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Douglas S. Allen
Iowa State University

Rudy C. Melfi
Ohio State University

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Improvements in Techniques for Aging Mammals by Dental Cementum Annuli

DOUGLAS S. ALLEN[1] AND RUDY C. MELFI

Department of Animal Ecology, Iowa State University, Ames, Iowa 50011

Department of Oral Biology, College of Dentistry, The Ohio State University, Columbus, Ohio 43210

Several modifications of commonly used histological techniques for the preparation of dental tissues for aging by dental cementum annuli are presented. Improvements in methods of tissue collection, preservation, decalcification, sectioning, and staining permit faster preparation of tissues and produce higher-quality, easily interpreted sections. Possible application of hard-tissue microtomes to sectioning of undecalcified or partially decalcified tissues is also discussed.

INDEX DESCRIPTORS: Aging techniques, dental cementum annuli, mammals.

In the 33 years since Sheffer (1950) first reported on the use of annulations in dental tissues for estimating the age of mammals, the technique has become the method of choice in most detailed mammalian population studies. Thorough reviews of both the structure of appropriate dental tissues and preparation methodologies are available (Klevezal and Kleinenberg, 1967; Morris, 1978; Grue and Jensen, 1979; Fancy, 1980). Although different authors have had varying degrees of success with the available techniques, primarily due to anatomical and dietary differences among the species investigated (Phillips et al., 1982), the consensus was, as stated by Fancy (1980:246), that "The accuracy of the cementum layer technique depends largely on the methods used to prepare the material." The purpose of the present paper is not to review the published methodological literature, but to present newly developed techniques and improvements in existing methods which markedly improve the quality of histological sections, and therefore, accuracy. Three major areas of improvement will be discussed: methods of preparation of fresh tissues, decalcification procedures, and staining and sectioning methods for decalcified, partially decalcified, and undecalcified preparations.

PREPARATION OF FRESH TISSUES

Extraction is the first source of potential error in age determination; even those teeth extracted by dentists typically have some degree of disruption of the cementum layer (Stott et al., 1982). If animals are dead, one or both halves of the mandible or a section of the mandible should be removed and preserved. This procedure is routinely performed at hunter check stations and can be accomplished with negligible damage to dental tissues by relatively unskilled persons. All fresh tissues should be rapidly preserved in a 10% formalin solution (preferably buffered) with at least a 10:1 preservative: tissue ratio. In large mammals, such as ungulates or carnivores, amounts of tissue to be preserved can easily be reduced by sawing off unneeded portions of the mandible (a hacksaw is suitable). The best preservation was obtained by daily changing of formalin for the first 2 days and later transferring tissues to 70% ethanol after formalin fixation was completed (1-2 weeks in most specimens). Preserved tissues can be stored in ethanol indefinitely and later processed without further treatment. Tissues stored in formalin must be cleared and transferred to ethanol prior to processing by any of the methods.

DECALCIFICATION

Decalcification of dental tissues may not be necessary because of the recent development of hard tissue microtomes. If chemical decalcification is unavoidable, a few simple modifications of existing techniques greatly improve the results obtained. Without complete decalcification, sections will be difficult to interpret.

To quicken decalcification, carefully trim all excess tissues in order to reduce the amount of tissue to be decalcified and the amount of both time and reagents required. For small mammals, the Isomet low-speed saw (Buehler, Ltd. Box 1459, Evanston, IL 60204) is ideal for trimming and is available in a dental research laboratory. For large mammals, a hacksaw or any other tool that will not damage the tissues is acceptable. Care should be taken to produce tissue blocks of similar size to provide similar demineralization times. Specimens from very small mammals have been decalcified successfully without trimming (Phillips et al., 1982).

Of many acceptable demineralizing solutions (reviewed by Fancy, 1980), the best results were achieved using a 5% formic acid-sodium citrate solution (1 part 50% formic acid plus 1 part 20% sodium citrate solution, both aqueous solutions). The slower rate of demineralization with this concentration may be partially offset by slow agitation throughout the decalcification process. Tissue blocks were immersed, either in cheesecloth bags (for larger specimens) or in plastic tissue cassettes (Technicon Corp., Tarrytown, NY 10591) in a vessel large enough to maintain at least a 10:1 reagent:tissue ratio in one-half of the vessel's capacity. The vessel should be covered to decrease evaporation and placed on a magnetic stirring plate. In addition to slow stirring, the demineralizing solution was changed often, preferably daily.

Complete decalcification can be detected with either a precipitant test (Lillie, 1965) or by radiography (Brain, 1966). The latter method is preferable, since any areas of calcium deposition will appear as easily recognizable, opaque spots on the radiograph. If radiography is used, we recommend running the initial series (or preferably a test series) of teeth or trimmed blocks of tissue through the decalcification process, with X-raying daily to determine the approximate time of near-completion of demineralization. Once this time has been estimated, subsequent batches may be X-rayed only during this critical period, rather than daily. Once decalcification is completed, trim excess tissue from the blocks with a sharp knife or razor blade prior to clearing and staining.

STAINING AND SECTIONING

Decalcified Materials

Staining is necessary to increase the contrast between the interlamellar substance and rest lines delineating growth increments in the cementum layer. Hematoxylin and eosin (H&E) or a metachromatic stain has been used most successfully in prior studies (Thomas, 1977;

[1]Present Address: Division of Sciences, Louisiana State University at Alexandria, Alexandria, Louisiana 71302

Fancy, 1980). H&E is the more complex of the two procedures, requiring, in the method we use (Harris' H&E), 16 separate steps and 40-45 min for completion. Quick toluidine, a simple, fast, metachromatic stain not tested by Thomas (1977), gives results comparable to those of toluidine-O, the stain recommended by Thomas, and is the better choice. Our quick toluidine procedure is as follows: 1) Five seconds of rapid dipping in quick toluidine stain (10ml 1% pyrinine B, 20ml saturated toluidine blue, and 20ml 2% sodium tetraborate mixed thoroughly and vacuum filtered); 2) distilled water, 2 sec more or less, depending on the degree of staining desired; 3) 95% ethanol, two repetitions of 1 sec dipping; 4) 100% ethanol, two repetitions of 1 sec dipping; 5) xylene, three repetitions of 2 min immersion, and 6) mount in mounting media (piccolyte), prior to placing the cover slip on the slide.

The superiority of quick toluidine over Harris' H&E can be seen on trimmed, whole-mandible sections from a 2-year-old Eastern gray squirrel (*Sciurus carolensis,* Linnaeus) (Fig. 1). Annuli in sections stained with H&E cannot be seen at 12.5X (Fig. 1A), are barely visible at 50X (Fig. 1B), and require a magnification of at least 125X (Fig. 1C) to be readily discernible. In sections stained with quick toluidine, the annuli are visible at the root apex at 12.5X (Fig. 1D), can be seen clearly at 50X (Fig. 1E), and are easily counted at 125X (Fig. 1F).

Partially Decalcified and Undecalcified Tissues

Preparing thin sections from untreated dental tissues usually has been done with several variations of the cutting, grinding, and polishing technique first used by Fisher and Mackenzie (1954). Low and Cowan (1963) were the first to report that none of these methods gave satisfactory results. Grinding methods gave inconsistent results and usually produced sections, like those made by Fogl and Mosby (1978) via razor-sectioning, that were difficult to observe and photograph. Thus, we concluded that grinding methods are inferior to those involving decalcification and conventional microtome sectioning.

The recent development of hard-tissue microtomes (e.g. Reichert-Jung Model 1140/Autocut, American Scientific Products, 1210 Waukegan Road, McGraw Park, IL 60085) has made possible thin sectioning of partially decalcified or untreated tissues. Reduction or elimination of the time required to demineralize tissues results in a significant decrease in preparation time. Goodman (1983), for example, was able to reduce the preparation time for human molars from 5-6 weeks to 1 week by using partially decalcified blocks. The technique has also been used successfully on incisors from Japanese macaques (*Macaca fuscata,* Lacepede) (Wada et al., 1978). Despite the high cost (approx. $17,000 for the microtome and $800 for the carborundum blade), these microtomes are now widely available in geological, metallurgical, dental, and medical laboratories and cooperative arrangements for their use could be easily arranged.

If hard-tissue equipment is to be used, it must be decided whether to partially decalcify the tissues prior to sectioning. Incompletely decalcified tissues must be fixed and embedded in a material able to withstand the stress of high-pressure sectioning. Partially decalcified tissues should be embedded in Paraplast (Sherwood Medical Instruments, Inc., St. Louis, MO 63103) (Goodman, 1983); a methacrylate medium should be used for embedding untreated tissues (Baron et al., 1983). Embedding with methyl-methacrylate requires at least 10 days to fix and infiltrate the tissues prior to embedding. Since partial decalcification usually requires considerably less than 10 days for all but the largest mammalian species, and Paraplast embedding is a comparatively simple procedure, partial decalcification would seem to be the most logical choice. All staining procedures are compatible with either sectioning method.

RECOMMENDATIONS

We recommend four technical variations which should enable investigators to achieve their goal of producing high-quality sections of mammalian dental tissues with minimal preparation time: 1) Use trimmed, whole mandible sections, whenever possible. 2) Partially or completely decalcify the tissues, depending on the method of sectioning to be employed, in a constantly stirred 5% formic acid-sodium citrate solution. 3) Verify the completion of the decalcification process via radiography. 4) Stain the sections with quick toluidine.

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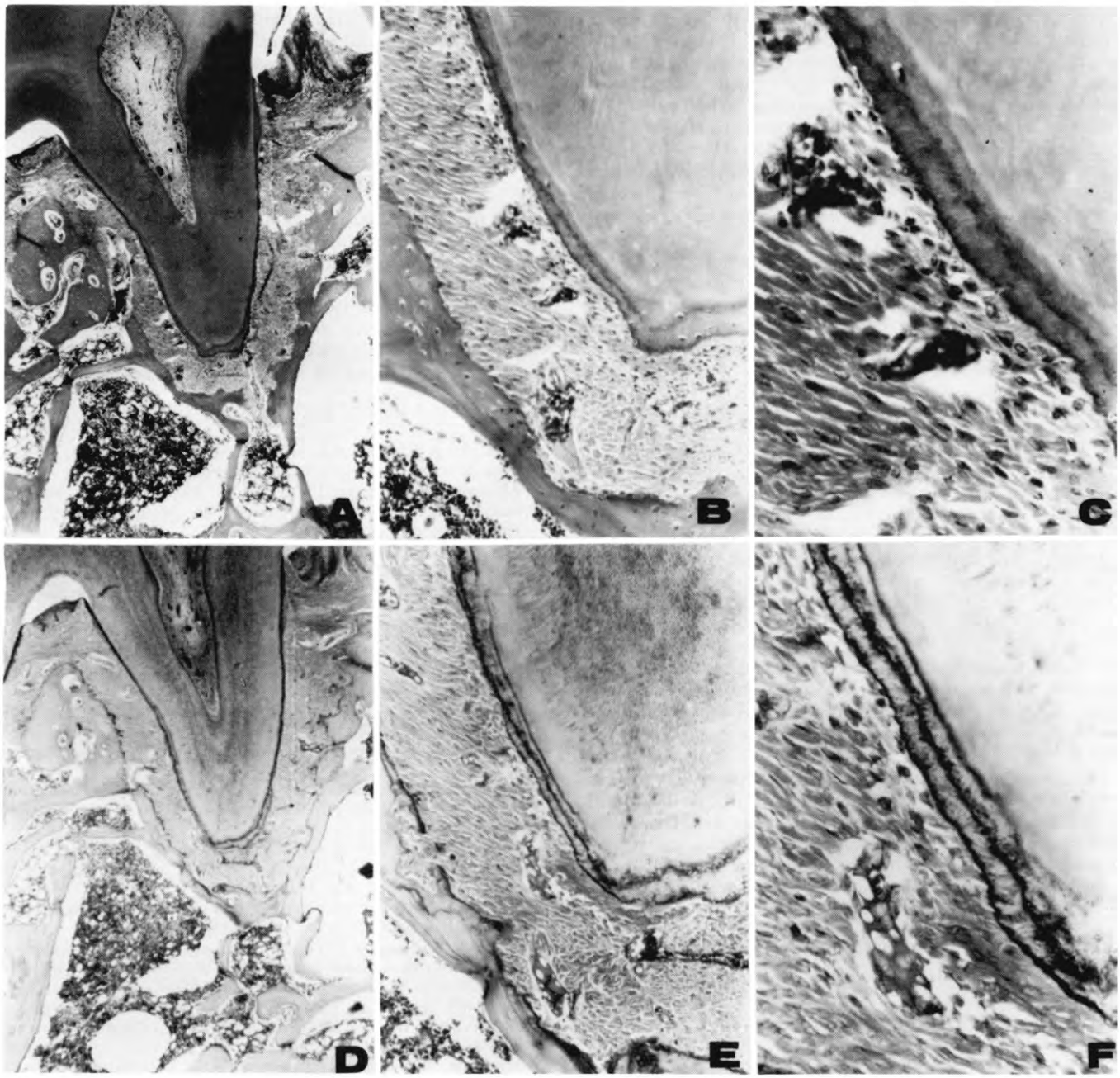

Fig. 1. Dental cementum annuli in a longitudinally sectioned molar from a 2 year-old Eastern gray squirrel. Sections A-C stained with Harris' hematoxylin and eosin, and Sections D-F stained with quick toluidine, are shown at 12.5, 50, and 125X.