wagner and Harkema, 2007); in this setting, the interest lies on *respiratory* epithelium (RE), situated proximal or anterior to SE. This complex gross and microscopic anatomy suggests that inaccurate sectioning might disallow identification of epithelial types. Additionally, even if RE-containing sections are successfully acquired, the underlying mucosa may still exhibit marked differences, related to the exact site it occupies (for instance, if it overlies cartilage or bone (Wagner and Harkema, 2007)); hence, accurate identification of nasal architecture is critical.

These factors collectively underscore the importance of appropriate decalcification and sectioning in allergic rhinitis rodent models. However, studies on the efficiency of diverse decalcification regimens, and on their effects on nasal epithelial histology, are lacking. This lack of standardization can cause considerable bias across studies. We therefore opted to minimize this bias: (i) by evaluating the decalcification *speed* and *efficiency* of different decalcifiers in a rodent AR protocol and (ii) by assessing the preservation of nasal *epithelial histologic* structures on hematoxylin and eosin (H&E)-stained sections.

2. Materials and methods

2.1. Animals

Six week-old male *Wistar* rats were used. They were housed at 22 + 2 °C and were fed ad libitum. All experiments were approved and conducted in accordance with the Institutional Animal Ethics EL 54 BIO 20, 13/174.

2.2. Allergic rhinitis protocol

Allergic rhinitis was induced as follows: briefly, throughout the first phase (*sensitization* phase) rats were sensitized by intraperitoneal (i.p.) administration of 1 ml of saline containing ovalbumin (1 mg) (*Grade V; Sigma Chemical Co., St. Louis, MO*) and alum (10 mg) (*aluminum hydroxide hydrate gel, Sigma Chemical Co., St. Louis, MO*) on two occasions (day 1 and day 3). Throughout the second phase (*exposure* phase), daily intranasal challenges were performed on three occasions (15th–17th day) by instillation of OVA–saline solution (10 μg/10 μl/nostril) into the nasal cavities by a micropipette. All animals ($n = 65$) underwent the same protocol.

2.3. Preparation of specimens

Twenty-four hours after the last intranasal challenge, rats (being 8 w.o. and weighing approximately 200–250 g) were sacrificed via an *i.p.* ketamine and xylazine overdose, and their heads were removed and fixed in 10% neutral buffered formalin for 48 h. Subsequently, the skin was striped, the lower jaw and eyes were removed and the skull was sectioned posteriorly, between the third upper molar tooth and the posterior opening of the pharyngeal duct (nasopharynx) (Fig. 1). Afterwards, the specimens were immersed in the decalcifying mediums.

2.4. Decalcification protocols

The decalcification agents were:

1. Formic acid in distilled water–10% concentration (FA10%);
2. Formic acid in distilled water–15% concentration (FA15%);
3. Anna Morse Solution (MS) composed of 50% formic acid and 20% sodium citrate) (Fernandes et al., 2007);

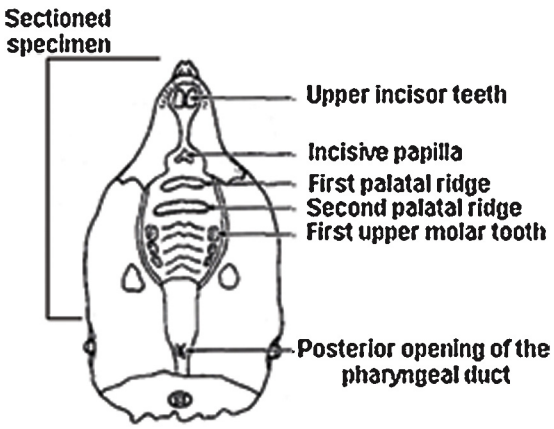

Fig. 1. Sectioning sites, and landmarks included in the sectioned specimen.

4. Ethylene diamine tetra-acetic acid/EDTA in distilled water-10% concentration (EDTA);

Each agent was utilized in 2 protocols (Table 1):

1. *A short one*, to investigate decalcification efficiency and epithelial histologic integrity at this stage. Duration of the short protocols was selected based on literature reports and our own pilot experiments.
2. *A long one*, to investigate potential additional decalcification efficiency, and adverse histologic effects. Duration of the long protocols was selected through our pilot experiments alone, due to lack of pertinent reports.

Therefore, specimens were randomly allocated to 8 groups ($n = 10$ for the groups of the short protocols and $n = 5$ for the long protocols; acronyms defined in Table 1).

A 9th group ($n = 5$) served as *positive* controls, via treatment with a strong acid; specimens were incubated for 5 days in 4% hydrochloric acid (HCl) in distilled water, a regimen expected to decalcify sufficiently but also to cause considerable histologic damage. Conversely, a group of *negative* controls was unfeasible, insofar as *non-decalcified* bone cannot undergo histological assessment without resorting to intricate techniques of snap-frozen hard tissue sectioning; this however requires considerable expertise, is not routinely carried out, cannot be easily applied to rat nasal turbinates due to their fragility (pilot study observations – data not shown) and it is not readily comparable to histologic analysis of *decalcified* tissue.

Each specimen was immersed in a distinct vial containing 100 ml of decalcifier, in line with the common practice of using a volume at least 5- to 10-fold the specimens size. The vials were kept at 21 °C, away from light. The decalcifier was replaced every 48 h during the first 10 days and every 72 h thereafter. No handling took place (stirring, heating, vacuum agitation, etc.).

Table 1
Protocol duration for each decalcification agent.

Decalcification medium	Protocol duration	
	Short	*Long*
Formic acid 10%	5 days (*FA10%5D*)	20 days (*FA10%20D*)
Formic acid 15%	5 days (*FA15%5D*)	15 days (*FA15%15D*)
Morse Solution	5 days (*MS5D*)	20 days (*MS20D*)
EDTA 10%	20 days (*EDTA20D*)	40 days (*EDTA40D*)