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A HISTOLOGICAL DEMONSTRATION OF NERVES IN SUBCHONDRAL BONE

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Several different staining procedures were carried out on decalcified histological sections from human femoral heads to demonstrate the nerves in subchondral bone. The femoral heads were obtained at surgery from patients with fractures of the femoral neck or osteoarthritic hip joints. The Bodian technique was found to be the most suitable. Serial sections were used in order to disclose the various sources of error. It was not possible to demonstrate nerves in the bone matrix, but they were easily seen in the subchondral bone marrow, aften related to the vessels. A comparison of the fracture and osteoarthritic cases revealed an obvious difference; more nerves were seen in osteoarthritis. The method described is considered suitable for further study of the nerves in osteoarthritic femoral heads.

Key words: innervation; subchondral bone; osteoarthritis

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Relatively few articles have been published, in recent literature, about the nerve supply of bone, and textbooks of histology hardly mention the subject. This is probably due to the difficulties encountered in working out a reliable technique. The presence of nerves in bone has been demonstrated in the periost, in the endosteum of the medullary trabeculae and in the bone marrow, by light microscopy (Sherman 1963, Leeson & Leeson 1970, Miller & McCuskey 1973).

The fact that nerves also occur in the Haversian canals has been shown by electron microscopy (Milgram & Robinson 1965), but it is still a matter of discussion whether or not nerve fibres are present in the bone matrix. Thus, Sherman (1963), Miller & Kasahara (1963), Milgram & Robinson (1965) and Cooper (1968) were unable to confirm the observations of De Castro (1930) and Hurrell (1937). De Castro described nerve fibres in growing bone in cats, often closely related to the osteocytes, and Hurrell demonstrated nerve fibres in the bone matrix of a fully grown cat. There have been no similar observations in human bone.

The nerves in bone appear to be mostly non-myelinated, but myelinated fibres have also been described (Cooper 1968). It is still not fully elucidated how the nerves end in bone.

This work is an attempt to find a suitable technique for demonstrating nerves in subchondral bone in human femoral heads and to describe some preliminary findings in osteoarthritic femoral heads.

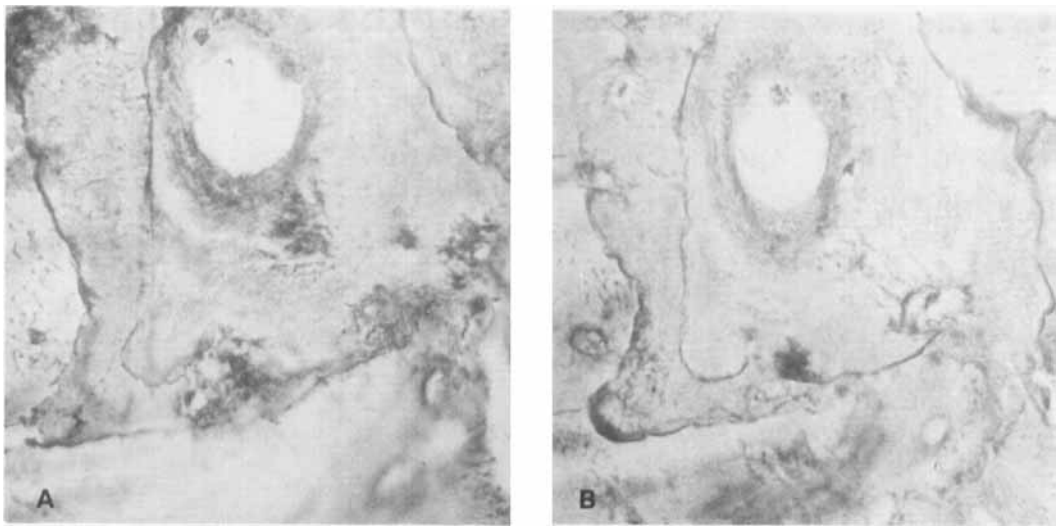


Figure 1 A. Subchondral bone from osteoarthritic femoral head illustrating staining of cement lines. Due to osteoarthritic alterations the arrangement of lamellae differ from that seen in normal bone. (Bodian, orig. magnification × 100). B. A serial section from the same sample at a distance of 6 μ. The cement lines are easily recognized.

MATERIAL AND METHODS

Twelve femoral heads were obtained from patients at the time of arthroplastic replacement after fracture of the femoral neck (two, control material) or osteoarthritis (ten). Immediately after operation, the specimens were preserved in 10 per cent buffered formaline phosphate. Equal slices, 10 mm thick, were obtained from each head with a hand saw. The slices included the circumference of the head with articular cartilage and subchondral bone from both weight-bearing and non-weightbearing areas. After decalcification in 10 per cent formic acid and embedding in paraffin, the samples were cut in serial sections at 6 μ and subsequently stained.

The Bodian method was used for axon staining and staining of the myelin sheaths was performed according to the method of Mahon and Weill. In addition, staining with haematoxylin-eosin and v. Gieson was performed.

The final evaluation was based on the histological sections stained with Bodian's method. In the present work a modification used routinely at the Laboratory of Paediatric Pathology, Rigshospitalet, was used. It is as follows:

1. Deparaffinize and hydrate with distilled water.
2. Place slides in protargol solution 1 per cent and add 6 g of clean copper shot per 100 ml of solution. Let stand at 37° C for 24 hours.
3. Rinse in distilled water, three changes.
4. Staining solution for approximately 5 minutes: 4 ml silver nitrate (10 per cent) with supersaturated sodium sulphite until clear. Add 3 ml of this solution to 300 ml gum arabic and shake for 5 minutes, then add 30 ml 2 per cent hydroquinone.
5. Rinse in distilled water, three changes.
6. Tone in gold chloride solution 1 per cent with addition of three drops glacial acetic acid per 100 ml solution. Tone for 5 minutes.
7. Rinse in distilled water, three changes.
8. Develop in oxalic acid (2 per cent) approximately 5 minutes.
9. Rinse in distilled water, three changes.
10. Sodium thiosulphate (5 per cent) for 5 minutes.
11. Rinse in distilled water, dehydrate in alcohol and clear in xylene.

It was necessary to work out methods which would eliminate the various sources of error, as structures other than nerve fibres in the bone become stained by the Bodian method (Figures 1 and 2); e.g., both canaliculi and lacunae become stained black. In doubtful cases, where canaliculi resembled nerves, it was possible to show by light microscopy (magnification × 1000) that the black colour in canaliculi appears as a lining and consequently the canaliculi are seen with a double outline enclosing a non-coloured zone (Figure 2). The cement lines between the different systems of lamellae were also stained by Bodian's method. When a black line appeared

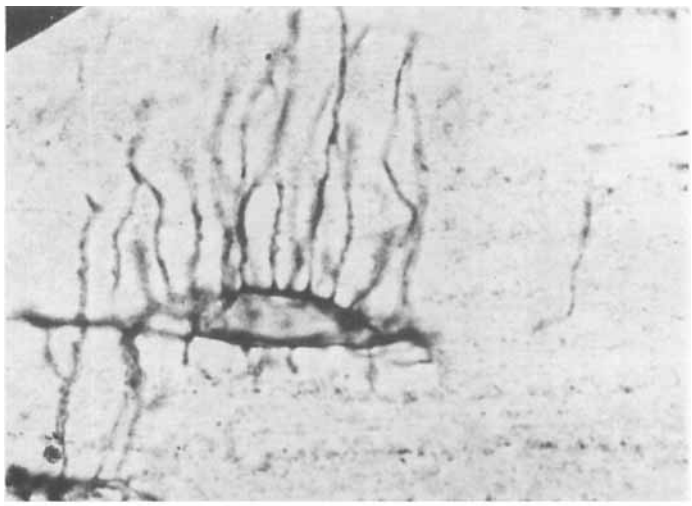


Figure 2. Osteocyte lacuna and canaliculi stained by Bodian. Canaliculi are seen with a double outline and a non-coloured zone in the centre. This can only be demonstrated by using high magnification and focussing (Bodian, orig. magnification × 1000).

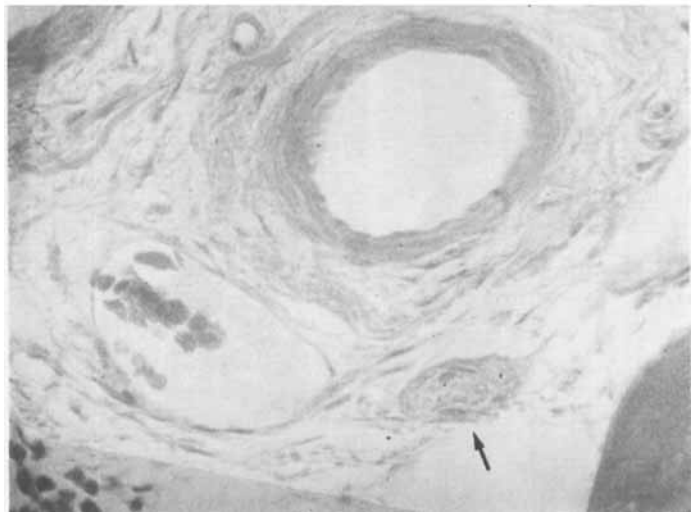


Figure 3. Subchondral bone and bone marrow from a femoral head from a patient with fracture of the femoral neck. Cross section of a small nerve (arrow) in association with vessels (Haematoxylin, eosin, orig. magnification × 100).

to cross the lamellae, it was necessary to study serial sections in order to find whether the lines were present in more than one of the serial sections (Figure 1, A and B).

Staining located to the endost, osteochondral junction and tidemark was never interpreted as nerves. Finally Sharpey's fibres which cross the lamellae should be mentioned, but the acid used for decalcification impairs the colour, as the fibres consist mainly of collagen.

RESULTS

Histological sections from the cases with fracture of the femoral neck: After elimination of the above-mentioned sources of error, it was not possible to demonstrate nerve fibres in the bone matrix by the Bodian method. However, in the bone marrow, nerves were easily demonstrated, usually closely associated with the vessels. Whereas the myelin sheath-staining gave negative results, staining with haematoxylin-eosin and v. Gieson showed some cross sections of nerves in the marrow related to the vessels (Figure 3).

Histological sections from osteoarthritic femoral heads: It was also not possible to demonstrate nerve fibres in the bone

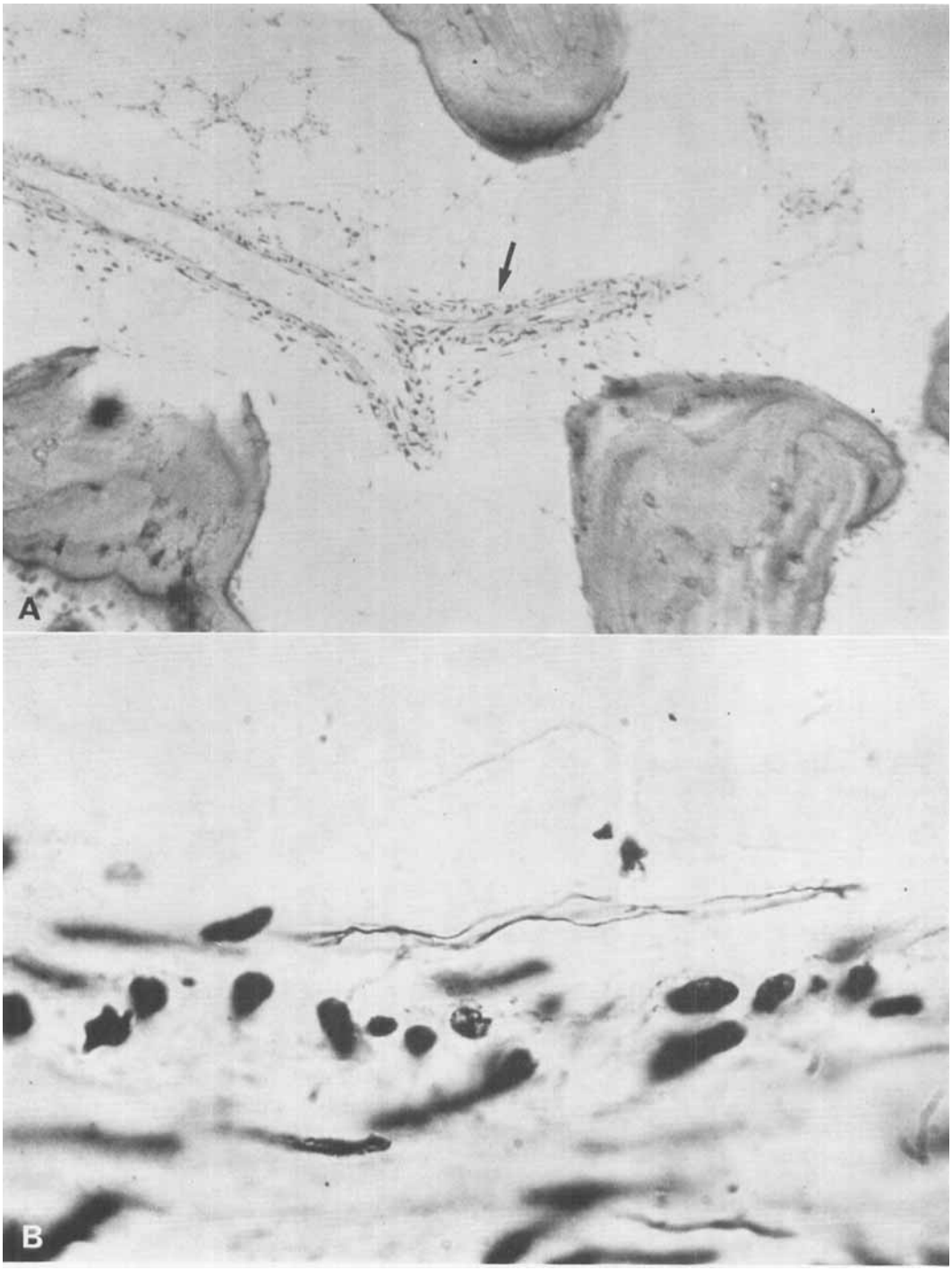
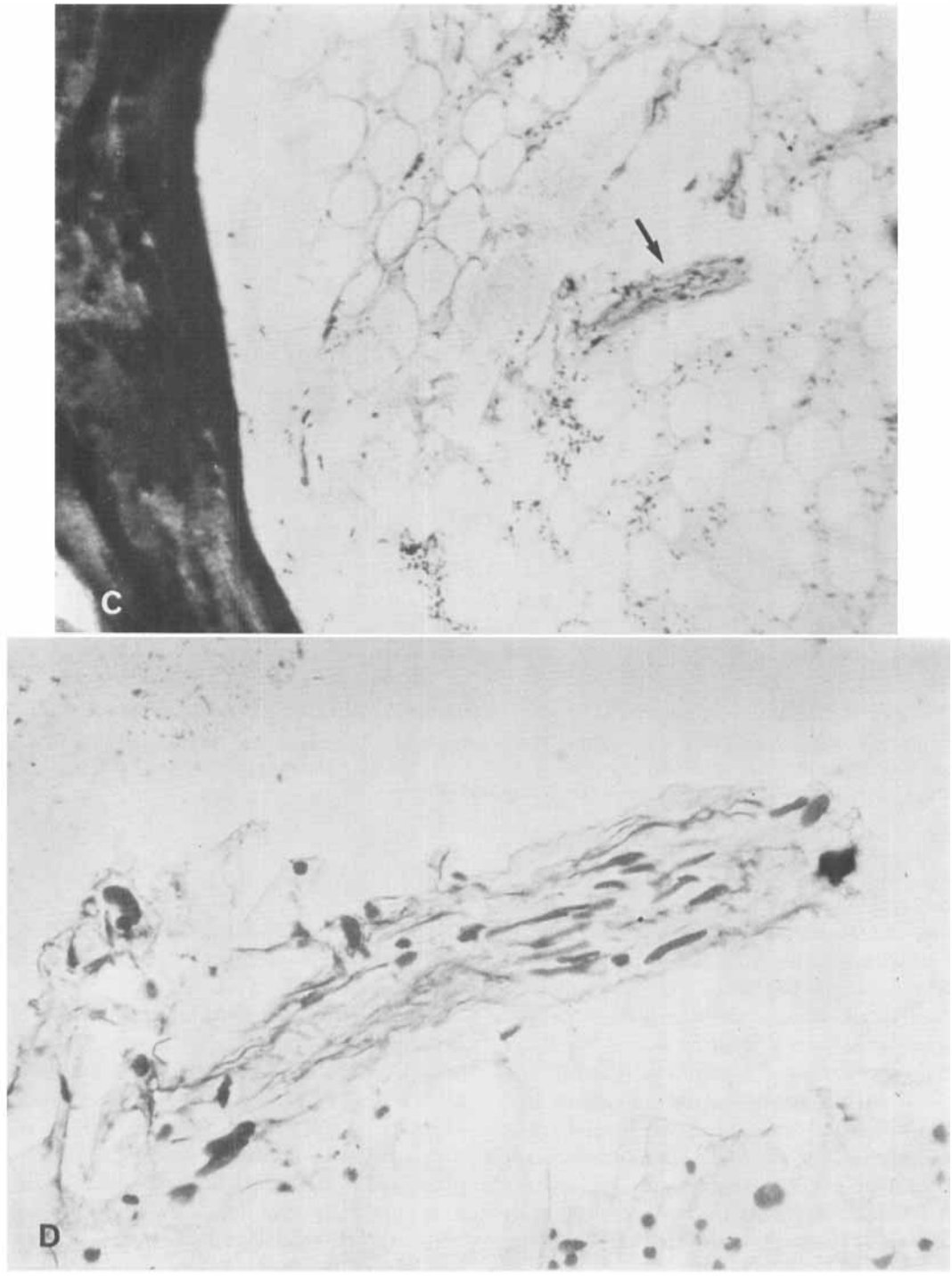


Figure 4. Subchondral bone from osteoarthritic femoral head. A. Longitudinal section of a nerve fibre (arrow) related to an arteriole (Bodian, orig. magnification × 100). B. Same as A (Orig.



magnification × 1000). C. Solitary nerve in the bone marrow (arrow) (Orig. magnification × 100). D. Same as C (Orig. magnification × 400).

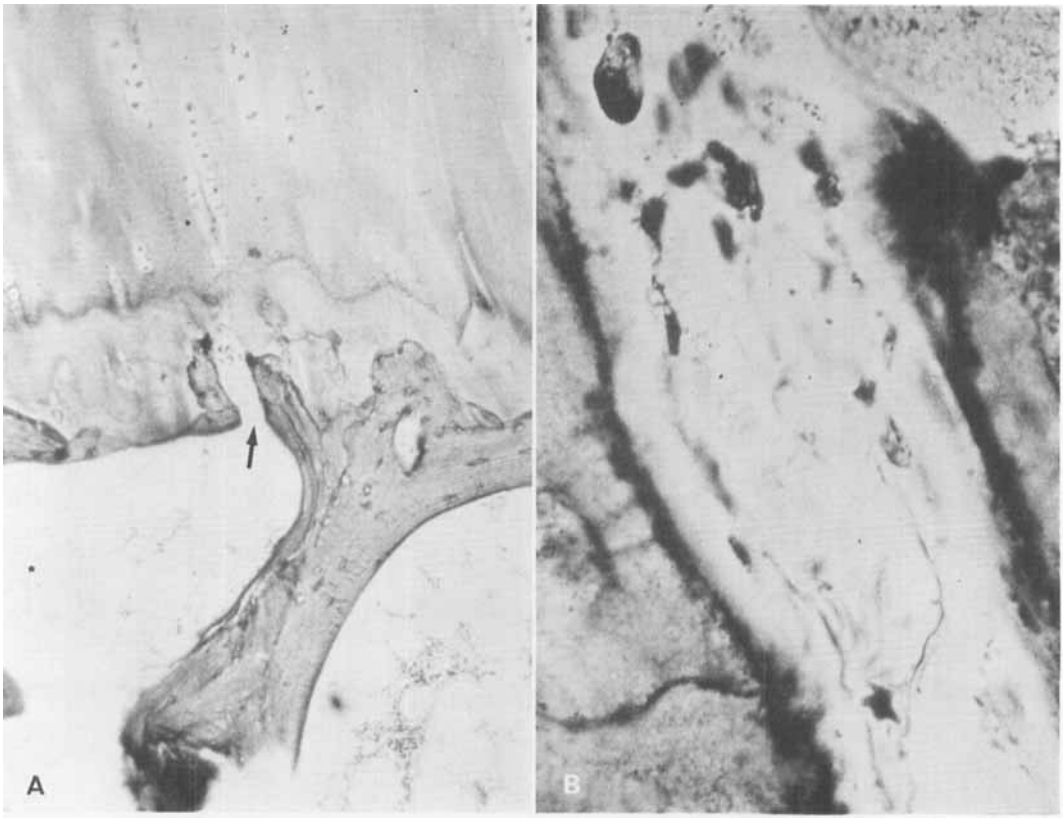


Figure 5 A. Subchondral bone and cartilage from osteoarthritic femoral head. Vessels penetrate from the subchondral marrow. Solitary axon is seen accompanying the vessels (arrow) (Bodian, orig. magnification × 100). B. Same as A (Orig. magnification × 1000).

matrix in these sections by Bodian's method. However, several nerve fibres, in longitudinal as well as cross sections, were seen in the bone marrow subchondrally where an abundance of vessels were also found (Figure 4 A, B, C, & D). As nerves were also demonstrated in sections with a proliferation of vessels into the calcified layer of articular cartilage (Figure 5 A & B), as well as in the subchondral granulation tissue (Figure 6 A & B) it was evident that there were more nerves in the osteoarthritic femoral heads than in the control cases.

The other staining methods gave results similar to those described for the sections from the fracture cases.

DISCUSSION

The distribution, character and ending of nerves in bone have not been fully elucidated.

In the present work the method of Bodian (1936, 1937)—an accepted axon staining method—was chosen as almost all nerve fibres in bone are unmyelinated (Cooper 1968) and, furthermore, as myelin sheath staining in our experience produced only negative results.

In analysing the decalcified sections of bone stained with Bodian's method, many considerations were taken into account concerning the method and results. Bodian staining is not specific for nerve fibres as many other structures in the bone

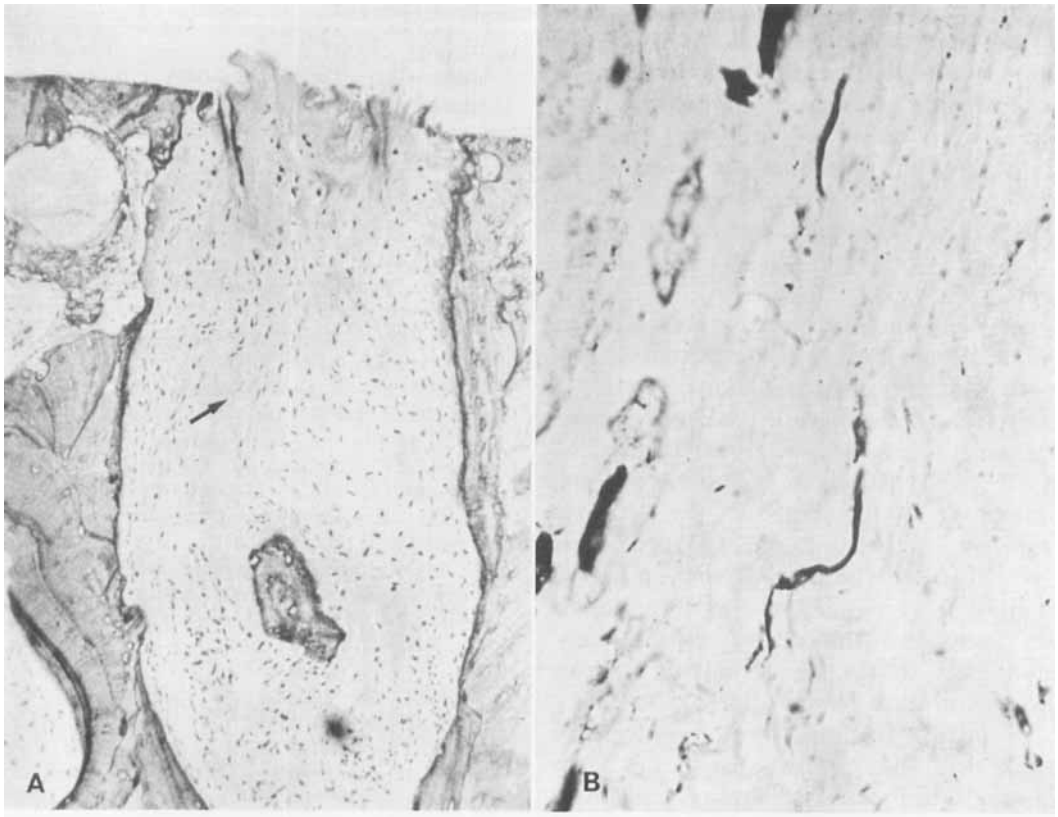


Figure 6 A. Subchondral bone (cartilage absent) from osteoarthritic femoral head. Nerve (arrow) is seen located in a cyst with granulation tissue (Bodian, orig. magnification × 100). B. same as A (Orig. magnification × 1000).

matrix are stained black in the same way as the nerves (Green et al. 1970). This applies to the osteocyte lacunae and particularly to canaliculi as they are lined with sulphated mucopolysaccharide (Ham 1969). According to Leeson & Leeson (1970), the black stained cement lines also contain mucopolysaccharide. The authors confirm this by staining the sections with Alcian Blue; an obvious green colour was then seen located to the osteocyte lacunae and to a minor degree to the canaliculi, and a diffuse green glow was seen in some sections of the bone matrix contrasting with the red colour of the bone.

According to Bodian (1937), treatment with acid should impair the colour of collagen and thus eliminate structures such as Sharpey's fibres. This was confirmed by staining sub-synovial fibrous tissue with Bodian after treatment with 5 per cent acetic acid.

Decalcification has been discussed. Hurrell (1937), who used 6 per cent nitric acid to decalcify, mentioned that it was necessary to use alum to prevent swelling of the matrix caused by the acid. This, on the other hand, interferes with the impregnation of the soft tissue. Rowles & Brain (1959) used 10 per cent formic acid to decalcify sections of teeth before staining nerve fibres with silver, and Gough (1970) stated that 10 per cent formic acid could be used to decalcify before silver impregnation.

In the present study, 10 per cent formic acid was also used. As in recent years undecalcified sections have been used routinely in many laboratories, sections were also prepared for Bodian staining after they had been embedded in methyl methacrylate; however, as details in the bone matrix were not so distinct, we preferred the decalcified sections.

Only subchondral bone from human femoral heads has been examined in this work. In the sections from "normal" bone it was not possible, after eliminating several sources of error, to demonstrate nerve fibres in the bone matrix, but only in the marrow. In sections from osteoarthritic femoral heads it was obvious that there was an increased number of nerves, as they were related to the increased amount of vessels subchondrally in the bone marrow and in the granulation tissue.

From this preliminary work it may be concluded that there seems to be a greater number of nerves in osteoarthritic subchondral bone than in normal bone, and this is probably by analogy with the increased vascularization. The subject may be used for further studies, as the various causes of pain in osteoarthritis are still not quite elucidated.

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REFERENCES

Bodian, D. (1936) A new method for staining nerve fibers and nerve endings in mounted paraffin section. *Anat. Rec.* **65**, 89–97.

Bodian, D. (1937) The staining of paraffin sections of nervous tissue with activated protargol. The role of fixatives. *Anat. Rec.* **69**, 153–162.

Cooper, R. (1968) Nerves in cortical bone. *Science* **160**, 327–328.

De Castro, F. (1930) Quelques observations sur l'intervention du système nerveus autonome dans l'ossification. Innervation du tissu osseux et de la moelle osseuse. *Trav. Lab. Rech. Biol. de l'Univ. de Madrid* **23**, 215–244.

Gough, N. G. (1970) The staining of nerves in serial sections. *J. Anat. (Lond.)* **106**, 437–448.

Green, W. T., Garland, N. M., Eanes, D. E. & Sokoloff, L. (1970) Microradiographic study of the calcified layer of articular cartilage. *Arch. Path.* **90**, 151–158.

Ham, A. W. (1969) *Histology*. 6th ed. p. 388. Pitman Medical Publishing Co., Ltd., London.

Hurrell, D. J. (1937) The nerve supply of bone. *J. Anat. (Lond.)* **72**, 54–61.

Leeson, T. S. & Leeson, C. R. (1970) *Histology*. 2nd ed. pp. 121–123. W. B. Saunders Co. Philadelphia, London, Toronto.

Milgram, J. & Robinson, R. A. (1965) An electronmicroscopic demonstration of unmyelinated nerves in Haversian canals of the adult dog. *Bull. Johns Hopk. Hosp.* **117**, 163–173.

Miller, M. L. & McCuskey, R. S. (1973) Innervation of bone marrow in the rabbit. *Scand. J. Haemat.* **10**, 17–23.

Miller, M. R. & Kasahara, M. (1963) Observations on the innervation of human long bones. *Anat. Rec.* **145**, 12–23.

Rowles, S. L. & Brain, E. B. (1959) An improved silver method for staining nerve fibres in decalcified sections of teeth. *Arch. oral. Biol.* **2**, 64–68.

Sherman, M. S. (1963) The nerves of bone. *J. Bone Jt Surg.* **45**-A, 522–528.

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