Optimization of nasal tissue decalcification technology in preclinical studies of inhaled drugs: applied to rat nasal mucosa tissue

Pathological examination

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Abstract: Objective To study the decalcification effect of different decalcification solutions on rat nasal tissue and its staining effect on pathological sections. Methods Three different decalcification solutions: 10% ethylene diamine tetraacetic acid (EDTA), 10% formic acid, and 5% nitric acid decalcification solution were selected to determine the decalcification of rat nasal tissue under room temperature and microwave conditions. The decalcification time and decalcification effect were compared and analyzed, and the quality of pathological slices made from bone tissue after different decalcification methods was comprehensively evaluated. Results: Under normal temperature conditions, nasal tissue meridians The decalcification time required by EDTA decalcification solution is the longest. The decalcification time of nasal tissue by nitric acid decalcification solution under microwave conditions is the shortest. The nasal tissue slices by EDTA decalcification solution The quality, HE staining, MASSON staining and immunohistochemistry staining were the best, the section quality of the nasal tissue with nitric acid decalcification solution was the worst, and the nasal tissue with formic acid decalcification solution had the worst quality. The tissue HE staining effect is better, while the quality of MASSON and immunohistochemical staining is slightly worse. Conclusion EDTA decalcification solution combined with microwave decalcification of nasal tissue, decalcification efficiency Significantly improved, with excellent sectioning and staining results.

Keywords: nose; decalcification; bone tissue; rat; histopathology; inhaled drugs

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Optimization of Nasal Tissue Decalcification Technique in Preclinical Studies of Inhaled Drugs: Histopathological Examination of Nasal Mucosa in Rats

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ABSTRACT: OBJECTIVE New inhaled formulations that act on the nose, mouth, respiratory tract, and whole body have received increasing attention. Meanwhile, the research and declaration of inhaled drugs have become hot spots amid infectious respiratory pandemic diseases worldwide. Due to the special anatomic structure of the nose, folds, grooves, and special structures may cause the specific uptake and deposition of inhaled substances. There are various epithelial tissues, glands, muscles, and cartilages in the vestibule, respiratory, and olfactory parts of the nose. Inhaled substances can generate irritating and toxic effects on various parts. The pathological diagnosis results from the preclinical safety evaluation of inhaled drugs are considered the gold standard for judging drug toxicology. The nose is composed of many bone components, and decalcification is required for the sectioning of hard bone tissues. Therefore, an efficient and high-quality decalcification method is the crucial pathological technique for evaluating inhaled drugs. **METHODS** In this study, 10% ethylenediamine tetraacetic acid(EDTA), 10% formic acid, and 5% nitric acid decalcification solutions were selected. Besides, the decalcification time and effect of these decalcification solutions for rat nasal tissues were compared and analyzed under static room temperature and microwave conditions. Moreover, the quality of pathological bone tissue sections prepared through different decalcification methods was comprehensively evaluated. **RESULTS** Compared with the decalcification method under normal temperature, the decalcification time under the treatment of KOS decreased significantly. The treatment with the EDTA decalcification solution had the longest decalcification time under normal temperature, while the treatment with the nitric acid decalcification solution had the shortest decalcification time under microwaves. During section evaluation, the EDTA decalcification solution had a higher quality score under normal temperature and microwaves, which indicated that the section quality was favorable. The nitric acid decalcification solution had a lower section quality score under microwaves, which indicated that the section quality was unfavorable. There was medium section quality for the formic acid decalcification solution under microwaves and normal temperature and for the nitric acid decalcification solution under normal temperature. The HE staining results suggested that there were incomplete nasal mucosa epithelia, fragmentation, and pink nasal bone tissues in the tissue sections treated by the nitric acid decalcification solution, presenting a peracid state. In the tissue sections treated by the formic acid decalcification solution and the EDTA decalcification solution, the nucleus of epithelial cells was blue-purple, the cytoplasm and interstitial components were pink, and the epithelial tissue structure of nasal mucosa was intact. The MASSON staining results suggested

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that in the tissue sections treated by the nitric acid decalcification solution, the whole section staining was red, the positive area was not obvious, and the epithelial cell differentiation was not prominent, with a fuzzy structure. In the tissue sections treated by the formic acid decalcification solution, the sections were slightly detached during staining, and slight cracks were observed in submucosa tissues. In the tissue sections treated by the EDTA decalcification solution, the structure of positive regions and epithelial mucosa regions was clear, and the nuclear and interstitial components were clearly distinguished. The immunohistochemical staining (Ki67) results suggested that in the tissue sections treated by the nitric acid decalcification solution, the staining of positive regions was uneven, and there were nonspecific negative reactions in some regions. In addition, local epithelial cells were unstained. In the tissue sections treated by the formic acid decalcification solution, the local regions were not clearly stained, and nonspecific negative and positive reactions appeared in some local regions. In the tissue sections treated by the EDTA decalcification solution, the positive regions were prominent, the boundaries between negative regions and positive ones were clear, and each region of the sections was stained evenly. **CONCLUSION** Among the three decalcification solutions in this study, the nitric acid decalcification solution had the shortest decalcification time while the poor section and staining quality. The decalcification time of nasal tissues through the EDTA decalcification solution combined with microwaves was significantly shorter than that through the EDTA decalcification solution at normal temperature. Furthermore, this decalcification method achieved favorable section and staining quality.
KEYWORDS: nose; decalcification; bone tissue; rat; histopathological; inhaled drugs

Inhalation drug delivery has been used for thousands of years [1]. Currently, There are a variety of inhalation devices used to deliver inhaled medications, including Pressurized dose inhalers, dry powder inhalers and nebulizers, etc. [2]. Medications delivered through inhalation devices reach the lungs, which are both local The target site of action is also the absorption part of systemic drugs. position, the advantage of this administration method is that systemic adverse reactions occur Low rate and quick onset of effect [3]. Acts on the mouth, nose, and exhalation by inhalation New dosage forms for the inhalation tract and the whole body have attracted much attention. At the same time, due to the global Infectious respiratory disease pandemic and other factors, inhaled drugs R&D has become a hot spot [4].

Inhaled drugs enter the lungs through the entire respiratory tract, the process Will be subject to multiple factors such as mechanical, chemical, immune and behavioral barriers impact[5]. Therefore, in preclinical safety evaluation and efficacy studies, It affects the nasal mucosa, oral mucosa, throat tissue, trachea and lungs Organs and other tissues were subjected to histopathological examination. Due to the structure of the nose Peculiarities, the folds, grooves and special structures in the nose may cause Specific uptake and deposition of inhaled substances [6]. vestibule of nose, The respiratory and olfactory regions contain a variety of epithelial tissues, glands, muscles and soft tissues. Bones and other tissues, inhaled substances can be irritating and toxic to various parts Sexual effects, pathological diagnosis of preclinical safety evaluation of inhaled drugs The results are the gold standard for determining drug toxicology.

The decalcification step is important for histopathological observation of the nose. and necessary steps. The nose contains more bone tissue, and the bone tissue contains Contains collagen and non-collagen proteins with high mineral content, mineral The substance is mainly composed of calcium and phosphorus insoluble salts (hydroxyapatite) [7]. Calcium and hydroxyapatite combine with organic protein matrix to provide bone tissue with Provide hardness [8]. Due to the hardness of bone tissue, the production of bone tissue slices More difficult. Therefore, in the process of making bone tissue slices, a It is difficult to find a decalcification method with both high efficiency and high quality. in progress "Decalcification" when slicing bone tissue is another key technical difficulty. Calcium effect directly affects pathological film production, film reading and result evaluation, and has also become a It is the speed-limiting link for many new drug applications.

In this study, rat nasal tissue was selected, and 10% ethylenediaminetetrakis Acetic acid (ethylene diamine tetraacetic acid, EDTA), 10% Formic acid, 5% nitric acid decalcification solution, 3 different decalcification solutions, static at room temperature and microwave conditions, to compare the decalcification time and decalcification effect. Analysis, comprehensive evaluation of bone tissue after different decalcification methods to make pathological sections Tablet quality, regulation for nasal bone tissue decalcification in drug preclinical studies Standardized methods provide scientific data.

1Materials and methods

1.1 Animals and materials

30 SPF male SD rats, body weight 180~220 g, Experimental animals were purchased from Beijing Weitonglihua Experimental Animal Technology Co., Ltd. Material production license number: SCXK (Beijing) 2021-0006. Animal feeding Conducted at the experimental animal facility of Shanghai Institute of Food and Drug Inspection Laboratory animal use license number: SYXK (Shanghai) 2021-0026. True nature Passed the Animal Ethics Committee of Shanghai Food and Drug Inspection Institute will be reviewed, and the animal ethics approval number is IACUC-SIFDC-21037.

Ulay sugar (batch number: 20210712), formic acid (batch number: 20220171), nitric acid (batch number: 20190382), EDTA (batch number: 20180103) and xylene (batch number: 20230607) were purchased from Sinopharm Tuan Chemical Reagent Co., Ltd.; Paraffin (Leica, Germany, batch number: 39601095); MASSON staining kit (batch number: 20221022) and immunohistochemistry secondary antibody kit (batch number: 20220184) were purchased from Beijing Jingsolaibao Technology Co., Ltd.; Ki67 primary antibody (batch number: ab15580) Purchased from Abcam Company.

KOS microwave rapid tissue dehydration machine (Milestone, Italy) Division); HistoCore tissue embedding machine, RM2255 rotary microtome, The ST5020 automatic tissue section staining machine was purchased from Leica, Germany. Division); ZEISSScopeA1 microscope (Zeiss, Germany).

1.2 Animal Disposal

Rats will be quarantined by veterinarians after entering the unit's animal facilities

The examination showed no obvious abnormalities. After 3 days of adaptive rearing,

Anesthetized by intraperitoneal injection of 20% Ule sugar solution (1 g·kgÿ1), and then sacrificed.

A systematic gross autopsy was performed, and nasal tissue was taken and placed in 10% neutral formaldehyde

Lin fixative.

1.3 Organizational processing

The above 30 nasal tissues were randomly divided into 6 groups, 5 in each group

The specimens were subjected to decalcification operations respectively. The grouping and decalcification reagents are shown in Table 1.

Table **1** Bone tissue decalcification procedure grouping

Tab. 1 Bone tissue decalcification of program

Group number	decalcification solution	Solvent	concentration/%	Microwave treatment
1	EDTA	4% formaldehyde solution	10	ÿ
2	Formic acid	4% formaldehyde solution	10	ÿ
3	Nitric acid	4% formaldehyde solution	5	ÿ
4	EDTA	4% formaldehyde solution	10	/
5	Formic acid	4% formaldehyde solution	10	/
6	Nitric acid	4% formaldehyde solution	5	/

After entering the decalcification process, remove EDTA and formic acid every 12 hours.

The bone tissue in the calcium solution is examined, and the nitric acid decalcification solution is checked every hour.

The bone tissue treated with medium microwave was inspected, and the needle penetrated into the bone without any friction.

Feeling is the end of decalcification.

1.4 Operation after decalcification is completed

After decalcification, use 10% sodium bicarbonate aqueous solution to neutralize

4 h, after completion of neutralization, perform histopathological preparation. Nasal group

The tissue was dehydrated through gradient ethanol, made clear in xylene, and immersed in wax (62°C liquid

paraffin), paraffin embedded and then tissue sectioning.

1.5 slice rating

During the sectioning process, the quality of the decalcified tissue was assessed.

analysis, including the integrity of the embedded tissue block, tissue fragmentation

condition, flatness of the spread, and integrity after drying. The above indicators are analyzed.

Different scores, ranging from 1 to 5, that is, 1 is the worst effect, 5 is the worst

For the best effect, count each score and total score to judge

No other method of decalcification makes it easier to make slices.

1.6 Slice staining

The sectioned bone tissue was subjected to routine section staining (HE),

Special staining (MASSON) and immunohistochemistry (Ki67) staining,

To evaluate the effect of bone tissue preparation under microscope after staining, the scoring method is the same as

Item "1.5" is used to determine which decalcification method has the best staining quality.

1.7 Bone tissue demineralization film evaluation

Conduct a comprehensive assessment of the contents of items "1.5" and "1.6",

Including scores, operation time and rationality, select the most suitable after evaluation

A comprehensive decalcification method will be used for subsequent research.

1.8 Data processing

Statistical analysis was performed using GraphPad Prism 5.01 software.

Graphs were drawn, and statistical data were expressed as $\bar{x} \pm s$. by Shapiro Wilk

The method tests whether the data is normally distributed, such as if the data is normally distributed,

Use *t* test between two groups. If the data is non-normally distributed, use

Rank sum test, P<0.05 means the difference is statistically significant.

2 results

2.1 Comparison of decalcification time

Compared with the normal temperature decalcification method, the KOS microwave decalcification method has

Decalcification time is significantly reduced. Among them, EDTA decalcification under normal temperature conditions

The decalcification time required for the liquid is the longest, and the nitric acid decalcification liquid under microwave conditions

The required decalcification time is the shortest, see Figure 1.

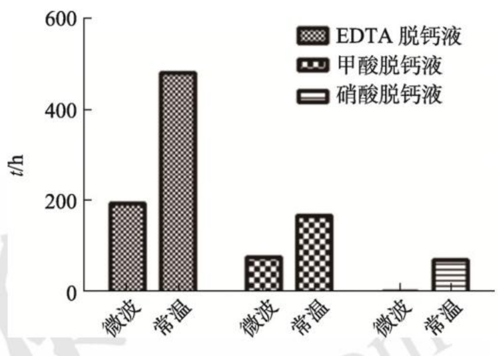

Figure **1** Comparison of decalcification time under different decalcification conditions

Fig. 1 Comparison of decalcification time under different decalcification conditions

2.2 Comparison of evaluation during slicing process

During the section evaluation process, EDTA decalcification solution is maintained at room temperature and micron

Under wave conditions, the slice quality score is higher, indicating that the slice quality

The quality of slices is good; nitric acid decalcification solution has better section quality evaluation under microwave conditions.

A low score indicates that there are certain problems with the quality of the production; A

Acid decalcification solution is cut under microwave, normal temperature conditions and nitric acid at normal temperature conditions.

The film quality is average, see Table 2.

2.3 Comparison of HE dyeing effects

HE staining shows that tissue sections that have undergone nitric acid decalcification solution,

The nasal mucosal epithelium is incomplete, fragmentation can be seen in sections, and the intranasal bones

The tissue is pink, showing a state of overacidity; it has been decalcified by formic acid.

and EDTA decalcified solution tissue sections, nuclei of epithelial cells

Blue-purple color, cytoplasm and interstitial components are pink, nasal mucosa

The epithelial tissue structure is intact, see Figure 2.

2.4 Special dyeing comparison

MASSON staining shows that tissue that has undergone nitric acid decalcification solution

The sections were stained red overall, and the positive areas were not obvious.

Epithelial cells are not clearly differentiated and have a fuzzy structure; after formic acid decalcification

The tissue sections were slightly detached during the staining process, and the submucosa group

Mild cracks can be seen in the tissue; tissue sections of EDTA decalcification solution are positive

The regional and epithelial mucosal regions have clear structures, and the nuclei and stroma are

The divisions are clearly distinguished, see Figure 3. It shows that nitric acid decalcification and formic acid decalcification combine

The abnormal conditions shown are artificial artifacts during the production process.

Table **2** Comparison of quality scores of tissues after decalcification under different conditions ($\bar{x} \pm s$, n=5)

Tab. 2 Comparison of tissue quality scores after decalcification under different conditions($\bar{x} \pm s$, n=5)

Scoring item	decalcification conditions	EDTA decalcification solution	formic acid decalcification solution	nitric acid decalcification solution
Embedded complete	microwave	5.0±0.0	4.6±0.5	3.6±0.51)3)
	Room temperature	5.0±0.0	4.6±0.5	4.2±0.41)
Tissue Disintegration	Microwave	5.0±0.0	4.0±0.01)	3.6±0.51)
	Room temperature	5.0±0.0	3.6±0.51)	4.0±0.01)
Spread flatness	microwave	5.0±0.0	4.8±0.4	3.2±0.42)3)
	Room temperature	5.0±0.0	4.8±0.4	3.4±0.52)3)
After drying,	microwave integrity is	5.0±0.0	4.2±0.4	3.8±0.41)
	Room temperature	5.0±0.0	4.6±0.5	4.0±0.03)

Note: Under the same decalcification conditions, compared with the EDTA decalcification solution group, 1) P < 0.05, 2) P < 0.01; Compared with the formic acid decalcification solution group, 3)P<0.05.

Note: Under the same decalcification conditions, compared with the EDTA decalcifying group, 1)P<0.05, 2)P<0.01; compared with the formic acid decalcifying group, 3)P<0.05.

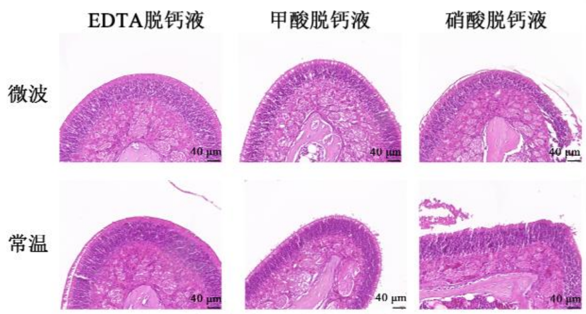

Figure **2** HE staining results of nasal mucosa under different decalcification conditions

Fig. 2 Results of nasal mucosa in different decalcification conditions with HE staining

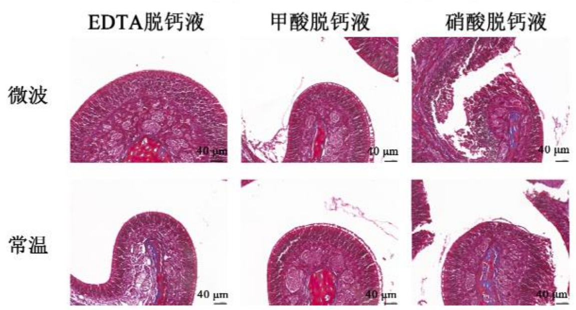

Figure **3** MASON staining of nasal mucosa under different decalcification conditions

Fig. 3 Results of nasal mucosa in different decalcification conditions with MASSON staining

2.5 Comparison of immunohistochemical staining

Immunohistochemical staining (Ki67) shows that after nitric acid decalcification

Tissue sections, positive areas are stained unevenly, and some areas are stained

Non-specific negative reaction, local epithelial cells appear unpigmented

Condition; tissue sections in formic acid decalcification solution, partial areas are not stained clearly,

Non-specific negative and positive reactions occurred in local areas; EDTA detachment

In tissue sections of calcium, the positive areas are more obvious, and the negative areas

The difference between the positive area and the positive area is clear, and all areas of the section are evenly stained, see

Figure 4.

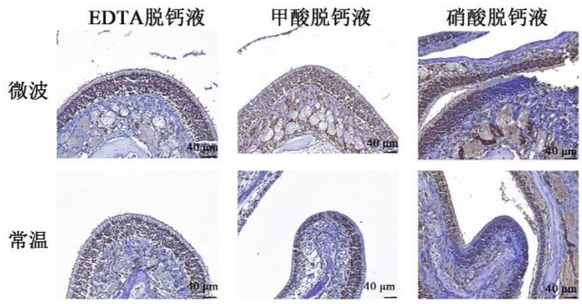

Figure **4** Immunohistochemical Ki67 staining results of nasal mucosa under different decalcification conditions

Fig. 4 Results of nasal mucosa in different decalcification conditions with IHC staining of Ki67

3 Discussion

Acidic decalcification solutions (formic acid and nitric acid) are commonly used decalcification solutions.

Increasing the concentration of acidic decalcification solution will also shorten the decalcification time.

The effect of calcium solution on decalcifying bone tissue of rodents, dogs and non-human primates

The results are better and the speed is faster. However, organizations using nitric acid decalcification solution will

Turns yellow, microwave can further accelerate the decalcification speed of acidic decalcification solution,

However, the quality of slices will be affected to a certain extent. In MASON staining,

In color and immunohistochemical staining, tissues using acidic decalcification solution are more

If it is easy to peel off and the color is not good, use formic acid decalcification solution.

HE staining of the treated tissue can keep it in better condition. in strong acid

Long-term decalcification can reduce antigen and enzyme activity, while weak acid or

Antigen reactivity, morphology and staining may be better preserved with chelating agents

color quality, thereby saving decalcification time [9]. However, long-term decalcification

It can also adversely affect dyeing quality [10]. Therefore, accurate control

The duration of decalcification improves sectioning and staining quality.

By using MASSON staining and immunohistochemical staining

(Ki67) evaluates the structure and cell tissue morphology of the nasal mucosa

analysis, MASSON mainly stains fibrillar collagen tissue.

After the nasal mucosal tissue is stimulated and damaged, the mucosa and submucosal fibers

Collagen deposition appears in connective tissue [11]. Not included in this study

Specific post-injury MASSON staining was positive, our institute

Changes such as tissue fragmentation and incomplete mucosal epithelium are all artificial.

phenomenon, so it can be judged that the use of formic acid and nitric acid decalcification solutions will have

MASSON staining of nasal mucosa.

Ki67 is extremely important in the process of tumor diagnosis and treatment and can accurately reflect

tumor cell proliferation activity [12]. Ki67 used in this experiment is a pair of

An indicator for evaluating nasal mucosal epithelial cells, because Ki67 is a
Antigens related to proliferating cells whose functions are closely related to mitosis
is indispensable in cell proliferation, so mucosal tissue
Epithelium can express Ki67 in large amounts, and mucosal tissue epithelium usually expresses
Positive. In this study, the use of Ki67 staining can better reflect the adhesion
The integrity of the membrane cells, and the nuclear tissue structure of the mucosal cells showed abnormal
Specific negative and positive expressions indicate that nitric acid decalcification solution has
The expression of histochemical Ki67 has a certain impact, so subsequent immunization
Decalcification solutions should be chosen carefully when performing immunohistochemistry or chemical special staining.

Non-acidic decalcification solution EDTA is a commonly used chelating decalcification solution.
agent, EDTA and hydroxyapatite crystals in bone tissue.
combine to form water-soluble nonionic compounds and promote the crystallization
The bound calcium in the inner body layer is transferred outward. Research has found that using EDTA
Decalcification solution re-sectioning quality, HE staining, MASSON staining machine free
showed good results in immunohistochemistry experiments, while under traditional conditions
Use EDTA decalcification solution for a longer time, even at low concentrations of EDTA
The decalcification process may take more than 2 months [13] when used under microwave conditions
EDTA decalcification solution can effectively shorten the decalcification time while maintaining
Certify the quality of decalcification and provide guarantee for subsequent research. Use this item to decalcify
Technology that can dissolve hydroxyapatite crystals in bone tissue in decalcification solution
Medium, when the pH value of the decalcification solution is neutral, it plays the role of chelating and decalcifying. EDTA
Decalcification properties, less damage to bone tissue, ensuring its internal enzyme activity
Preservation of specificity and antigenic characteristics for subsequent production of immunohistochemistry/fluorescence and
Histochemical staining provides assurance.

In summary, different decalcification solutions can affect the quality of paraffin-embedded bone.
Completeness and staining quality of tissue sections. Three species were used in this study
Among the decalcification solutions, the nitric acid decalcification solution has the shortest decalcification time, while slicing and
Poor staining quality, nasal tissue using EDTA decalcification solution and microwave
Tissue decalcification, the decalcification time is significantly shorter than that of EDTA decalcification at room temperature.
Calcium time, and both sectioning and staining results are good.

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