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Novel polyvinyl alcohol (PVA) based cryoprotection method that facilitates cutting frozen sections of decalcified human trabecular bone

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Summary. Processing adult human trabecular bone to obtain tissue sections suitable for research or diagnostic purposes has always been challenging, particularly in the preparation of adult bone specimens for advanced immunohistochemistry applications. In contrast to the majority of soft tissues, decalcified bone samples perform poorly under standard paraffin embedding techniques and immunolabeling protocols fail frequently, due to the loss of protein antigenicity observed. We report on a new, PVA based infiltration method that avoids excessive heat exposure to tissue samples during embedding. The developed PVA based infiltration medium provides sufficient structural support to the heterogenic morphology and distinct architecture of subchondral trabecular bone and adjacent articular cartilage. Furthermore, the addition of bovine serum albumin (BSA) to this infiltration solution guaranteed safe attachment of cryosections to glass slides. The protocol allows the preparation of high quality sections of adult human trabecular bone tissues which can be used for both classical histochemical stains and for immunohistochemistry, since protein antigenicity is satisfactorily preserved.

Key words: Cryoprotection, Cryomicrotomy, Human trabecular bone, Immunohistochemistry, Polyvinyl alcohol

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Introduction

For most bone tissue specimens the standard histological procedure involves formalin fixation, followed by decalcification and paraffin embedding. Paraffin is inert and does not react with tissues, a fundamental advantage of this standard embedding medium. For this reason, paraffin embedding is a common tissue processing technique used for soft tissues and for decalcified bone samples. However, some antigenic epitopes are damaged, sequestered or altered by aldehyde fixation and processing to paraffin wax. Therefore, the search for a simple and effective antigen retrieval (AR) method has become a hot topic in immunohistochemistry protocols. Recent studies have revealed that AR techniques may be successful by partially or fully reversing the alterations caused by chemical fixation and paraffin embedding (for review see Shi et al., 2011). However, the technical difficulty remains in obtaining larger serial sections suitable for microscopic examinations from adult human trabecular bone tissues embedded in paraffin. During the embedding procedure adult trabecular bone tissues become brittle and paraffin does not provide sufficient structural support to obtain usable sections.

The second common embedding medium used for histological study of the human bone is celloidin. Although celloidin permits good preservation for morphologic assessment, the use of this technique has a number of profound disadvantages. The time needed for embedding is extremely long (typically a few months for human specimens) and the study of proteins by immunostaining is difficult or impossible due to the difficulty in removing celloidin from the sections

(Keithley et al., 2000).

Alternative techniques have used plastic embedding material such as methyl-methacrylate (MMA) or cryosectioning of non-decalcified bone tissue. MMA has been used to embed non-decalcified bone for more than forty years, delivering good histology (Schenk, 1965). This method is widely used for bone histomorphometry. In contrast to paraffin embedding, plastic embedding can lead to severe chemical alterations of the tissue because resin monomers are able to form covalent bonds with biological material during polymerization, particularly with proteins. This may cause destruction of antigenic sites or hides those sites with a cover of resin (Kellenberger et al., 1987). Although the modification of acrylate polymerization methods on undecalcified bone and bone marrow samples (Wolf et al., 1992), and antigen retrieval protocols (for review see Brorson, 2001, 2002) have significantly increased the number of antigens detectable on tissue embedded in plastic, staining results have been weaker or even negative in comparison to paraffin-embedded material after decalcification (Gaiser and Bernhards, 2007).

On the other hand, cryosectioning of non-decalcified bone tissue involves labour-intensive procedures using special tapes and glues to obtain usable sections and expensive modifications to a standard cryostat (Kawamoto, 2003).

Thus, from a practical point of view, we tried an alternative way to produce good quality sections and preferred to decalcify the bone before freezing and microtomy. Investigation of related literature revealed that the cutting of decalcified rodent bone tissue is possible using the conventional procedure of infiltration with sucrose as a cryoprotectant before freezing (Tokuyasu, 1973; Begum at al., 2010). Unfortunately, our initial attempts to produce usable cryosections of human trabecular bone using this technique failed. For this reason, the primary goal of this study was to develop a new method for processing human articular cartilage and trabecular bone for microscopy studies.

In this article, we report that a polyvinyl alcohol based infiltration medium in combination with propylene glycol and BSA is superior to standard paraffin embedding. Our data demonstrate that the new embedding protocol allows trouble-free cryosectioning of adult human bone samples. In addition, the antigenicity and morphologic structure of human trabecular bone tissues is well preserved.

Materials and methods

Source of specimens

Fresh bone specimens of human femoral heads were obtained from four patients undergoing a total hip arthroplasty (THA) at the University Hospital of Basel. Average age was 74.5 (SD 8.6) years, all were male. Informed, written consent was obtained specifically for the purposes of this study in addition to consenting to the procedure. All specimens were collected using a standard, transgluteal approach. All procedures were carried out by a single surgeon (AMN). After preparation and partial resection of the hip capsule, the hip was disarticulated. Subsequently, an osteotomy of the femoral neck was performed according to preoperative computer-aided planning, and the specimens were processed for immediate analysis by the Musculoskeletal Research Group.

Fixation

The samples were washed in PBS and cut into 6 mm thick slices using a low speed diamond saw (Isomet, Buehler). Chemical fixation of bone slices was initiated by immersion in ice-cold 10% formalin in PBS for 48 hours and aldehyde crosslinking was completed by post-fixation in the same fixative at RT for 2 hours.

Degreasing

After fixation, specimens were rinsed in PBS for 20 min and the degreasing process was started by incubation in a graded series of ethanol (50%, 1 h; 70%, 1 h; 100%, 2 x 1 h) at room temperature, followed by extraction overnight in a chloroform/ethanol (50%/50%; v/v) mixture at 37°C. Thereafter, bone segments were rinsed twice in 100% ethanol (1 h) followed by 70% ethanol (1 h) and then fully rehydrated in water (1 h).

Decalcification

Degreased specimens were immersed in 5% formic acid and decalcification was performed for 3-5 days at room temperature under vacuum with daily changes of decalcification solution. Decalcification solution should be at a volume 10 times greater than the tissue sample. During decalcification, bone segments effervesced when calcium remained in the sample. To ensure completion, decalcification was continued for 12 hours after effervescence had ceased. The specimens were then rinsed using running tap water for 1 hour and neutralized by incubation in 20 mM Hepes buffer pH 7.4 for 2 hours.

Infiltration

The specimens were placed into polyethylene cups containing a sufficient amount of infiltration solution: 12% PVA (MW 13000-23000, 87-89% hydrolyzed, Sigma-Aldrich St. Louis, MO, USA), 3% propylene glycol, 0.5% BSA, and 0.01% sodium azide in 20 mM Hepes buffer pH 7.4. The cups were tightly closed and put into the warming cupboard at 37°C. Initially, specimens floated on the surface but gradually sank to the bottom of the cups with progressive infiltration. After sinking to the bottom of the cups (generally after 12 h), the specimens were gradually evacuated at room temperature for 10 min in a vacuum chamber. After

vacuum treatment, specimens were put back into the warming cupboard at 37°C for further infiltration. The total time of this infiltration process was 24 hours. Afterwards, specimens were cooled at 4°C for 1 hour. The bone samples were then removed from the chilled infiltration solution and plunged into isopentane cooled to -65° C in liquid nitrogen and quick frozen for 15 min. After rapid freezing, samples were stored at -20°C until cryosectioning was performed.

Cryomicrotomy

Samples were mounted to the cryotome stage using infiltration solution. Cryostat sections (10-16 μm thick) were cut using a CryoStar NX70 cryostat (Thermo Scientific) at -23°C, mounted on SuperFrost microscope slides (ThermoFisher) and air dried.

Pretreatment of cryosections

Sections were incubated in 80% ethanol, 20 mM Hepes buffer pH 7.4 for 30 min at room temperature. At this stage, PVA but not BSA will be extracted from bone sections. To achieve optimum adhesion of the sections to the microscope slides, the sections were then fixed in solution of 10% Formalin, 80% ethanol and 20 mM Hepes buffer pH 7.4 for 1 hour at room temperature and subsequently washed in tap water for 10 min.

Histological stains

The following standard staining methods were used in the study: Hematoxylin and eosin (H&E); Safranin-O (0.05 % Safranin-O in 0.5% glycolic acid /NaOH, pH 3.5 for 8 min); Safranin-O/Fast Green (0.01 Fast green in 0.5% glycolic acid /NaOH, pH 3.5 for 3 min and 0.05% Safranin-O in 0.5% glycolic acid / NaOH, pH 3.5 for 8 min); Periodic acid-Schiff staining (PAS).

Immunohistochemistry

Free aldehyde groups were quenched by incubation in 50 mM NH4Cl for 10 min and non-specific protein binding was blocked with an incubation buffer containing 1% normal goat serum, 3% BSA, 0.2% Triton X-100 in PBS for 30 min, subsequently. Primary and secondary antibodies were diluted at a final concentration of 1:100 in the same incubation buffer. Sections were then incubated in rabbit polyclonal anti-laminin (Sigma (St. Louis, MO, USA), overnight at 4°C. After thoroughly washing three times in PBS, sections were incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch, Roche, Switzerland) as secondary antibody for 2 h at room temperature. Immunoreaction was visualized with a BCIP (5-bromo-4-chloro-3'-indolyphosphate) / NBT (nitro-blue tetrazolium chloride) alkaline phosphatase substrate system (0.57 mM BCIP, 0.30 mM NBT in AMPD buffer (50 mM 2-Amino-2-methyl-1,3-propanediol, 5 mM $MgCl_2$, 100 mM NaCl, adjust to pH 9.5 with 1M HCl) at room temperature for 10 min. For controls, the primary antibody was omitted. After rinsing in PBS for 5 min, glass coverslips were mounted using Mowiol.

Microscopy and Image Acquisition

Digital image acquisition was performed using a Leica DFC 420 camera set-up with a 5 Megapixel CCD resolution (Leica Microsystems, Heerbrugg, Switzerland).

Results

In light of the frequent failures of standard sucrose cryoprotection, we first tested a variety of alternative reagents, among those glycerol, ethylene glycol, propylene glycol, polyethylene glycol (PEG), polyvinylpyrrolidone (PVP) and polyvinylalcohol (PVA) in various combinations. After many attempts, we found that infiltration with solution of 12% PVA (MW 13,000-23,000) /15% sucrose improved cryoprotection and we were able to successfully cut frozen sections from a block of human femoral head or tibia plateau. Unfortunately, we encountered some difficulty in getting the cryosections to adhere to slides (including the use of "plus" slides, poly-L-lysine coated slides and slides coated with various concentrations of porcine gelatin). While testing 12% PVA solution in combination with propylene glycol, we observed that the addition of 3% of propylene glycol facilitates cutting and improved adherence of the sections to the slides. However, the best results were obtained with 12% PVA, 3% propylene glycol and 0.5% BSA in infiltration solution, as described in detail above.

The method reported here allows the preparation of large and thin sections (down to 6-8 μm) from any kind of bone sample, including adult, osteoporotic human bones. We successfully cut sections from a large block (1.5x1 cm), taken from an adult human femur head, without cutting artifacts (Fig. 1A). The whole section stained with hematoxylin and eosin remains attached to the microscope slide and is free of distortion. There was excellent preservation of all cellular detail of cartilage, bone and bone marrow. This is more evident at a higher magnification (Fig. 1B-D). Similarly, the protocol used for PAS (Fig. 2), Safranin-O (Fig. 3) and immuno-staining for laminin (Fig. 4) yielded very satisfactory results. All sections are well preserved and there is no background staining.

Discussion

Performing adult human bone histology is technically challenging, particularly with large size trabecular bone specimens. Sectioning of adult bone tissue is difficult, because of the variation in hardness between the cartilage covering the articular surfaces,

adjacent densely collagenous cortical and trabecular bone including the tender red - and the predominantly fatty marrow. Our aim was to obtain suitable sections of human trabecular bone for standard histological staining and immunolabeling using a commercially available cryomicrotomy set-up and disposable blades. In order to achieve this, we have developed a PVA based supporting medium and effective infiltration method that provides excellent quality, even of large sections of trabecular bone. Sections obtained were sufficiently uniform in low magnification survey micrographs as well as for structural detail at high magnification.

Lubkin and Carsten (1942) were the first investigators to use polyvinyl alcohol for preparing tissues for microscopic studies. Following their method, tissue samples are infiltrated through an ascending series of aqueous PVA-glycerol solutions and embedded in aqueous PVA which is slowly dried to produce a firm block. This solid block was used for histochemical studies of lipids and enzymes. However, the embedding process takes weeks, and there is considerable shrinkage of specimens (Feder, 1962). Probably for this reason, infiltration of tissues with PVA according to this method was rarely used in the past. Lubkin and Carsten (1942) avoid (intentionally) degreasing of tissues before infiltration with supporting medium. Thus, in our opinion, this is an important disadvantage of their method for histological purposes. High lipid content and general adipose tissue inhibit the diffusion of hydrophilic reagents into the specimen. Consequently, it is difficult or not possible to adequately fill all spaces within the non-degreased bone sample with aqueous

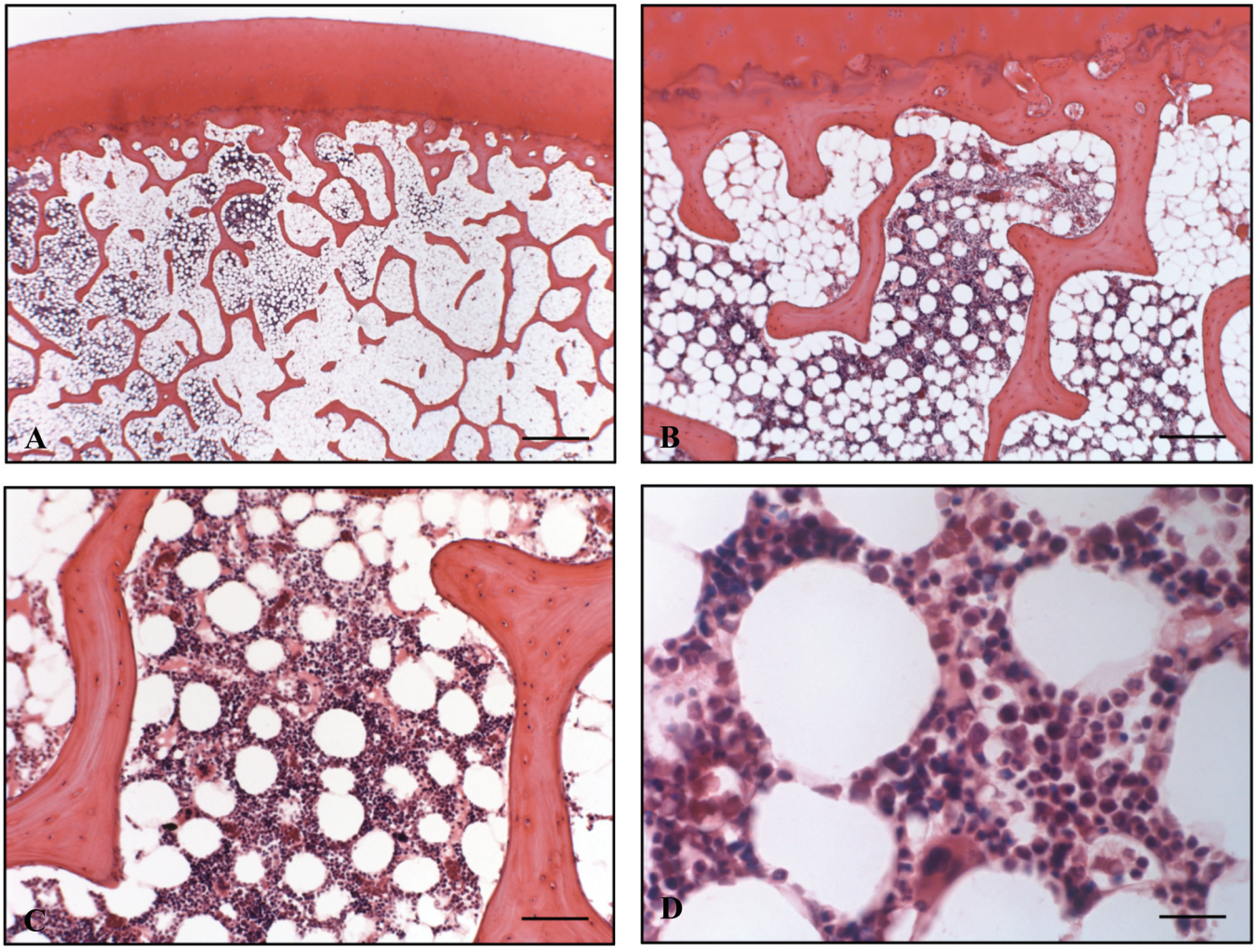

Fig. 1. Cryosections of adult human femur head. **A.** Hematoxylin & eosin shows articular cartilage and subchondral bone can be studied over a wide area as shown by a survey at low magnification. **B, C.** More morphologic details and dimensions are clearly revealed at higher magnification. **D.** Delicate zones of yellow and red bone marrow are well preserved, allowing hematopoiesis to be evaluated on the cellular level at high magnification. Bars: A, 1 mm; B, 250 μm; C, 100 μm; D, 25 μm.

infiltrating medium. Another disadvantage of the method developed by Lubkin and Carsten (1942), or a similar method as described by Feder (1962), is the difficulty in removing hardened PVA from the sections. Similar to celloidin, the medium cannot be washed out, but interferes with stains and immunolocalization of proteins is difficult or impossible.

In our method, specimen preparation, fixation, decalcification and neutralization were achieved by steps comparable to conventional paraffin embedding protocols. However, unlike paraffin the exposure of tissue to heat has been avoided. To facilitate the infiltration, we degreased bone samples in an ascending series of ethanol solutions and finally in ethanol/chloroform mixture (1:1) at 37°C. Thorough degreasing of bone samples was a critical step to achieve complete infiltration with our supporting medium and a prerequisite for satisfactory and reproducible results.

Initially, we tested PVA in combination with polyethylene glycol (PEG) of various molecular weights. Although PEG is widely used as a plasticizer in most embedding media for cryomicrotomy (Cocco et al., 2003), the results obtained with PEG were rather disappointing. Sections were very often severely compressed or did not descend at all behind the antiroll plate during cryosectioning. In the light of the frequent failures with PEG, we first tested PVA solutions in combination with sucrose. By applying this combination we obtained good sections, but this medium showed a nonhomogeneous, mottled appearance on freezing.

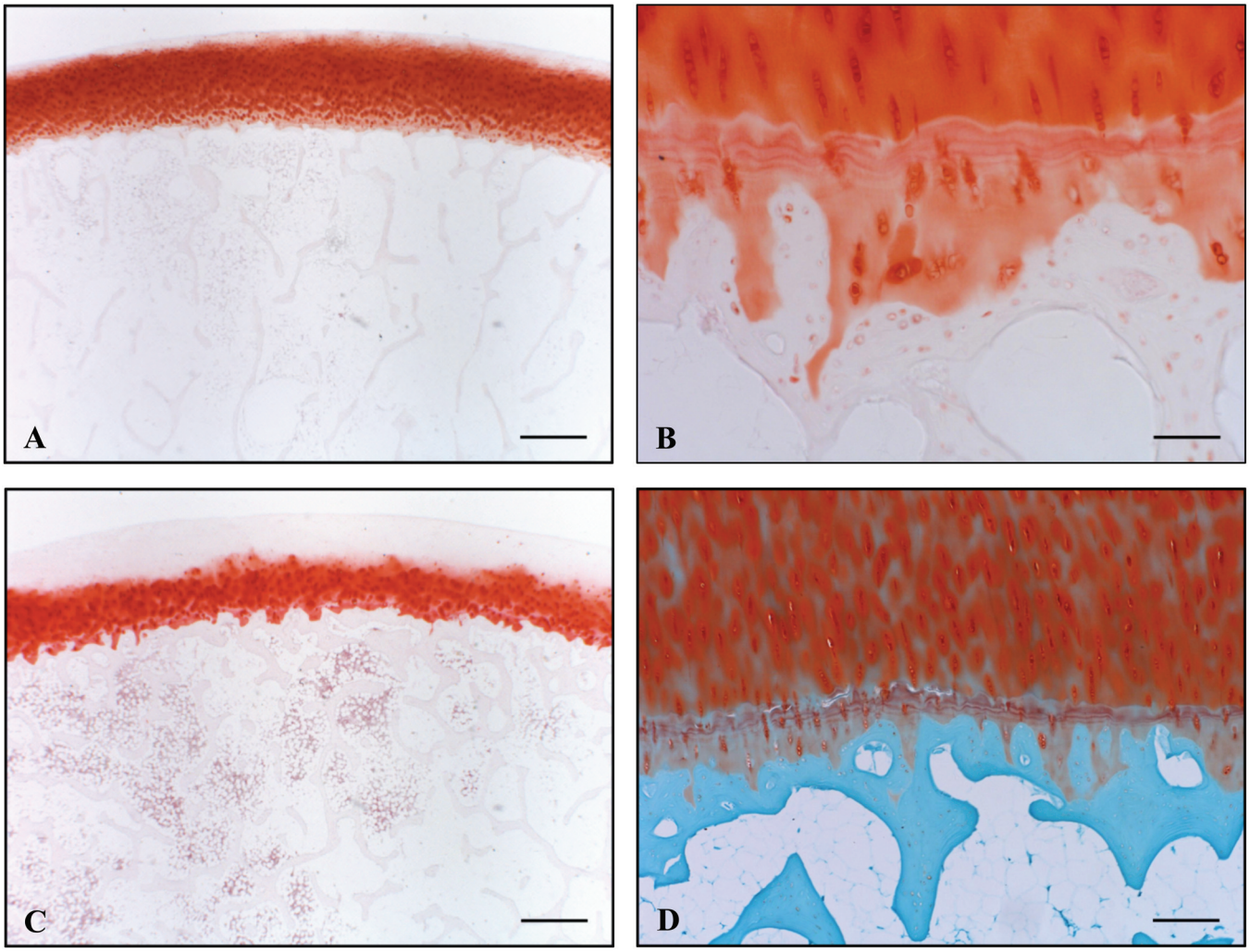

Fig. 2. Safranin-O staining of different human femur head specimens. **A.** As expected, proteoglycans are stained by Safranin-O throughout the normal articular cartilage layer. **B.** The border (tidemark) between calcified and hyaline cartilage zones is clearly resolved by Safranin-O at higher magnification. **C.** Cartilage damage of superficial layers can be easily verified on cryosections by loss of Safranin-O binding, due to the lack of proteoglycans. **D.** Counterstaining of protein with Fast-Green, a standard dye in bone histology, confines the subchondral bone which is negative for proteoglycans. Bars: A, C, 1 mm; B, 25 μm; D, 100 μm.

Additionally, the occurrence of sucrose crystals on sections after air drying was irritating. The adhesion of the sections to the microscope slides was poor and sections frequently became detached from slides during staining. We suspect that the growth of the sucrose crystals during air drying of the cryosections accelerated the detaching of the sections from slides. Finally, we tested the performance of the PVA solution in the presence of propylene glycol. The innovative combination described in this report resulted in homogeneous, white appearance of frozen medium, with similar or equal hardness and density to the decalcified bone. Adhesion of the sections to slides had improved, but was still not satisfying for a routine application. However, we were able to solve this problem by adding BSA to the infiltration medium. Following postfixation of air dried sections with formalin as described above, adhesion of the sections to the slides improved dramatically. In the first step, according to our protocol, PVA will be extracted in the ethanol solution from bone sections. During the second step however, BSA (insoluble in 80 % ethanol solution) still remains in the

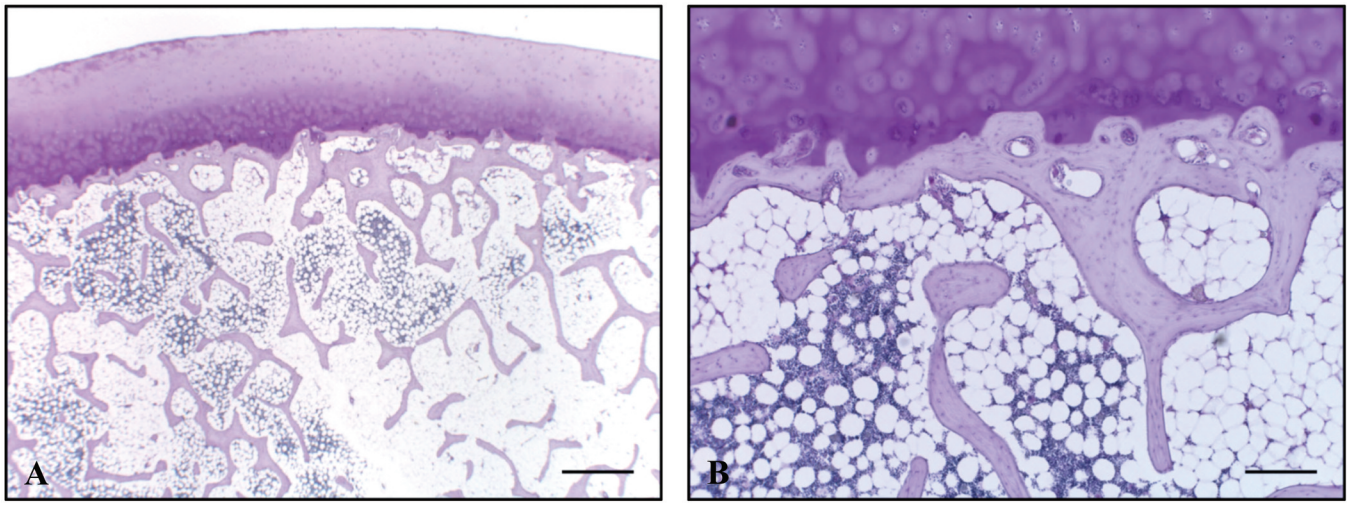

Fig. 3. PAS-staining of adult human trabecular bone. **A.** Classical PAS staining method demonstrates the general suitability of cryosections from human bone specimens to well established standard histochemical protocols. **B.** Subchondral bone plate delineates clearly from cartilage by PAS at higher magnification allowing precise morphometric studies of thin frozen sections. Bars: A, 1 mm; B, 250 μm.

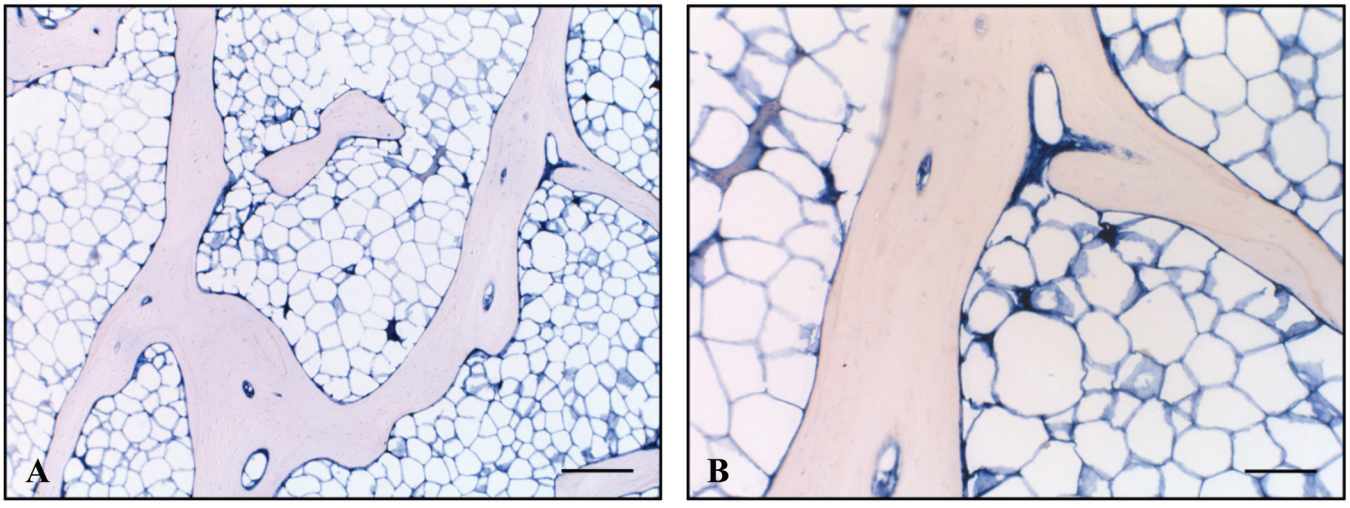

Fig. 4. Immunostaining of cryosections of adult human femur head. Expression of laminin is detected in the basement membranes of fat cells and of blood vessels and the signal was developed by alkaline phosphatase system. Obviously, there is no background staining observed in these cryosections. Bars: A, 100 μm; B, 50 μm.

sections and will be fixed by formalin. At this stage, a fine network of cross-linked BSA is formed ensuring durable adhesion of the sections to the microscope slides.

The method described in this paper offers several advantages over conventional paraffin-based tissue processing methods. Firstly, the high section quality with excellent structural preservation. Secondly, the easy application of a variety of histological stains, and finally, since protein antigenicity in the bone tissue sample is maintained and antigen retrieval protocols can be avoided, sections are directly suitable for immunolabeling.

Therefore, we believe that this new PVA based infiltration method has the potential to add significantly to the study of human bone and is an alternative choice for processing human bone specimens for both histomorphometry and immunohistochemistry.

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