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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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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.

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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-Fukuda 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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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);

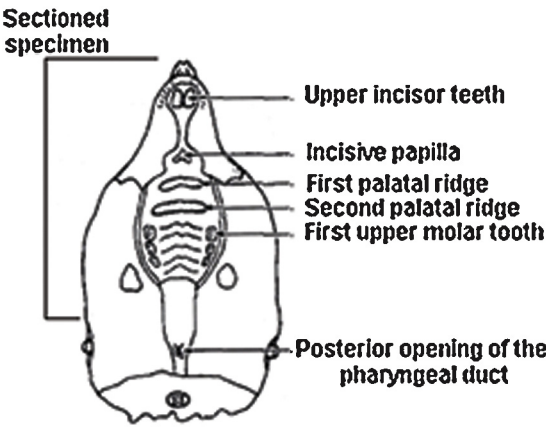


Fig. 1. Sectioning sites, and landmarks included in the sectioned specimen.

- 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)

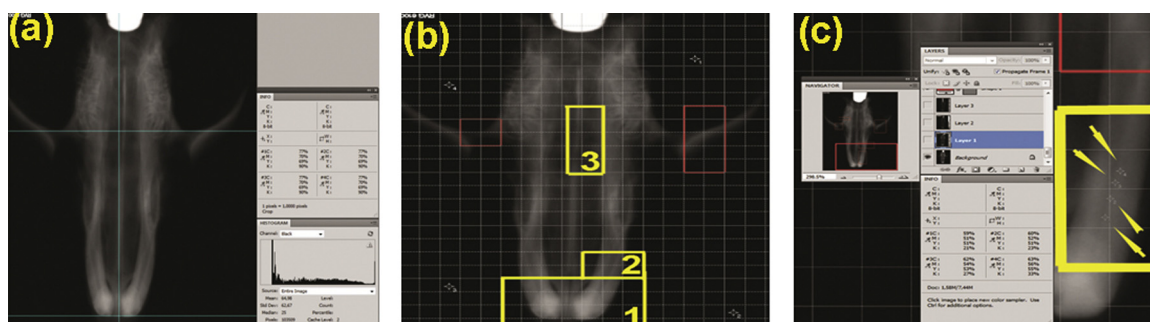


Fig. 2. (a) X-ray of a specimen is calibrated on horizontal and vertical axes, in order for all previous and later X-rays of this particular specimen to completely overlap. (b) The enveloped and numbered areas were selected for initial evaluation of the progress of decalcification. *1st area*: at the level of the upper incisor teeth and immediately posterior to it. *2nd area*: posterior to the upper incisor teeth and at the level of the incisive papilla. *3rd area*: posterior to the second palatal ridge, at the level of the first and second molar teeth and into the posterior region of the ethmoid recess. (c) The four sites (spots) within the 2nd area (posterior to the upper incisor teeth) which were selected for initial evaluation of the progress of decalcification (arrows).

2.5. Assessment of decalcification

To ensure decalcification, one can use chemical, radiographic or physical tests (Mattuella et al., 2007; Verdenius and Alma, 1958). We used X-ray testing and Photoshop™ analysis, on the basis of a protocol designed by us. Specifically, specimens underwent X-ray

imaging on days 0, 1, 3, 5, 8, 12, 15, 20, 25, 30, 35, and 40 (unless their protocol was concluded earlier). Each specimen was placed on a firm surface and an X-ray was taken with the arm of a DeGotzen x-mind™ (DeGotzen, VA, Italy) put 5 cm over the specimen and at a 90° angle. For each distinct specimen, each X-ray was analyzed in the Adobe Photoshop™ application (Adobe Corporation, San Jose, CA);

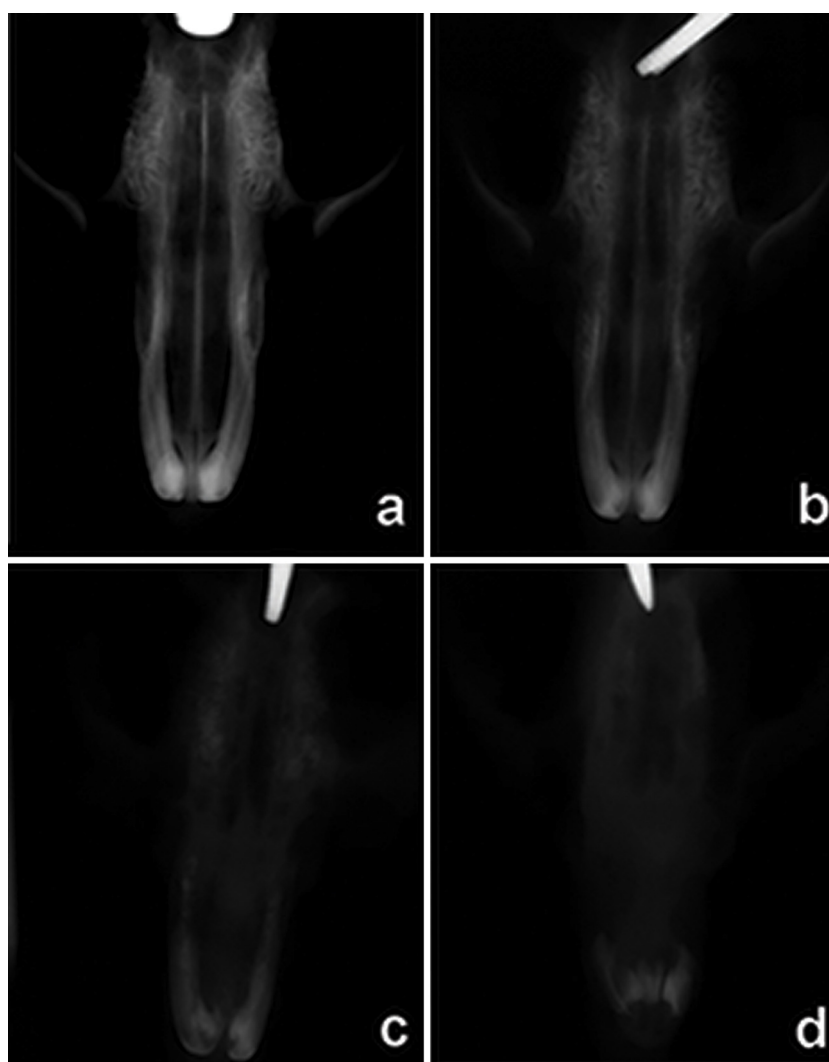


Fig. 3. Progress of decalcification in subsequent X-rays of a specimen. Structures in the 1st area (upper incisor teeth) are the last to decalcify regardless of decalcification medium.

it was calibrated to fit exactly the previous X-rays of this particular specimen (Fig. 2a) and was compared against them, so that the progress of decalcification could be assessed.

2.5.1. First phase

More specifically, for the *initial* phase of evaluation of decalcification, 3 inset, fixed areas were selected for each specimen (Fig. 2b). Four inset sites (spots) were further selected in each area (Fig. 2c) bringing the sum of unique sites included in the initial assessment to 12 for each specimen. Decalcification at each spot was evaluated against same-spot readings in later X-rays. Our findings indicated that the last area to decalcify is the area housing the upper incisor teeth (1st area in Fig. 2b; and Fig. 3) (data not shown), whose roots extend posteriorly (Fig. 4). Therefore, the 1st area was selected as the criterion for complete decalcification, in the next phase of assessment.

2.5.2. Second phase

In the next phase, decalcification was defined as follows: in each X-ray, spots that did not correspond to bone (clear space) were consistently found to have a *K* (black) index of 90% in the *CYMK* palette. Therefore, a *K* index of roughly 90% *within the 1st area*, would define adequate decalcification. However, we found that in formic-acid based decalcification (FA10%, FA15% and MS), *K* index tended to plateau at 80% (Fig. 5). Therefore, for formic acid-based decalcifiers, we arbitrarily defined the threshold of adequate decalcification at a *K* index of 80%. For EDTA we set a threshold of 90%, because *K* index in these specimens did not plateau at 80%.

2.6. Histological analysis

Decalcified bone was transversely sectioned posteriorly to the upper incisor teeth and anteriorly to the incisive papilla, and specimens were embedded in paraffin, thin-sectioned at 4 μ m and stained with hematoxylin and eosin (H&E). We selected this site as it is representative of all types of nasal epithelium (Mery et al., 1994) (Fig. 4): importantly, respiratory epithelium, the type appropriate for AR investigations, overlies the septum, the proximal nasoturbinate and the proximal maxilloturbinate. Therefore, a site on the septum was selected for our study (Fig. 4), and blinded histological assessment of RE ensued, by the following six criteria: *height of epithelial cells, cell borders, cilia, cytoplasm, nuclei, and goblet cells* (Table 2). The mean histologic score for each group was calculated, and each group was assigned in one of three histologic grades on the basis of this score (Table 3).

2.7. Statistical analysis

Normality of histologic scores was demonstrated on Q–Q plots, and one-way ANOVA and independent sample Students *t*-test were used to compare differences in means among groups. A two tailed *p* value of <0.05 was considered statistically significant.

3. Results

3.1. Decalcification

In Fig. 5 we present the progress of decalcification. *K* index was roughly 80% for all 3 formic-acid based decalcifiers (FA10%, FA15% and MS) at the end of the short protocol (5 days). This was the threshold we had defined for these agents as adequate decalcification. Indeed, decalcifying activity plateaued from the 5th day onwards throughout the respective long protocols (15 days for FA15%; 20 days for FA10% and MS) (Fig. 5); this indicates that further incubation in these mediums was redundant.

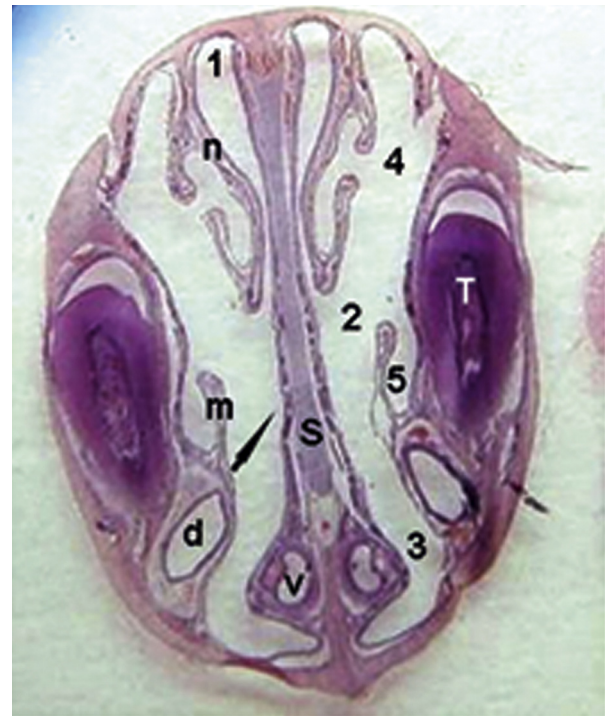


Fig. 4. Transverse section of the nasal structures of the rat at the level posteriorly to the upper incisor teeth and anteriorly to the incisive papilla. H&E stain. Squamous epithelium can be found within the ventral meatus, transitional epithelium covers the lateral surface of the nasoturbinate, the dorsal lateral meatus and the lateral surface of the maxilloturbinate, while olfactory epithelium appears in the dorsal meatus. Arrow marks the site where analysis of the epithelium was undertaken. 1, dorsal meatus; 2, middle meatus; 3, ventral meatus; 4, dorsal lateral meatus; 5, ventral lateral meatus; n, nasoturbinate; m, maxilloturbinate; d, nasal lacrimal duct; v, vomeronasal organ; T, root of the upper incisor tooth; S, septum.

In regard to EDTA decalcification, the 90% *K* index defined as threshold was reached at the end of the short protocol (20 days), and remained at this range throughout the long protocol (40 days) (Fig. 5). This indicates that incubation in EDTA after 20 days, was superfluous.

3.2. Histological analysis

Mean histological scores are presented in Table 3. *Short* decalcification regimens (5 days) in the formic acid-based agents (FA10%,

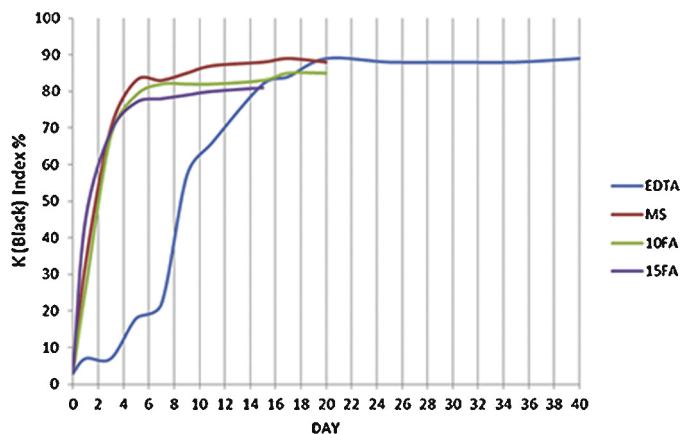


Fig. 5. Decalcification progress in the 1st nasal area for all decalcifying agents. *x*-axis: days of incubation in the decalcifying medium. *y*-axis: *K* (black) index of the 1st area in the specimen X-ray. A threshold of roughly 80–90% (depending on the decalcifying agent), was defined as acceptable decalcification.

Table 2

Grading system for the preservation of the features of the nasal respiratory epithelium, following decalcification. Samples received a 0–2 grade for each criterion, bringing the total score for each sample to a 0–12 range.

Features of epithelial cells	Grading
Height	
<10 μ	0
11–15 μ	1
16–20 μ	2
Cell borders	
No borders	0
Obscure borders	1
Clear borders	2
Cilia	
Absence	0
Adhered together	1
Clear	2
Cytoplasm	
Colorless	0
Weakly colored	1
Colored	2
Nuclei	
Shadowy/indistinct	0
Colorless	1
Colored	2
Goblet cells	
Absence	0
Obscure	1
Clear	2

FA15% and MS) and the EDTA (20 days), caused no appreciable damage to the epithelium (Fig. 6). Conversely, long incubation periods in the acid-based agents led to pronounced epithelial damage. In contrast, the long EDTA protocol (40 days) did not compromise the histological analysis (Grade 1: appropriate), albeit it did cause modest epithelial deterioration and a statistically significant drop in the histologic score, as compared to the short protocols (Fig. 6 and Table 4). The EDTA40D protocol was unique, in that its results were significantly different from all other regimens, demonstrating that it was less safe than the short protocols but was superior to the other long protocols (Table 4). Incubation in 4% HCl for 5 days caused severe epithelial deterioration (Fig. 6c). One-way ANOVA demonstrated significant differences among groups ($P < 0.001$); Students *t*-test indicated that there were no significant

Table 3

Mean histologic scoring and grading of groups. Grading was defined as follows: Score 0–4 = 3rd grade: decalcification regime unacceptable – noxious for the epithelium; Score 5–8 = 2nd grade: decalcification regime inappropriate – harmful for the epithelium; Score 9–12 = 1st grade: decalcification regime appropriate – safe for the epithelium.

Regime	Final score (mean and standard deviation)	Grade
EDTA20	Mean = 11.8 S.D. = 0.422	1st
EDTA40	Mean = 9 S.D. = 0.707	1st
FA10%5D	Mean = 11.7 S.D. = 0.483	1st
FA10%20D	Mean = 5 S.D. = 0.707	2nd
FA15%5D	Mean = 11.3 S.D. = 0.675	1st
FA15%15D	Mean = 5 S.D. = 0.707	2nd
MS5D	Mean = 11 S.D. = 0.667	1st
MS20D	Mean = 5.2 S.D. = 0.837	2nd
HCL5D	Mean = 3 S.D. = 0.707	3rd

differences among protocols of the same duration (i.e. long and short) except for the EDTA40D regimen, but there were significant differences between the short and long protocols for each agent (Table 4).

4. Discussion

In the present study, we appraised the efficiency of 10% EDTA, 10% and 15% formic acid and Morse Solution, on the decalcification of rat nasal bone; we also compared their effects on the histologic integrity of nasal respiratory epithelium.

Acidic demineralizers such as aqueous formic acid are widely used (Kiviranta et al., 1980). Nevertheless, they are likely detrimental to the tissue, as they can cause collagen distortion and decrease the affinity of histological stains, effects largely dependent on the solution's acidity and duration of decalcification (Mattuella et al., 2007). Variable formic acid protocols are used in AR models: Farraj et al. (2006) implemented a 7-day protocol with a 13% FA solution for 6 w.o. mice, Harkema et al. (1987) employed a 7–10 day protocol of the same concentration for bonnet monkeys and a 4-day protocol for 10–14 w.o. rats (Harkema et al., 1999), and Hotchkiss et al. (1998) and Wagner et al. (2002) used this solution for 4 days, for adult rats. Yamasaki et al. (2001) decalcified the skulls of 2 w.o. guinea pigs in a 5% FA solution, albeit they do not report protocol duration.

Conversely, Morse Solution (MS) is not generally used in AR protocols. This is rather surprising, as MS requires short incubation periods, minimally affects cellular morphology, and lacks the tendency of formic acid to cause cellular edema (Fernandes et al., 2007; Yamamoto-Fukud et al., 2000). In our study, 5 days of MS yielded excellent H&E results (1st grade). However, at variance to our findings, Fernandes et al. (2007) reported diminished tissue preservation and affinity for H&E in maxillae of 8 w.o. rats, after 72 h of MS use. In light of this data, we concede that, less than 5 days of incubation may have produced better histologic results than those we report here. However, in our study, X-ray imaging revealed that the nasal structures were not adequately decalcified before 5 days; in fact, our pilot attempts to section the specimens after a 3-day MS protocol were laborious and often unsuccessful, due to structural collapse (data not shown). Inasmuch as the nasal turbinates (as opposed to maxillae), are extremely fragile, inadequate decalcification can severely compromise their integrity during sectioning. Therefore, we favor a 5-day protocol for MS decalcification for 8 w.o. rats and we extend a note of caution on long use of MS, as the long protocol led to marked epithelial deterioration (2nd grade) without further facilitation of sectioning.

EDTA is a tetradentate chelating agent which extracts calcium from the bone (Kiviranta et al., 1980), and is widely used as it preserves intercellular integrity and staining affinity and minimizes histological artifacts (Kiviranta et al., 1980). Nevertheless, extended contact with EDTA may still distort tissue structure and reduce affinity for some histological stains (Trudel et al., 2000). To date, very few investigators have employed EDTA in AR models and their protocols were highly variable. A 20% EDTA–Na solution was used for 3 days (Iwasaki et al., 2003) and 7 days (Nakaya et al., 2006) to decalcify mice skulls, and a 2.5% EDTA–Na solution was used for 6–10 w.o. mice (Yamatomo et al., 2001), albeit duration was not reported. Accordingly, in a previous work of ours, we have used 10% EDTA solution for 15 days to decalcify nasal bone of 11 w.o. rats (Guibas et al., 2013); although we were able to efficiently conduct the analyses in this work, tissue sectioning and mounting on glass slides was cumbersome. This provided the impetus for the present study in which we assessed the decalcifying efficiency and potential adverse effects of 20 and 40 days of EDTA. The specimens were adequately decalcified upon 20 days; from thereon,

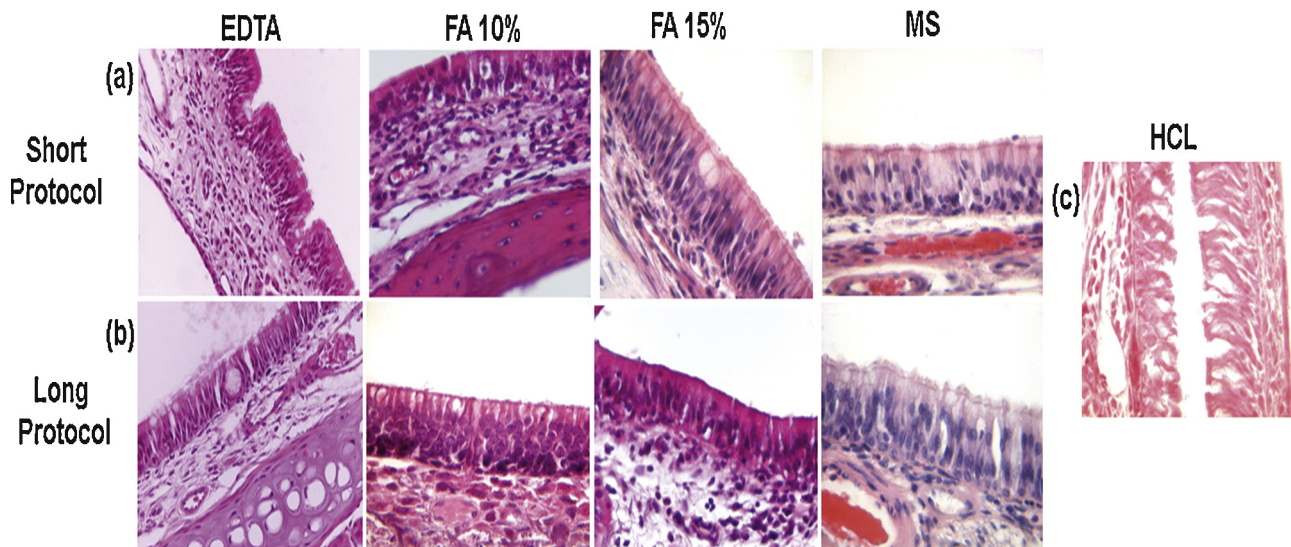


Fig. 6. H&E stained sections of the respiratory nasal epithelium following decalcification of the nasal osseous structures in different decalcifying mediums for variable duration. FA 10%, formic acid 10%; FA15%, formic acid 15%; MS, Morse Solution; HCL, hydrochloric acid

Table 4
Results of pairwise comparisons of mean histologic scoring of the groups. Students *t*-test. Bold indicates statistical significance. Levenes test indicated equality of variance for all pairs. *P* < 0.05 considered significant; M.D. = mean difference; –, presented elsewhere in the table.

Protocols	Short protocols				Positive control	Long protocols			
	EDTA20D	FA10%5D	FA15%5D	MS5D	HCL5D	EDTA40D	FA10%20D	FA15%15D	MS20D
EDTA20D	X	M.D. = 0.1 <i>P</i> = 0.62	M.D. = 0.5 <i>P</i> = 0.062	M.D. = 0.5 <i>P</i> = 0.10	M.D. = 8.8 <i>P</i> < 0.001	M.D. = 2.8 <i>P</i> < 0.001	M.D. = 6.8 <i>P</i> < 0.001	M.D. = 6.8 <i>P</i> < 0.001	M.D. = 6.6 <i>P</i> < 0.001
FA10%5D	–	X	M.D. = 0.4 <i>P</i> = 0.14	M.D. = 0.4 <i>P</i> = 0.20	M.D. = 8.7 <i>P</i> < 0.001	M.D. = 2.7 <i>P</i> < 0.001	M.D. = 6.7 <i>P</i> < 0.001	M.D. = 6.7 <i>P</i> < 0.001	M.D. = 6.5 <i>P</i> < 0.001
FA15%5D	–	–	X	M.D. = 0.0 <i>P</i> = 1.00	M.D. = 8.3 <i>P</i> < 0.001	M.D. = 2.3 <i>P</i> < 0.001	M.D. = 6.3 <i>P</i> < 0.001	M.D. = 6.3 <i>P</i> < 0.001	M.D. = 6.1 <i>P</i> < 0.001
MS5D	–	–	–	X	M.D. = 8.3 <i>P</i> < 0.001	M.D. = 2.3 <i>P</i> < 0.001	M.D. = 6.3 <i>P</i> < 0.001	M.D. = 6.3 <i>P</i> < 0.001	M.D. = 6.1 <i>P</i> < 0.001
HCL5D	–	–	–	–	X	M.D. = 6 <i>P</i> < 0.001	M.D. = 2 <i>P</i> = 0.002	M.D. = 2 <i>P</i> = 0.002	M.D. = 2.2 <i>P</i> = 0.002
EDTA40D	–	–	–	–	–	X	M.D. = 4 <i>P</i> < 0.001	M.D. = 4 <i>P</i> < 0.001	M.D. = 3.8 <i>P</i> < 0.001
FA10%20D	–	–	–	–	–	–	X	M.D. = 0 <i>P</i> = 1.00	M.D. = 0.2 <i>P</i> = 0.69
FA15%15D	–	–	–	–	–	–	–	X	M.D. = 0.2 <i>P</i> = 0.69
MS20D	–	–	–	–	–	–	–	–	X

further incubation was neither required nor effective. H&E yielded excellent results (1st grade) in the short and acceptable (albeit statistically inferior) results in the long protocol, suggesting that for H&E staining, the incubation period can be extended for up to 40 days, with little epithelial deterioration. Thus, this agent is clearly appropriate for AR protocols, when histologic assessment is sought and urgency is not a critical factor.

Although our findings can support suggestions on appropriate decalcification regimens for experimental allergic rhinitis, certain limitations disallow complete standardization. For instance, the different bone mass between the skulls of mice and rats clearly affects decalcification times, since small specimens can be demineralized rapidly due to smaller penetration fields (Fernandes et al., 2007; Kiviranta et al., 1980). Furthermore, animal age affects the duration of decalcification, as it correlates with solubility and crystallinity of bone minerals (with younger bone minerals being more soluble/less crystalline than older ones) (Horvath, 2006). Temperature, solution mass, stirring of the solution and other factors, also affect decalcification. Thus, the exact time required may vary widely among different experimental settings.

Overall, our study is the first to attempt a standardization of the decalcification process in experimental allergic rhinitis, a field where this procedure is largely carried out tentatively via considerably diverse protocols. Based on our findings we favor the use of EDTA for 20 days for 8 week-old rats, when urgency is not an issue. However, a longer protocol may be required for older/bigger animals; such a protocol may extend up to 40 days with little epithelial deterioration. When there is urgency, formic-acid-based agents appear to be appropriate decalcifiers. Since epithelial features were preserved for either concentration of FA (10% and 15%), we favor the former, which is likely to cause less soft tissue degeneration. However, extended incubation in all formic acid-based agents will severely compromise histological results. We content that our findings provide a basis for the standardization of decalcification procedures in the experimental allergic rhinitis setting.

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Conflict of interest statement

The authors declare no pertinent conflict of interest.

Author contributions

George Guibas conceptualized, designed and carried out the study, collected and collated data, conducted the statistical analysis and the literature search, designed and drafted the manuscript, and approved the final manuscript as submitted; Sotiris Lakis collated data, conducted the histologic analysis and the literature search, critically revised the manuscript and approved the final version; Christoforos Gkimpas collated data, conducted the radiographic analysis and the literature search, critically revised the manuscript and approved the final version; Marianthi Manda carried out the study, collected data, critically revised the manuscript and approved the final version, Dorothea Kapoukranidou collated data, critically revised the manuscript and approved the final version; Evangelia Spandou designed and supervised the study, critically revised the manuscript and approved the final version as submitted.

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