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Application of Hydrochloric Acid Decalcification Solution in Preparation and Section of Intervertebral Disc in Mice

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Abstract

The pathological section of bone tissue can observe the whole picture of bone tissue, which is very important for determining the clinical prognosis. However, the rich calcium salt in bone tissue makes it difficult to make a complete film. At present, the 10%EDTA decalcification method is a generally accepted method that takes a long time but is not suitable for clinical diagnosis. Acid decalcification is increasingly becoming the first choice for the treatment of bone specimens. The intervertebral discs of mice were collected and embedded in paraffin sections after two different decalcification methods. H&E staining, Safranine O-Fast Green staining, Picrosirius Red-Alcian Blue staining and immunohistochemistry were performed respectively. The results showed that the two methods could clearly show each layer of tissue and the shape of intervertebral disc was complete. The Aggrecan positive protein could be expressed in all of them. In this experiment, 8% hydrochloric acid decalcification solution was applied to the preparation and staining process of mice intervertebral disc, in order to popularize and apply in the treatment of clinical bone lesions.

Keywords

Intervertebral Disc; Decalcified Solution; H&E Staining; Safranine O-Fast Green Staining; Picrosirius Red-Alcian Blue Staining.

1. Introduction

Intervertebral disc (IVD) degeneration is considered a primary contributor to disc degeneration disease and is associated with lower back pain [1], which has become a serious public health issue and causes an enormous economic burden [2]. Mice is one of the most commonly used experimental animals in orthopedic research. Because of the similar anatomical structure between its caudal vertebra and human intervertebral disc, researchers often use mouse caudal intervertebral disc to simulate normal human intervertebral disc [3]. Histological observation is commonly used in clinical diagnosis and basic research of intervertebral disc degeneration, and plays an important role in in-depth discussion of its pathogenesis and treatment [4]. However, because the bone tissue contains a large amount of calcium salt precipitation, the hardness of the bone tissue is very high, which makes it difficult to make complete slices, especially the production of large sections [5]. EDTA is an excellent calcium mixture, its decalcification solution is mild, does little damage to the tissue, can maintain the intact structure of the tissue, especially the microchemical change of the cell, and can preserve the activity of antigens in the tissue [6]. Studies have shown that it takes more than 3 weeks for EDTA decalcification solution to decalcify rat tibia, which can not meet the needs of rapid clinical diagnosis [7]. In clinic, calcium salt is often separated from collagen fiber by adding acid according

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to the solubility of calcium salt [8]. Different preparation solutions have different solubility coefficient of calcium salt, dissolution effect of calcium salt and separation degree of collagen fibers. Therefore, it is necessary to optimize the formula of decalcification solution to achieve rapid and effective decalcification and relatively less damage to bone tissue cells [9]. In order to explore a more ideal decalcification solution and decalcification method, we used 8% hydrochloric acid decalcification solution to decalcify the bone tissue of mice tail intervertebral disc and achieved good results.

2. Materials and Methods

2.1 Animals and Experiment Protocol

Twelve mice were purchased from Zhejiang Weitong Lihua Experimental Animal Technology Co., Ltd. All animal experiments were performed according to the guidelines, and animal procedures were approved by the Institutional Animal Care and Use Committee of Jinan University. After abdominal anesthesia, the mice were killed by cervical dislocation, and then the skin, fascia and muscle layer of the tail of the mice were stripped, and the specimens of circular caudal intervertebral disc were taken out and immersed in 4% paraformaldehyde solution for 24 hours. Rinse the intervertebral disc with PBS and put it in EDTA decalcification solution (pH7.2) and 8% hydrochloric acid decalcification solution respectively. If the tissue can be bent, such as a rubber band or needle, it can be judged to be decalcified completely.

2.2 Paraffin Embedding

IVD were fixed in 10% phosphate buffered formalin for 24–48 h at room temperature. Fixed IVD were trimmed into appropriate size and shape and placed in embedding cassettes. The paraffin embedding schedule was as follows (total 16 h): 5 h 70% ethanol, one change, 5 h each 80% ethanol, one change, 2 h 90% ethanol, two changes, 2 h 95% ethanol, two changes, 1 h 100% ethanol, two changes, 1 h each xylene, two changes, 1.5 h each paraffin wax (58–60 °C), two changes, 2 h each embedding tissues into paraffin blocks. In the process of transparency, naked-eye observation was used to determine whether the tissue was transparent or not. If it is amber, it indicates that transparency is successful. If it is not transparent, it should be extended for an appropriate time until it is transparent. The fixed and dehydrated tissues were immersed in paraffin wax twice (30 min/treatment) and then embedded into paraffin blocks until use. Paraffin tissue sections with a thickness of 4-6 μm were routinely made.

2.3 H&E Staining

The slices were baked for 1h at 60℃, then sections were deparaffinized in xylene, followed by 100%, 95%, 90% and 80% ethanol washes successively. After the slides were stained with hematoxylin and eosin successively, the slices were sealed with neutral resins and photographed under the light microscope.

2.4 Safranine O-Fast Green Staining

H&E staining method was used in the process of slice dewaxing to dehydration, hematoxylin staining for 15min, hydrochloric acid alcohol separation for 15s, double distilled water washing, 0.02% solid green solution dyeing for 30min, glacial acetic acid washing to remove residual solid green dye solution, 0.1% fuchsin staining 10min.

2.5 Picrosirius Red-Alcian Staining

H&E staining method was used in the process of slice dewaxing to dehydration, hematoxylin staining for 15min, hydrochloric acid alcohol separation for 15s. And Alcian Blue 8GX (PH2.5) was dyed for 15 min. Rinse with running water for 2min. The slides were stained with Picrosirius Red for 1 h and differentiated with hydrochloric acid for 15s.

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2.6 Immunohistochemical Staining

The sections of the paraffin-embedded nucleus pulposus tissue or mice IVD were deparaffinized using xylene and rehydrated by decreasing concentrations of ethanol (100%, 90%, 80%, and 70%), followed by immersion in sodium citrate buffer and heating in a steamer for 30mins for antigen retrieval. Then, 3% hydrogen peroxide was used to remove endogenous peroxidase activities, and the sections were blocked with normal goat serum at room temperature for 60mins. The sections were incubated with the Aggrecan primary antibody(1:100, Abcam)overnight at 4°C. The goat anti rabbit IgG-HRP secondary antibody (1:200, CST) was applied at 37°C for 60mins. Then, the sections were stained with DAB for 1min and hematoxylin for 10s. Cells were visualized using a microscope (Leica, Germany). Experiments were repeated three times independently. The results of immunohistochemical staining were analyzed using the Image J software.

3. Detection Index

The morphological integrity of cartilage endplate, annulus fibrosus and nucleus pulposus in intervertebral disc, as well as the distribution of Aggrecan protein in mouse caudal intervertebral disc were observed under microscope.

4. Results

4.1 Decalcification Time Result

At room temperature, the decalcification time of mouse tail intervertebral disc with 8% hydrochloric acid decalcification solution was significantly less than that with mild 10%EDTA decalcification solution. The decalcification time of 10%EDTA decalcification solution is 20±2 days, while that of 8% hydrochloric acid decalcification solution is only 2±0.5 h. The tissue sections of the two groups had complete structure and no obvious knife marks.

4.2 H&E Staining Result

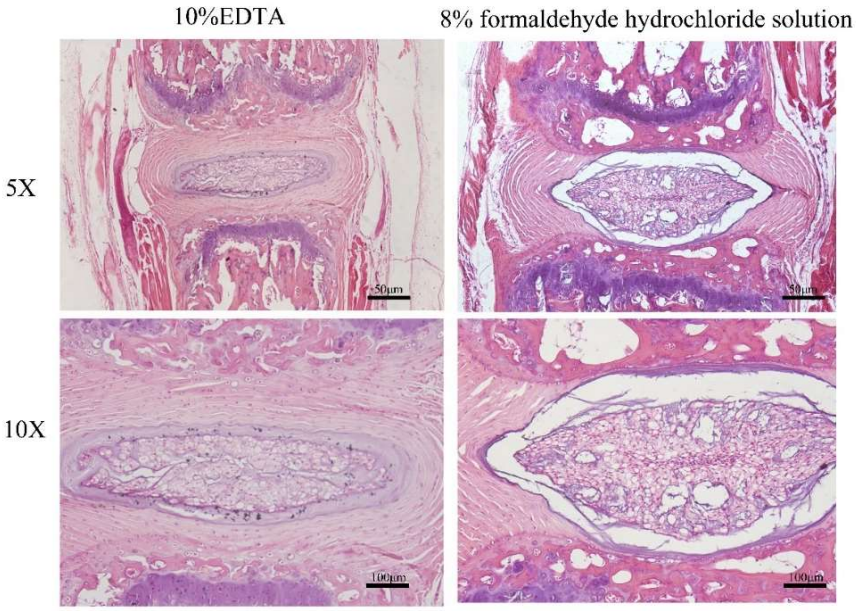

Figure 1. H&E staining of intervertebral disc sections of mice with different decalcification solutions in two groups.

H&E staining is usually used to identify various tissue types. Hematoxylin is an alkaline dye, blue-purple, which can color the nucleus and so on. Eosin is the most suitable general tissue structure staining combined with hematoxylin [10]. It can distinguish the cytoplasm of different types of cells through proper differentiation, as well as different types of connective tissue fibers and matrix, stained

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with different shades of red and pink. H&E staining showed that the intervertebral space of the mice was normal and the fibrous annulus was arranged neatly in the two methods. The structure of fibrous annulus is relatively complete, and there is a clear distinction between the cartilage-like structure of cartilage endplate and the bony structure of vertebral body. The outer AF contains slender fibroblast-like cells, and the inner AF cells are spherical, similar to articular chondrocytes. The vacuoles of nucleus pulposus cells were obvious. The annulus fibrosus is arranged in a ring, the arrangement of fibrin is soft and regular, and the boundary between the annulus fibrosus and the nucleus pulposus is clear. It shows that the two methods are suitable for common histological staining.

4.3 Safranine O-Fast Green Staining Result

Safranine O-Fast green staining showed that the structure of intervertebral disc in 10%EDTA decalcification solution group was clear, and the dividing line between annulus fibrosus and nucleus pulposus was almost clear. The staining results showed that there was a sharp contrast between red and green, indicating that 10%EDTA decalcification solution had less damage to cartilage matrix. In the 8% hydrochloric acid decalcification solution group, the fibrous annulus was slightly red stained, the cartilage layer was slightly stained with Safranine O, and the nucleus was slightly green stained. The overall staining effect of 8% hydrochloric acid decalcification solution was similar to that of 10%EDTA group.

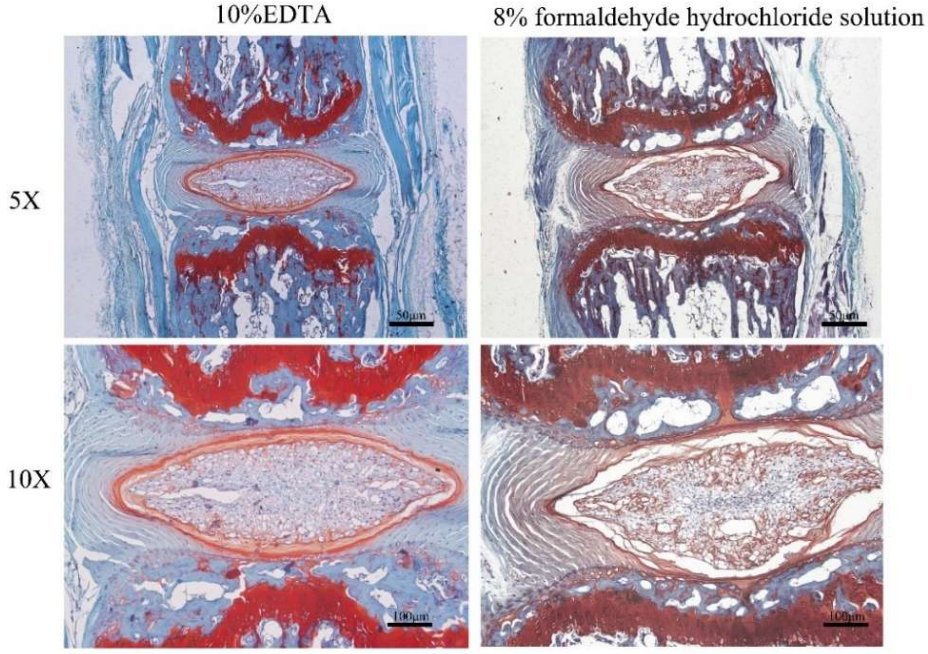

Figure 2. Safranine O-Fast Green staining of intervertebral disc sections of mice with different decalcification solutions in two groups.

4.4 Picrosirius Red- Alcian Blue Staining Result

Alcian Blue is a cationic dye that can dye mucopolysaccharide blue and cartilage blue in bone staining. Picrosirius Red is an acid dye that reacts with alkaline collagen fibers to produce obvious red collagen fibers, while the nucleus is green and the rest is yellow. The staining results showed that the two kinds of decalcification solution could show the normal shape of intervertebral disc structure, and there was no significant difference. The cartilage endplate was rich in proteoglycans, and it can be seen that the cartilage was obviously stained blue, and there was also a slight blue staining inside the nucleus pulposus. And the annulus was stained red.

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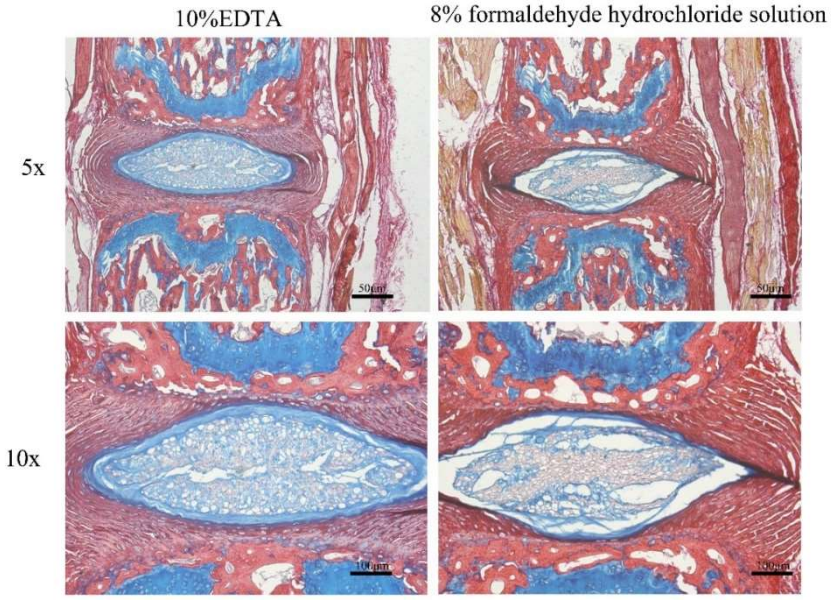

Figure 3. Picrosirius Red-Alcian Blue staining of intervertebral disc sections of mice with different decalcification solutions in two groups.

4.5 Immunohistochemistry

Immunohistochemistry is based on the principle of specific combination of antigen and antibody, through the chemical reaction to make the chromogenic agent of the labeled antibody to determine the tissue intracellular antigen, and to study its localization, qualitative and quantitative. Aggrecan is the most abundant proteoglycan in nucleus pulposus of intervertebral disc. It aggregates with hyaluronic acid and has negative side chain groups. These negative charges make nucleus pulposus hydrophilic and highly hydrated. The immunohistochemical results of the decalcified solution in the two groups showed that the strong brown of Aggrecan specific histochemical positive signal was expressed in most of the area of nucleus pulposus tissue in the decalcified solution in the two groups. The results showed that the decalcification of 8% hydrochloric acid decalcified solution for 2 hours would not destroy the antigenic substance in the intervertebral disc, nor would it affected the effective expression of antigens in immunohistochemical staining.

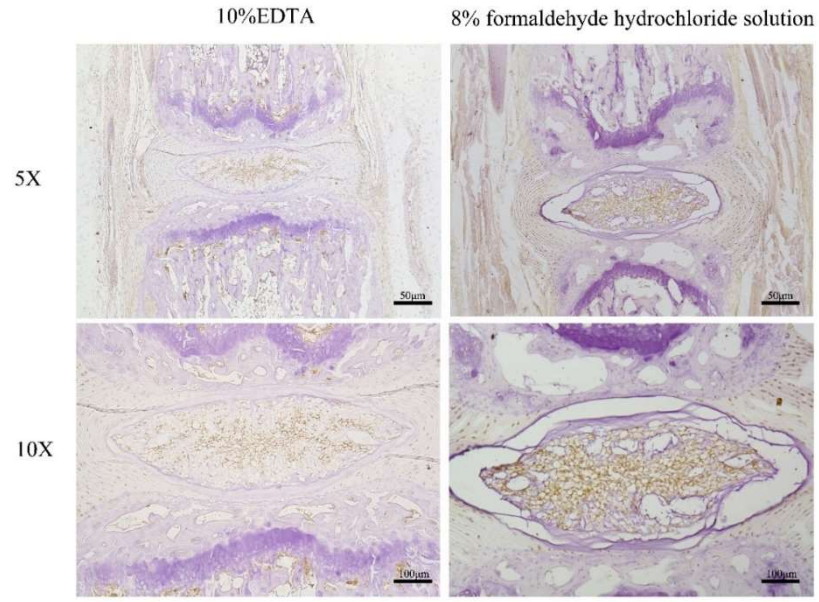

Figure 4. Expression level of proteoglycan in two groups of intervertebral discs.

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5. Conclusion

Decalcification is the use of chemical or physical methods to separate calcium salts and collagen fibers from bone tissue. The purpose of softening bone tissue is to obtain complete bone sections. The selection of decalcification solution is an important link in the production of bone slices, that is, to cut out a complete section, but also to be able to completely preserve the integrity of the internal components of bone tissue. It has a certain protective effect on enzymes and antigens in the tissue. Otherwise, excessive or insufficient decalcification will affect the dyeing effect and seriously affect the interpretation of the results. The time of fixation and decalcification should not be too long, and try to keep it in a relatively cold environment to avoid the loss of tissue immune activity. If the decalcification time is too short, it is easy to separate the tissue structure of the nucleus pulposus and annulus fibrosus and destroy the layered structure of the annulus fibrosus, so that in the study of intervertebral disc degeneration, it is easy to be confused with the destruction of annulus fibrosus structure caused by fibrous annulus puncture degeneration model. The 8% hydrochloric acid decalcification solution used in this experiment contains concentrated hydrochloric acid 8ml, glacial acetic acid 2ml, 10% neutral formalin 46ml, sodium chloride 10g, aluminium chloride 10g and water 44ml. 10% neutral formalin is a commonly used fixation solution for specimen tissues, which has strong penetration and shrinking of tissue, so that most tissues can be well preserved.. After the addition of hydrochloric acid, formaldehyde hydrochloride was prepared, which can not only fix the bone tissue, but also play the role of decalcification, thus shortening the tissue fixation and pre-treatment time. The addition of glacial acetic acid can make the dense bone tissue expand and dissolve lipids. The decalcification solution can penetrate into the bone tissue smoothly, which is beneficial to the precipitation of calcium salt and speed up the decalcification rate. At the same time, it has the characteristics of preventing tissue autolysis and can better preserve the chromosome structure. At the same time, it can not only prevent tissue autolysis, but also preserve the chromosome structure and reduce the damage of acid to the nucleus. To sum up, the method of making intervertebral disc sections with 8% hydrochloric acid decalcification solution is simple, time-consuming, and staining meets the requirements of morphological observation. This is an ideal decalcification solution to meet the needs of rapid clinical diagnosis. It is suitable for clinical and scientific research to observe the morphology of large specimens of bone tissue and the effect of chemotherapy of bone tumor. It lays a foundation for the molecular detection of bone lesions and is worth popularizing.

Acknowledgments

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