An effective and practical immunohistochemical protocol for bone specimens characterized by hyaluronidase and pepsin predigestion combined with alkaline phosphatase-mediated chromogenic detection

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Summary. The aim of this study was to provide an effective procedure for immunohistochemistry (IHC) investigations of bone specimens. Samples from rat femoral and human vertebral bone were processed with a detailed and effective IHC protocol summarized here. First, a novel antigen retrieval (AR) method of hyaluronidase combined pepsin predigestion (H+P) was established and the optimal concentration and pH value for AR of bone specimens were determined. Second, the newly developed method was compared with existing AR methods (boiling in sodium citrate, hyaluronidase predigestion (H) and pepsin predigestion (P), with PBS only as the negative control) using two chromogenic detection systems (horseradish peroxidase (HRP) and alkaline phosphatase (AP)) to evaluate their efficacy in obtaining the best IHC results for bone samples. Considering the drawbacks of significant shrinking and detachment from slide for heat retrieval methods and the only moderate immunolabeling for H and P, H+P was the optimal AR method for IHC of bone specimens with the advantages of both good morphological preservation and strong immunoreactivity. Moreover, AP-mediated chromogenic detection was superior to HRP-labeled chromogenic detection due to significantly less non-specific staining. In conclusion, we presented an effective and practical IHC protocol for bone specimens characterized by H+P predigestion combined with AP-

mediated chromogenic detection. Finally, a detailed troubleshooting guide was provided for common mistakes that occur during IHC processing of the bone tissue samples.

Key words: Immunohistochemistry, Bone tissue, Antigen retrieval, Hyaluronidase, Alkaline phosphatase-mediated chromogenic detection

Introduction

Bone is the dense, rigid and slightly elastic form of connective tissue constituting most of the skeleton. It is composed chiefly of cells embedded in an organic collagenous matrix (predominantly type I collagen fibers, 25% by weight) providing tensile strength, and an inorganic mineral component (75% by weight) of calcium phosphate (primarily in the form of hydroxy-apatite crystals) which provides stiffness under compression (Clarke, 2008). The organic matrix and inorganic mineral are in close association, forming the tough bone matrix (Camozzi et al., 2010). Hence, it is necessary to remove the inorganic hard minerals (a process termed decalcification) to enable 3~5µm thick sections to be cut for histological examination of bone specimens (Valentine and Piper, 2012). Immunohisto-chemistry (IHC) has become a crucial technique widely used in many medical research laboratories, it is also an essential tool for clinical diagnostics (Leong et al., 2010; Leong and Leong, 2011). IHC is especially useful in diagnostic surgical pathology as it allows analysis of

protein subcellular localization (Mohd Omar et al., 2010). However, IHC analysis of bone tissues is severely hampered by technical difficulties associated with sectioning calcified tissue, preservation of tissue morphology and remaining antigen integrity (Klein and Memoli, 2011). Consequently, IHC of bone specimens is often challenging and it is problematic to find an ideal IHC procedure for histological and histochemical studies of bone (Schoen, 1991).

Choosing an antigen retrieval (AR) method and subsequent detection system are two important factors for successful IHC (D'Amico et al., 2009; Bratthauer, 2010). The masking of antigens by chemical fixation, processing, and embedding media interactions produce weak or even false negative staining for immunohisto-chemical detection of certain proteins (Leong and Leong, 2007). Multiple different approaches for re-exposing epitopes for successful antibody binding exist (Shi et al., 2011). Functional identification of antigen antibody binding can only be observed through the use of a reporter molecule, predominantly enzymes conjugated to secondary antibody, the most common being horseradish peroxidase (HRP), and alkaline phosphatase (AP). Each enzyme has corresponding chromogenic substrate solutions with which it can react to produce a colored product visualized through the use of selected instruments, including the microscope (Bratthauer, 2010). However, evaluation studies of the efficacy of different AR and chromogenic detection methods using IHC for bone tissue samples are largely lacking.

In this study, we have developed a detailed protocol for IHC staining for routine biopsies of bony tissue. First, an effective and practical AR approach with hyaluronidase and pepsin (H+P) predigestion was developed. Subsequently, the efficacy of four different AR methods and two chromogenic detection systems (HRP and AP-mediated) were compared to investigate which was optimal for antigen detection in IHC of bone specimens. Finally, causes and possible solutions related to common problems identified during bone IHC were discussed.

Materials and methods

Solutions, reagents and apparatus

Collagen I monoclonal antibodies (ab6308) and β-Amyloid precursor protein (APP) polyclonal antibodies (ab68896) were all purchased from Abcam (Cambridge, UK), the non-biotin horseradish peroxidase detection system was purchased from Dako (Carpinteria, CA, USA), hyaluronidase and pepsin were purchased from Maixin Biotech CO., LTD (Fuzhou, China). All other chemicals and solutions were purchased from Zhongshan Golden Bridge Biotech CO., LTD (ZSGB-Bio, Beijing, China) unless otherwise stated. All instruments were from Thermo Fisher SCIENTIFIC Inc. (Waltham, MA, USA) unless specified otherwise.

Detailed protocol for IHC of bone specimens

Sample preparation

All the experiments were approved by the Institutional Animal Care and Use Committee (Approval No: IACUC-2012-0504) of Sun Yat-sen University and were performed according to EU Directive 2010/63/EU. Sprague-Dawley (SD) rats (male, age 20 weeks, initial body weight 200-250g) were euthanized by intraperitoneal injection with sodium pentobarbital (100 mg/kg), and the femur removed, stripped of soft tissue and immediately placed in periodate-L-Lysine-paraformaldehyde (PLP) fixative. Vertebral trabecular bone specimens were obtained from needle biopsies of patients undergoing percutaneous vertebroplasty (PVP) following vertebral compression fractures. Specimens were collected using a Spacer (internal diameter 11G) from the PCD System (SI Medical, Wonju-si, Gangwon-do, Korea), and immediately immersed in PLP fixative. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University (Approval No: 2012-2-72) and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Fixation

PLP fixation utilizes periodate to oxidize sugars in the tissue to create aldehydes, which are then cross-linked by lysine, whilst the paraformaldehyde cross-links protein.

(a) PLP fixative preparation. i) Solution A: 8% paraformaldehydes (w/v) were solubilized at 60°C in phosphate buffered saline (PBS) containing 0.5 mM NaOH, and particulates were removed by filtration. ii) Solution B: 0.1 M L-lysine was dissolved in PBS. iii) Working solution: 100 ml solution A and 300 ml solution B were mixed, then 856 mg sodium periodate was added, pH was adjusted to 7.4 and solutions were stored at 4°C.

(b) Tissue blocks were placed in freshly prepared working solution of fixative, at approximately a 1:10 ratio of specimen to fixative (v/v). Fixation time varied with the size of the blocks: needle biopsies of 0.3 cm^3 required approximately 12 hours fixation, rat femoral bone specimens of 2×0.5×0.3 cm required approximately 24 hours fixation. Normally, small biopsies may be ready after 12-24 hours, large specimens may be up to 48 hours or more.

Decalcification

(a) Following fixation specimens were immersed sequentially in the following solutions, for 12 hours respectively at 4°C. i) 5% glycerol (v/v) in PBS. ii) 10% glycerol (v/v) in PBS. iii) 15% glycerol (v/v) in PBS.

The specimens were moved to Ethylene-diaminetetra-acetate-glycerol (EDTA-G) solutions: 14.5% (w/v) EDTA and 3M NaOH were dissolved in distilled water containing 15% (v/v) glycerol, pH was adjusted to 7.3.

(b) At 4°C at a 1:20 ratio of specimen: EDTA-G for decalcification for 2-30days (dependent on the volume and size of the specimens, approximately 2 days for needle biopsies of 0.3 cm^3 and 1 month for adult rat femoral specimens of 2×0.5×0.4 cm. Bone specimens obtained from young mice (e.g. less than 10 days old) can be processed without this step). Decalcification solution was replaced every 2-3 days, the decalcification process was monitored by probing with a needle and terminated when the bone specimens resembled soft tissue.

(c) Following decalcification bone specimens were rinsed sequentially in the following solutions for 12 hours for each solution at 4°C. i) 15% sucrose (w/v) in 7.5% glycerol-0.01 M PBS. ii) 15% sucrose (w/v) in 0.01M PBS. iii) 7.5% sucrose (w/v) in 0.01M PBS

(d) Bone specimens were stored in 0.01M PBS at 4°C.

Dehydration and embedding

Specimens were dehydrated as follows: i) Graded ethanol series (50%, 70%, 80%, 95%, 95%, 100%, 100%): 25-40 min for each ethanol concentration. ii) Xylene: 20-40 min. iii) Xylene: 20-40 min. iv) Soft wax: 25-40 min (fusion temperature 52-54°C). v) Hard Wax: 25-40 min (fusion temperature 60-62°C).
Notes: xylene time varied depending on tissue type, approximate time for bone tissues was 30 min.

Cutting and mounting of sections

All sections used for comparative experiments were obtained from serial sections of the same tissue block embedded in paraffin. 3 μm sections were prepared using a microtome (HM 340E, Thermo) with a blade (MX35 ULTRA, Thermo). Subsequently, sections were floated on a water bath maintained at 55°C to ensure that they were well stretched and wrinkle-free, and then sections were mounted on SUPERFROST® PLUS glass slides. Two sections widely separated were placed per slide so that one of the sections served as an on-slide no antibody control. Finally, the slides were dried completely at room temperature for 5-15 minutes by draining them vertically, and then heated to 65°C in an oven for 2 hours.

Deparaffinization and rehydration of sections

i) Paraffin was removed by incubating sections in 3 changes of xylene, 5 min each; ii) Sections were rehydrated in 2 changes of 100% ethanol for 3 min each, then through a graded series of ethanol: 90%, 80% and 70% for 3 min each; iii) Sections were washed in 3 changes of PBS / 0.025% Triton X-100 (or 0.2% Tween 20) for 3 min each with gentle agitation;

Antigen retrieval

Sections were encircled with a hydrophobic "PAP" pen to prevent solutions spreading on the slide; the circle was 3mm wider than the specimen in radius to reduce the surface tension to the antigen. Slides were divided into 4 groups and subjected to one method of AR per group as follows:

Solutions were prepared as follows: i) Hyaluronidase solution: hyaluronidase was dissolved in deionized water at concentrations of 1.5-2.5 mg/ml, pH was adjusted to 4.5-6.0 and solution was stored at -20°C until use. ii) Pepsin solution: pepsin was dissolved in aqueous 17% HCl at concentrations of 0.3-0.5% (w/v), pH was adjusted to 1.0-2.5 and solution was stored at -20°C until use.

Method 1 - Hyaluronidase combined with pepsin predigestion. i) Hyaluronidase solution was pre-heated to 37°C and added (50-100 μL) to the sections ensuring coverage of the entire bone tissue biopsy sample, and digested at 37°C for 30min in a humidified chamber (optimal incubation time varied depending on tissue type and degree of fixation). ii) Sections were washed in PBS / 0.025% Triton X-100. iii) Pepsin was pre-heated to 37°C and added (50-100 μL) to the sections ensuring coverage of the entire bone tissue biopsy samples, and incubated at 37°C for 30 min in a humidified chamber (optimal incubation time varied according to tissue type and degree of fixation).

Method 2 - Hyaluronidase predigestion. i) Hyaluronidase solution was pre-heated to 37°C and added (50-100 μL) to the sections ensuring coverage of the entire bone tissue biopsy sample, and digested at 37°C for 30min in a humidified chamber.

Method 3 - Pepsin predigestion. i) Sections were transferred to 0.4% pepsin (w/v) in 0.1 M HCl and then were incubated in pepsin working solution for 30 minutes at 37°C.

Method 4 – Heat mediated. i) Sections were immersed in sodium citrate pH7.4 in a pressure cooker according to the manufacturer's instructions, and were incubated for 2 min from the point steam was produced from the outlet valve, and then allowed to cool naturally to room temperature.

Method 5 - No treatment. i) Sections were incubated with PBS only for 30 minutes at 37°C.

Washing

Sections were rinsed in 3 changes of PBS / 0.025% Triton X-100 for 3 min each.

For each AR group 2 methods of chromogenic detection were tested: HRP-mediated detection and AP-mediated detection. These two detection methods required separate blocking, second antibody incubation, detection, dehydration and mounting protocols as described below.

Blocking

HRP-mediated detection: i) Sections were incubated in 3% H_2O_2 in the dark for 10min at room temperature to block endogenous peroxidase, and rinse in 3 changes of PBS / 0.025% Triton X-100 for 3 min each. ii) Non-specific antibody binding was blocked by incubation in 10% normal serum with 1% BSA in TBS for 30 min at room temperature. iii) Slides were held vertically to drain for several seconds (do not rinse) and the area around the sections was gently dried with tissue paper.

AP-mediated detection: i) Non-specific antibody binding was blocked by incubation in 10% normal serum with 1% BSA in TBS for 30 min at room temperature. Endogenous phosphatase activity was eliminated by the addition of 0.24 mg/ml levamisole to the blocking solution. ii) Slides were held vertically to drain for a few seconds (do not rinse) and were wiped around the sections with tissue paper.

Primary antibody incubation

i) Primary antibody dilutions were prepared in PBS / 1% BSA. ii) Sections were incubated with primary antibody at appropriate dilution overnight in a humidified chamber at 4°C. iii) Sections were rinsed with 3 changes of PBS / 0.025% TritonX-100 for 3 min each.

Second antibody incubation

HRP-mediated detection: i) Goat anti-rabbit HRP conjugated secondary antibody was added to the slide according to the manufacturer's instruction (GK500505A, Dako, Carpinteria, CA, USA), and incubated for 30 min at room temperature. ii) Slides were rinsed with 3 changes of PBS / 0.025% Triton X-100 for 3 min each.

AP-mediated detection: For detection of primary antibody with AP the Avidin-Biotin Complex (ABC) method was used as follows: i) Sections were incubated with biotin-labeled secondary antibody at 37°C for 15min. ii) Slides were washed with PBS / 0.025% Triton X-100. iii) Sections were incubated with AP-labeled streptavidin for 15 min at 37°C. iv) Slides were rinsed with 3 changes of PBS/0.025% Trixton X-100 for 3 min each.

Detection

HRP-mediated detection: i) Sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-imidazole buffer (pH7.6), until brown staining was visible under microscopy (about 5-10 seconds). ii) The reaction was terminated by rinsing the sections with distilled water.

AP-mediated detection: i) Sections were incubated with Fast Red TR at 37°C for 30 min.

Counterstaining

Slides were counterstained with Mayer's hematoxylin for 15 seconds and then rinsed in flowing tap water for 20 minutes;

Dehydration and Mounting

HRP-mediated detection: i) Sections were dehydrated through graded ethanol (70%, 80%, 90% and 100%) for 3 min each. ii) Slides were dried in an oven at 60°C for 2 min and then were cleared in 3 changes of xylene, for 5 min each. iii) Slides were mounted in resin (containing xylene) mounting medium.

AP-mediated detection: i) Slides were dried in an oven at 60°C for 2 min, and then were mounted in glycerin mounting medium.

Viewing and assessment of staining

All slides were viewed and photographed using a microscope (DM4000B, Leica, Heidelberg, Germany). Staining intensity and non-specific staining were assessed visually: the intensity of the positive reactions were graded independently using the four tier criteria of – negative, + weak, ++ moderate and +++ strong. Because APP is a protein widely expressed in cell membranes (Kai et al., 2012, Zhang et al., 2012), positive reactions occurring in the extracellular matrix of bone tissues and in the matrix of bone marrow were served as non-specific staining controls; non-specific staining was divided into three categories: none, some, and obvious.

Results

Exploration of the optimal pH value and concentration of hyaluronidase and pepsin for AR of bone specimens

Because both hyaluronidase and pepsin digestion are proven AR methods for IHC (Brown, 1998; D'Amico et al., 2009), we proposed that hyaluronidase and pepsin digestion combined was an effective AR method for bone tissue specimens. We then determined the optimal combined concentration and pH value for both hyaluronidase and pepsin.

Nine combinations of hyaluronidase and pepsin (Table 1) were tested for collagen I expression and the immunostaining outcomes are presented in Figure 1. The morphological structures for panel A-I were all well

preserved, but the intensity of immunostaining varied dependent on different combinations. Very weak immunolabeling of collagen I was detected in the hyaluronidase 0.5-1.5 mg/ml pH 3.5-5.0 / pepsin 0.5-0.7% pH 1.0-2.5 (Fig. 1D) and hyaluronidase 1.5-2.5 mg/ml pH 3.5-5.0 / pepsin 0.5-0.7% pH 2.5-4.0 (Fig. 1E) treatment group, while slightly more intense immunostaining was observed using hyaluronidase 1.5-2.5 mg/ml pH 2.0-3.5/ pepsin 0.1-0.3% pH 2.5-4.0 (Fig. 1B). Moderate immunostaining of collagen I was found in hyaluronidase 0.5-1.5 mg/ml pH 2.0-3.5 / pepsin 0.1-0.3% pH 1.0-2.5 (Fig. 1A), hyaluronidase 2.5-3.5 mg/ml pH 2.0-3.5 / pepsin 0.1-0.3% pH 4.0-5.5 (Fig. 1C), hyaluronidase 2.5-3.5 mg/ml pH 3.5-5.0 / pepsin 0.5-0.7% pH 4.0-5.5 (Fig. 1F), hyaluronidase 0.5-1.5 mg/ml pH 4.5-6.0 / pepsin 0.3-0.5% pH 2.5-4.0 (Fig. 1G) and hyaluronidase 2.5-3.5 mg/ml pH 4.5-6.0 / pepsin 0.3-0.5% pH 4.0-5.5 (Fig. 1I). The most intense and specific staining was observed using hyaluronidase 1.5-2.5mg/ml

Table 1. Exploration of the optimum concentration and pH value of hyaluronidase and pepsin for antigen retrieval of bone specimens.

Solution A: Hyaluronidase		Solution B: Pepsin			
Concentration (mg/ml)	pH value	Concentration (%)	pH value	Representative Images	Staining Intensity
0.5-1.5	2.0-3.5	0.1-0.3	1.0-2.5	Fig. 1A	moderate
1.5-2.5	2.0-3.5	0.1-0.3	2.5-4.0	Fig. 1B	weak
2.5-3.5	2.0-3.5	0.1-0.3	4.0-5.5	Fig. 1C	moderate
0.5-1.5	3.5-5.0	0.5-0.7	1.0-2.5	Fig. 1D	weak
1.5-2.5	3.5-5.0	0.5-0.7	2.5-4.0	Fig. 1E	weak
2.5-3.5	3.5-5.0	0.5-0.7	4.0-5.5	Fig. 1F	moderate
0.5-1.5	4.5-6.0	0.3-0.5	2.5-4.0	Fig. 1G	moderate
1.5-2.5	4.5-6.0	0.3-0.5	1.0-2.5	Fig. 1H	strong
2.5-3.5	4.5-6.0	0.3-0.5	4.0-5.5	Fig. 1I	moderate

Type I collagen was detected in rat bone femoral bone samples by incubation at 37°C firstly with solution A (Hyaluronidase of different concentrations and pH values) and secondly with solution B (Pepsin of different concentrations and pH values), followed by standard IHC protocols using monoclonal mouse anti-collagen I. Representative images of each combination are presented in Fig. 1.
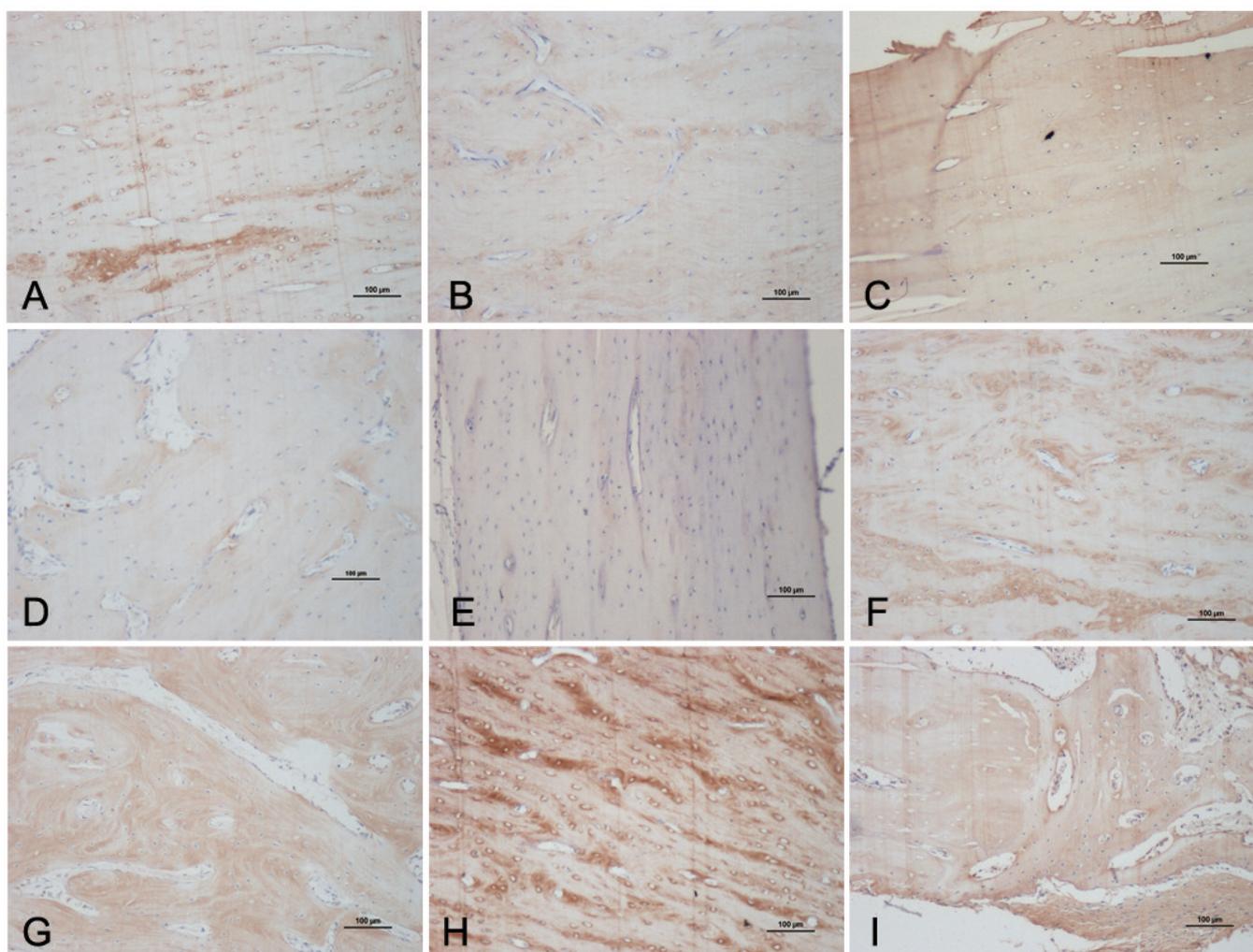
Fig. 1. Exploration of the optimal concentration and pH value of hyaluronidase and pepsin for antigen retrieval of type I collagen in rat femoral bone specimens. AR solutions used are in detailed in Table 1. All experiments were repeated three times with similar results and representative photomicrographs of each combination **(A-I)** are presented. Bars: 100 μm.
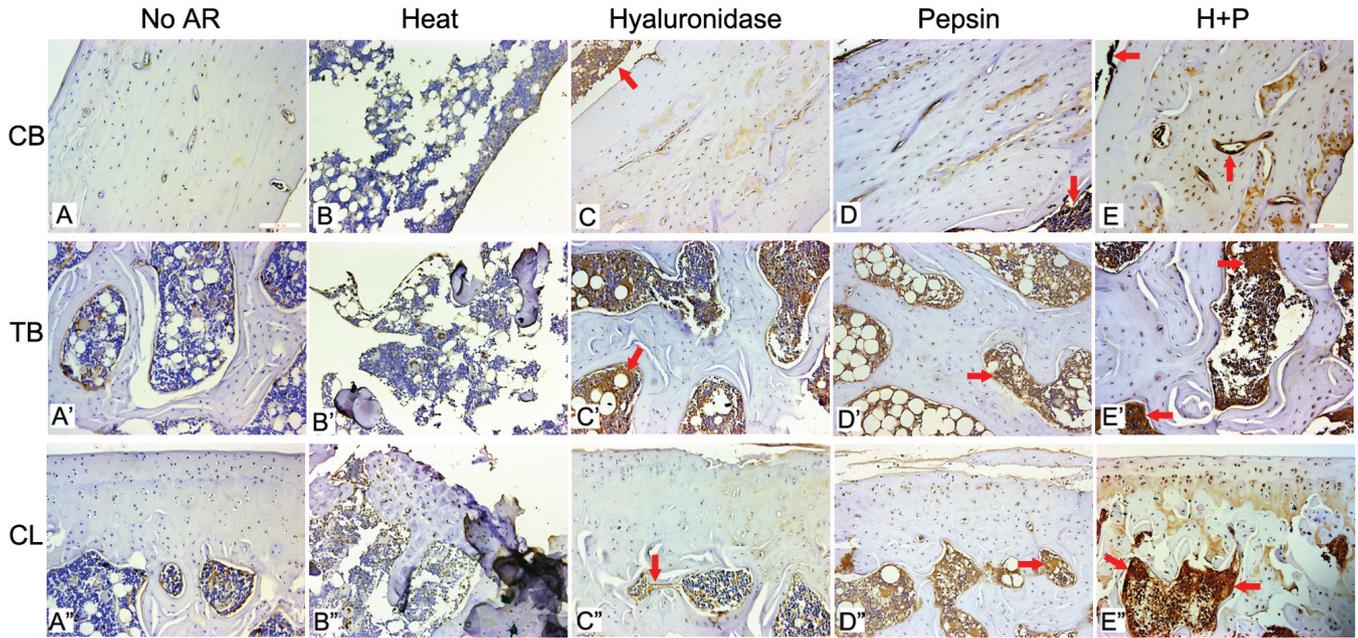
Fig. 2. Representative photomicrographs APP staining in rat femoral specimens comparing different antigen retrieval protocols in combination with HRP-mediated chromogenic detection (brown coloration). Hyaluronidase in combination with pepsin predigestion gave optimal staining results in cortical bone (CB), trabecular bone (TB) and cartilage (CL). No AR, no antigen retrieval (AR); Heat, AR using sodium citrate heated in a pressure cooker; Hyaluronidase, AR with hyaluronidase predigestion; Pepsin, AR with pepsin predigestion; H+P, AR with hyaluronidase and pepsin predigestion. Non-specific staining is indicated by red arrows. Bars: 100 µm.
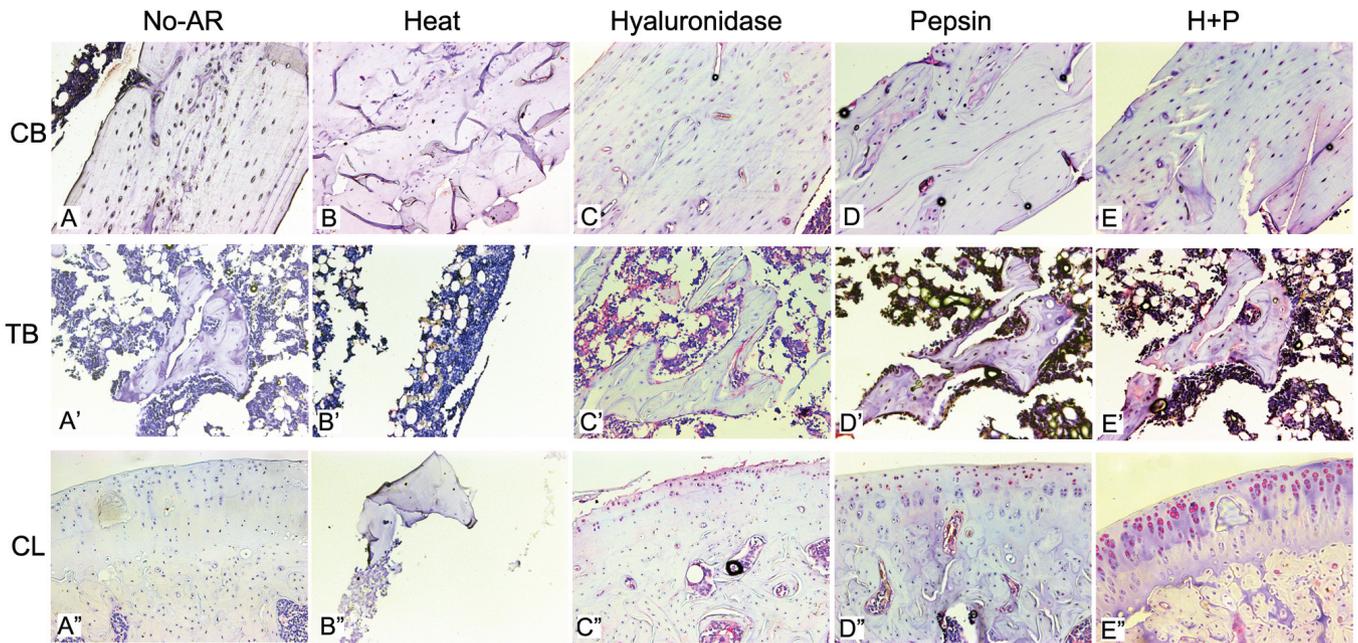
Fig. 3. Representative photomicrographs of APP staining in rat femoral specimens comparing different antigen retrieval protocols in combination with AP-mediated chromogenic detection (red coloration). Hyaluronidase in combination with pepsin predigestion gave optimal staining results in cortical bone (CB), trabecular bone (TB) and cartilage (CL). No AR, no antigen retrieval (AR); Heat, AR using sodium citrate heated in a pressure cooker; Hyaluronidase, AR with hyaluronidase predigestion; Pepsin, AR with pepsin predigestion; H+P, AR with hyaluronidase and pepsin predigestion. × 200

pH 4.5-6.0 in combination with pepsin 0.3-0.5% pH 1.0-2.5 (Fig. 1H), hence, this was considered as the optimal combination resulting in successful AR of bone tissue for IHC.

Effect of different AR protocols on IHC staining of rat femoral specimens with HRP-mediated chromogenic detection

Following determination of the optimal concentration and pH levels of hyaluronidase in combination with pepsin for AR on rat bone specimens, we then processed to assess how this method performed in comparison to other commonly used AR methods.

We examined expression of APP in rat femoral samples (cortical bone, CB; trabecular bone, TB; and cartilage, CL) by IHC using heat mediated AR, hyaluronidase alone, pepsin alone, and our optimized hyaluronidase and pepsin protocol (H+P), with detection

by HRP-labeled secondary antibodies (positive reactions indicated by brown staining, Fig 2, Table 2). Incubation with PBS only (No AR) was included as a negative control. High temperature treatment has been one of the most successfully used AR methods (Krenacs et al., 2010), therefore we tested heat mediated AR by heating sections in a pressure cooker. Heating did improve in staining relative to No AR, however, the tissues were badly damaged with poor morphology, sections were torn and wrinkled and many detached from the slides (Fig. 2B,B',B", Table 2). Enzymatic treatments have been effectively applied to unmask certain antigens, in particular pepsin has been reported to improve detection of many kinds of antigens (MacIntyre, 2001). We determined whether incubation with hyaluronidase or pepsin alone would improve IHC APP immunolabeling. As shown in Fig. 2C,C',C",D,D',D" and Table 2, hyaluronidase or pepsin pretreatment resulted in moderate antigen exposure and good preservation of

Table 2. The effects of different AR methods and detection systems on APP IHC staining of bone tissue samples from rats.

	Horseradish Peroxidase-mediated Chromogenic Detection															Alkaline Phosphatase-mediated Chromogenic Detection														
	Cortical Bone					Trabecular Bone					Cartilage					Cortical Bone					Trabecular Bone					Cartilage				
AR	N	C	H	P	B	N	C	H	P	B	N	C	H	P	B	N	C	H	P	B	N	C	H	P	B	N	C	H	P	B
Integrity	W	L	W	W	W	W	L	W	W	W	W	L	P	P	W	W	L	W	W	W	W	L	W	W	W	W	L	P	W	W
Intensity	N	M	M	M	S	W	M	M	M	S	W	M	M	M	S	W	W	M	M	S	N	W	M	M	S	W	W	M	M	S
NSS	N	S	O	O	O	N	S	O	O	O	N	S	O	O	O	N	N	N	N	N	N	N	S	S	N	N	N	S	N	N

AR, antigen retrieval; Integrity, Tissue integrity; Intensity, Staining Intensity; NSS, non-specific staining; N, no AR; C, AR with pressure cooker in sodium citrate solution; H, AR with hyaluronidase predigestion; P, AR with pepsin predigestion; B, AR with hyaluronidase and pepsin predigestion. The abbreviations representing different degrees of tissue integrity/staining intensity/non-specific staining are as follows. Integrity: L, almost lost; P, partial lost; W, well preserved. Intensity: N, negative; W, weakly positive; M, moderately positive; S, strongly positive. Non specific staining: O, obvious; S, some; N, none. Representative images for each staining regimen are presented in Fig. 2 (HRP) and Fig. 3 (AP).
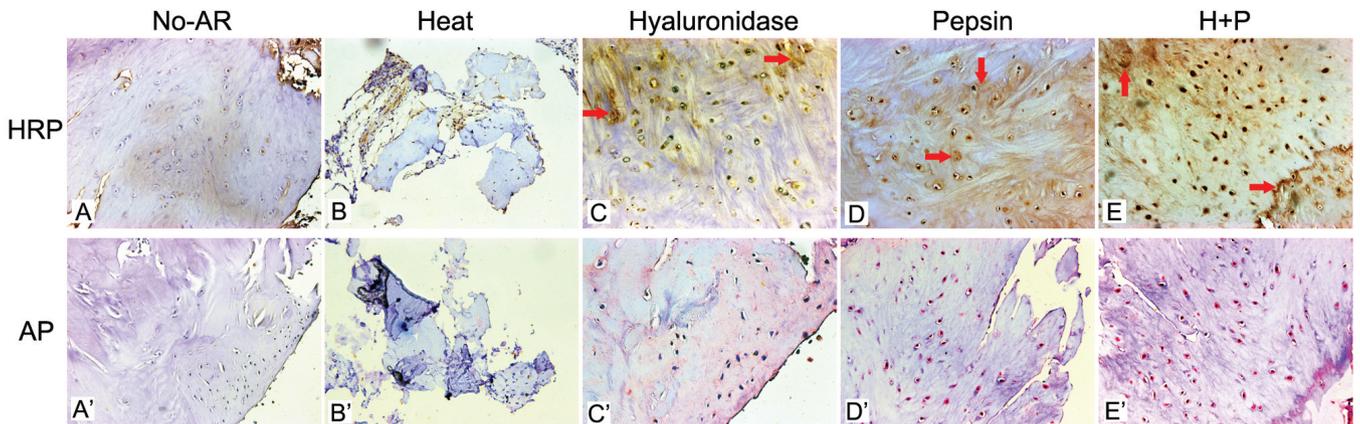
Fig. 4. Representative photomicrographs of APP staining in human vertebral bone specimens comparing different antigen retrieval protocols in combination with either HRP (brown color) or AP-mediated chromogenic detection (red color). HRP, horseradish peroxidase-mediated chromogenic detection; AP, alkaline phosphatase-mediated chromogenic detection; No AR, IHC without antigen retrieval (AR); Heat, AR using sodium citrate heated in a pressure cooker; Hyaluronidase, AR with hyaluronidase predigestion; Pepsin, AR with pepsin predigestion; H+P, AR with hyaluronidase combined pepsin predigestion. Red arrows indicate non-specific staining. × 200
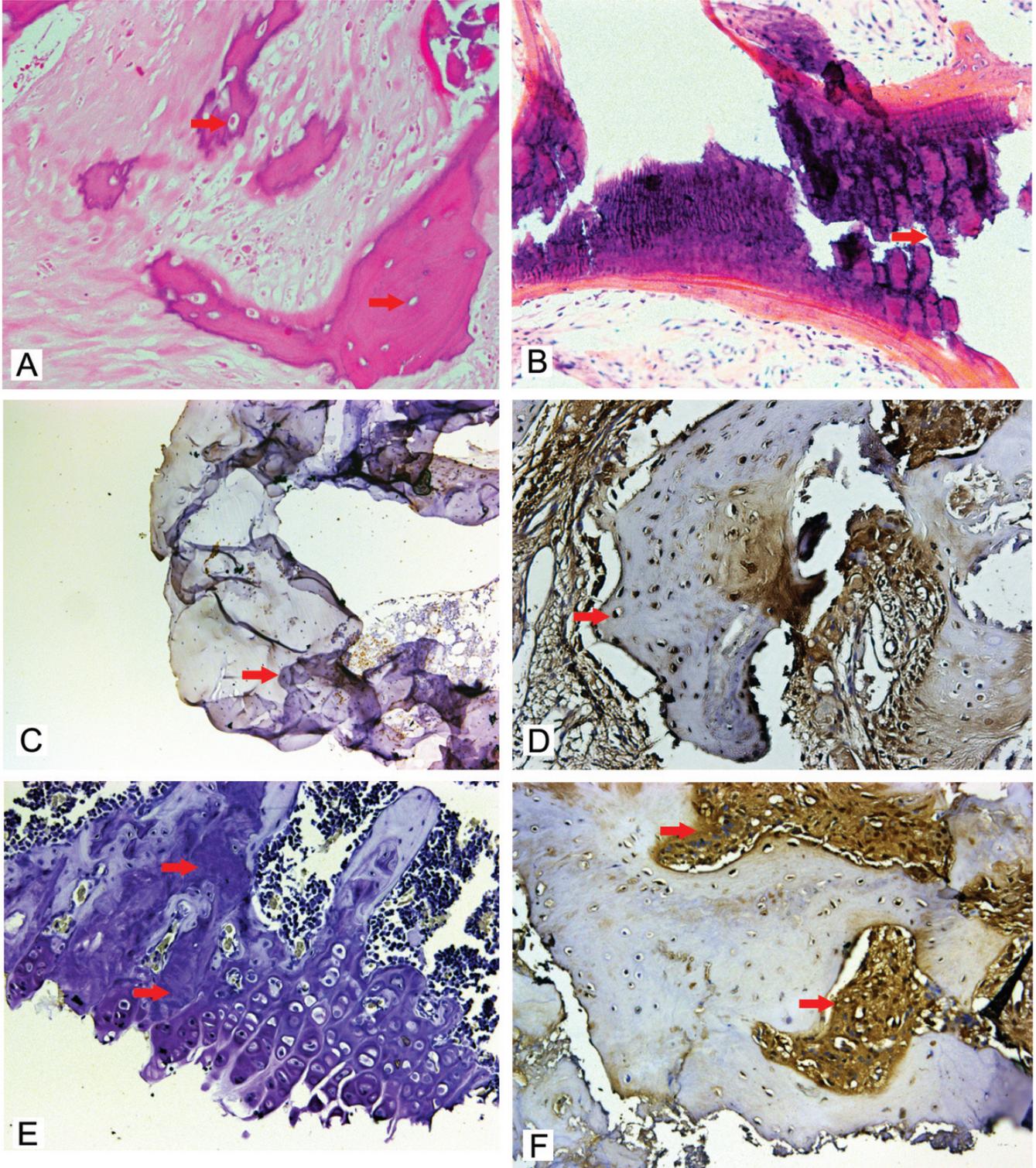
Fig. 5. Common mistakes and drawbacks observed during IHC of bone specimens. **A.** Over-decalcification. **B.** Poor decalcification. **C.** Detachment from slide and overlapping. **D.** Over-incubation with DAB. **E.** Over-counterstaining. **F.** Non-specific staining. Arrows indicate defects. x 200

section morphology and tissue structure, but the non-specific staining in bone marrow was obvious even after blocking with 10% normal serum and quenching endogenous peroxidase activity with 3% H_2O_2. Finally, we examined the utility of our optimized H+P method for unmasking APP antigen (Fig. 2E,E',E", Table 2). As expected, it yielded the most intense immunolabeling but also resulted in well preserved structure and morphology of bone tissues. Unfortunately, non-specific staining was still strong in the bone marrow even after blocking and quenching endogenous peroxidase activity.

Collectively, H+P pretreatment achieved the best results for IHC of APP in bone tissue specimens under HRP-mediated chromogenic detection. However, it was not considered to be optimal due to significant levels of non-specific staining.

Effect of different AR protocols on IHC staining of rat femoral specimens with AP-mediated chromogenic detection

Considering the drawback of non-specific staining with HRP-mediated chromogenic detection, we further examined whether this shortcoming also occurred under AP-labeled chromogenic detection (positive reactions indicated by red staining) which is also commonly used in IHC.

Sections of rat femoral bone were immunostained using the AR protocols described above and utilizing AP-labeled secondary antibodies. Weak staining was observed in the No AR sections, and heating in a pressure cooker (Fig. 3B,B',B") had no beneficial effect on immunostaining intensity or tissue morphology. Hyaluronidase (Fig. 3C,C',C") or pepsin (Fig. 3 D,D',D") pretreatment resulted in only moderate staining while H+P yielded the best IHC results with strong staining intensity, good morphology integrity and much reduced non-specific staining (Fig. 3E,E',E") when compared with the HRP-mediated chromogenic detection system (Fig. 2E,E',E"). The comparative results of AP versus HRP across all AR methods in the rat femoral samples are summarized in Table 2.

Accordingly, we concluded that H+P predigestion in combination with AP-mediated chromogenic detection is the optimal protocol for bone tissue IHC in rats.

Effect of different AR protocols on IHC results of human vertebral trabecular bone specimens with HRP- and AP-mediated chromogenic detection

To verify that the IHC protocol optimized in rat femoral sections of H+P predigestion in combination with AP-mediated chromogenic detection was also suitable for human bone specimens, we performed IHC on needle biopsy samples of vertebral trabecular bone from patients undergoing PVP.

As expected, No AR led to very weak APP immunolabeling (Fig. 4A,A'). Heat mediated AR caused the sections to detach from the slide and also produced significant issues with overlapping and folding of the sections (Fig. 4B,B'). Hyaluronidase (Fig. 4C,C') or pepsin (Fig. 4D,D') predigestion alone resulted in good preservation of tissue morphology but only moderate intensity of immunostaining. As expected, H+P predigestion produced the best IHC results for both tissue structure preservation and intensity of positive staining (Fig. 4E,E'). Consistent with our observation in rat femoral sections, high level of non-specific staining was observed in human bone samples using HRP-mediated chromogenic detection (Fig. 4C-E) when compared with AP-labeled chromogenic detection (Fig. 4C'-E'). The comparative results of AP versus HRP across all AR methods in the human samples are summarized in Table 3.

In summary, H+P predigestion combined with AP-mediated chromogenic detection gave consistent positive results for human bone specimens, indicating that this protocol is suitable for IHC staining of bone tissue samples both from humans and rodents (rat).

Common mistakes encountered during IHC processing of bone tissue specimens

In order to improve understanding and quality of

Table 3. The effects of two detection systems and different AR methods on the IHC staining of APP in bone tissue samples from human.

Antigen Retrieval	Horseradish Peroxidase-mediated Chromogenic Detection					Alkaline Phosphatase-mediated Chromogenic Detection				
	N	C	H	P	HP	N	C	H	P	HP
Tissue Integrity	W	L	W	W	W	W	L	W	W	W
Staining Intensity	W	W	M	M	S	W	W	M	M	S
Non-specific Staining	S	S	O	O	O	N	S	S	N	N

N, no antigen retrieval (AR); C, AR with pressure cooker in sodium citrate solution; H, AR with hyaluronidase predigestion; P, AR with pepsin predigestion; HP, AR with hyaluronidase and pepsin predigestion; The abbreviations representing different degrees of tissue integrity/staining intensity/non-specific staining are as follows. Integrity: L, lost; P, partially lost; W, well preserved. Intensity: N, negative; W, weakly positive; M, moderately positive; S, strongly positive. Non specific staining: O, obvious; S, some; N, none. Representative images for each staining regimen are presented in Fig. 4.

bone tissue IHC processing, we have provided some example images of common problems resulting in poor quality IHC of bone specimens.

(1) Over-decalcification (Fig. 5A, HE staining), note the loss of tissue architecture, and loss of nuclear details as the hematoxylin fails to form a bond with the cell nucleus and thus staining was almost totally eosinophilic;

(2) Poor decalcification (Fig. 5B, HE staining), note the fuchsia calcium nodules indicating a failure of decalcification and the fragments resulting from chattering when the blade encounters densely calcified areas within the sections;

(3) Detachment and overlapping/folding (Fig. 5C), note the sections were wrinkled and torn with disrupted appearance;

(4) Over-incubation with DAB (Fig. 5D), note the nearly black immunostaining in place of the expected brown immunolabeling in a normal positive reaction;

(5) Over-counterstaining (Fig. 5E), counterstaining with Mayer's hematoxylin for too long (more than 2 minutes). Note the extensive blue staining of the cytoplasm and extracellular matrix of osteocytes/chondrocytes, the nucleus has become almost black instead of blue for normal counterstaining;

(6) Non-specific staining (Fig. 5F), note the intense false positive staining in the bone marrow instead of in the bone tissues.

Discussion

In this study, we found that predigestion firstly with hyaluronidase (1.5-2.5 mg/ml, pH 4.5-6.0) and then with pepsin (0.3-0.5%, pH 1.0-2.5) was an effective AR method with intense staining for IHC of bone tissue specimens. Furthermore, AP-mediated chromogenic detection was superior to HRP-labeled chromogenic detection with its distinct advantage of reduced non-specific staining. Hence, we have produced an effective and practical IHC procedure for routine biopsies of bone tissues characterized by H+P predigestion in combination with AP-mediated chromogenic detection. This detailed protocol will greatly facilitate the use of IHC on bone sections, thus providing a convenient and reliable method for visualizing antigen expression in bone tissues for both experimental research and clinical histocytopathology diagnosis.

AR is an essential step for IHC protocols and significantly increases the sensitivity of the immunohisochemical detection of epitopes (D'Amico et al., 2009). However, the mechanism underlying AR is not fully elucidated (Bogen et al., 2009). It seems most likely that formalin or other aldehyde fixation forms protein cross-links that mask the antigenic sites in tissue specimens, such crosslinking or denaturation produces changes in secondary and tertiary structure of the protein, which may render the antigenic epitopes against which the antisera are directed inaccessible or unrecognizable (Ramos-Vara, 2011). There are several

ways to break the formalin induced bonds including heat, enzymatic digestion, and ultrasound. Combinations of these methods are an alternative approach to unmasking antigens if individual methods are unsuccessful. It is especially useful when performing double or triple labeling of two or more antigens simultaneously (Frost et al., 2000). Heat induced epitope retrieval (HIER) is the most commonly used AR method, this technique involves the application of heat for varying lengths of time in an aqueous solution (commonly referred to as the retrieval solution) (Yamashita, 2007). Microwave oven, pressure cooker and steamer are the most commonly used heating devices. The heating length of 20 minutes appears to be optimal and cooling usually takes a further 20 minutes. Citrate buffer of pH6.0 is the most popular retrieval solution and is suitable for most antibody applications (Shi et al., 2001). In the study, HIER was used for comparison but we found that tissues were inevitably damaged or destroyed after heating. We speculate this destruction occurred due to mechanical tissue disruption from the steaming and boiling, or due to tissue being expelled from the solution by the boiling. Proteolytic induced epitope retrieval (PIER) is another method commonly applied, this has been reported as restoring immunoreactivity to tissue antigens with different degrees of success (Brooks, 2012). Several enzymes have been used including proteolytic enzymes, such as proteinase K (Mori et al., 2011), trypsin (Momose et al., 2011), pepsin (Franciosi et al., 2007), nucleases (Takagi et al., 1993), and hyaluronidase (Ahrens and Dudley, 2011). In our study, we proposed a new AR method of hyaluronidase and pepsin combined predigestion for bone tissues and obtained good results with higher sensitivity of immunoreactivity when assessed by microscopy in comparison with hyaluronidas or pepsin predigestion only. Moreover, in our study there were no adverse effects on tissue morphology or epitope destruction using enzyme digestions such as has been described by other researches (Pileri et al., 1997; D'Amico et al., 2009). This discordance may possibly be due to our careful optimization of concentration, pH value, incubation time and temperature for enzymatic digestion, and highlight the need for these parameters to be comprehensively tested before application.

What is the underlying AR mechanism of H+P predigestion? D'Amico postulated that digestive enzymes can interrupt crosslinking between proteins, thereby facilitating the antigen exposure and then the antigen-antibody binding (D'Amico et al., 2009). Furthermore, Suurmeijer and Boon summarized the possible mechanism of AR as follows (Suurmeijer and Boon, 1993; Shi et al., 1997): (a) breaking of the formalin-induced cross linkage between epitopes and unrelated proteins; (b) extraction of diffusible blocking proteins; (c) precipitation of protein; and (d) rehydration of the tissue sections, allowing better penetration of antibodies and increasing accessibility of epitopes. In our study, we found heating was an aggressive method

for IHC of bone tissues with the biggest problem of detachment of the sections from the slide. Therefore, we adopted a moderate approach of enzymatic predigestion. We speculate that hyaluronidase is largely responsible for re-exposing extracellular matrix proteins/antigens (Sabit et al., 2001) while pepsin is mainly used for unmasking cytoplasmic proteins/epitopes, so the AR procedure of predigestion firstly with hyaluronidase and secondly with pepsin is a good solution for both serious detachment and insufficient antigen exposure problems of IHC for bone tissues.

The method of antigen detection via antibodies labeled with a protein having enzymatic activity are common, and predominantly utilize HRP and AP (Bratthauer, 2010). However, non-specific staining is very common and must be blocked to generate high quality images. Peroxidase has an oxidative function that permits the use of chromogens that, when oxidized, not only change color but also precipitate to render a permanent label. There are many oxidizable compounds that precipitate as a permanent color, the most common and widely used is DAB, which produces a brown color when in solution with peroxidase and hydrogen peroxide. This material is insoluble in alcohol and xylene and therefore sections can be routinely dehydrated and cleared without loss of chromogen. The major shortcoming of DAB is its close resemblance to endogenous pigments like melanin, lipofuchsin, hemosiderin and formalin pigments, thus resulting in difficulty in identifying true-immunoreaction products over background. In that case, it may be necessary to try different molecules that precipitate as an alternate color in these situations, such as 3-amin-9-ethylcarbazole (bright red), 4-chloro-1-naphthol (blue) and tetramethylbenzidene (dark blue-black). Another common enzyme used is AP, which removes phosphates off the donor molecule, which in turn acts as a mediator of a color change involving a third molecule. This system is favorable as it amplifies the color producing molecules per enzyme molecule relative to peroxidase, leading to better sensitivity. It is especially sensitive for investigating protein or nucleic acid blots with enzyme labels. A major problem for AP-mediated detection is the presence of endogenous AP in the tissue being examined, which needs to be quenched by using levamisole in the blocking step, but the exact conditions for successful inactivation vary depending on the tissue. Here, we found levamisole was satisfactory in eliminating endogenous AP in bone tissues. Although the endogenous AP is usually destroyed in processing to paraffin wax (Brooks, 2012), the underlying mechanism needs to be explored further. In addition, a biotin-avidin detection system was used in our study, so it was necessary to block unwanted avidin binding to endogenous biotin by the pretreatment of unconjugated avidin which is then saturated with biotin. AP acts on many substrates, each producing a different color product. In our study, we used Fast Red TR/Naphthol AS-MX which precipitates as a red color at the site of

AP localization. And much less non-specific staining was observed when compared with DAB incubation, however, this chromogen has a drawback of being soluble in alcohol, thus the tissue sections cannot be dehydrated and cleared as commonly done, and an aqueous mounting medium like glycerin has to be used.

In view of the common problems that occur during IHC of bone tissue specimens that we described (Bussolati and Leonardo, 2008), improvement, if not absolute resolution, of the key issues are required (Taylor, 2006). Particular issues include:

1) Tissue detachment off slides (or loss of tissue sections or poor attachment). This is the biggest challenge for success of IHC for bone tissue sections and there are some techniques for avoiding this as follows. (1) Positively charged slides or amino-propyl-tri-ethoxy-silane (APES) coated slides help hold the sections on. Gelatin coated slides have also been used with good results; (2) Cut thin sections at 3 μm or 3.5 μm; (3) Let the slides stand upright in an oven at 65-70ºC for two hours before deparaffinizing the sections, this time and temperature do not affect the immunorecognition of the antigens (Jones et al., 2001); (4) Use proteolytic induced AR instead of heat induced AR; (5) Reduce the incubation time in proteolytic/microwave induced AR as short as possible; (6) Use PBS containing 0.02 % Triton X-100 as a washing buffer instead of PBS only and avoid agitating the slices when washing between steps.

2) Fixation. The aim of fixation is to get a snapshot of the tissue as it is when still functioning in the body, prompt and adequate fixation is essential to ensure the preservation of tissue architecture and cell morphology (van der Loos, 2007). Prolonged fixation may significantly diminish the antibody binding capability and under-fixation can lead to edge staining, the ideal fixation time varies depending on the size of the tissue block and the type of tissue, but fixation between 18 to 24 hours with 4% paraformaldehyde in 0.1M phosphate buffer seems to be ideal for most applications (Merrell et al., 2005).

3) Decalcification. The primary advantage of acid decalcification is speed, which is important in diagnostic pathology for patients, but it is known that these reagents often have negative effects on morphological preservation, antigenicity retention, and the integrity of DNA (Sanjai et al., 2012), so it is recommended to use the moderate chelating agent of EDTA in pH neutral solution to obtain the best quality instead of harsh acid (Alers et al., 1999; Serper and Calt, 2002). It is important to rinse the specimens thoroughly to remove all decalcification solution before proceeding with processing. Avoid over decalcification and under decalcification by assessing the end-point of decalcification with physical testing by probing with a needle until it resembles soft tissues or chemical detection or X-ray radiography (Sanjai et al., 2012; Valentine and Piper, 2012).

4) Washing. Multiple washing steps are recommended between each staining step for the reason

that it is critical not only to remove unbound antibody, but also to wash away antibodies that are weakly bound to nonspecific sites.

5) Over-staining. Monitor the progress of color development by periodic examination using a microscope. Stop development when specifically labeled structures show proper color and before nonspecific background labeling begins to occur (Brooks, 2012).

In summary, we have found an effective and practical immunohistochemical protocol for bone specimens characterized by H+P predigestion combined with alkaline phosphatase-mediated chromogenic detection. This method produced superior results when compared with other commonly used AR methods. In addition, this procedure considerably enhanced the immunolabeling that can be obtained in fixed sections without any evident deleterious effect on tissue integrity at microscopic level. This method should be widely applicable for labeling antigens in aldehyde fixed tissue from both normal and pathological states. We believe that the procedure presented here has the potential to be a sensitive tool for investigations ranging from basic research to diagnosis, especially for obtaining data which prove difficult to obtain with standard histological techniques.

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