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THE STAINING AND REFRACTIVE PROPERTIES OF BONE

C. J. DREYER

THE change in the refractive and staining properties of bone which occurs after pressure is applied to it was first described by Dreyer[1] who applied pressure to the skulls of dead Vervet monkeys by strapping elastic bands under tension around them. The refractive and staining properties of the inner and outer tables of the calvaria of untreated monkeys differ in many sections cut from these bones. The inner table is usually non-birefringent and stains green with Masson's stain, while the outer table tends to be birefringent and to stain brown. These differences are probably due to the muscular forces which act on it.

This paper is a report on further investigations on the effects of stress on bone and was undertaken to find out: (*a*) what changes occurred as a result of stress applied *in vitro* on bone; (*b*) if similar changes could be induced *in vivo* by functional stresses and (*c*) whether these changes were permanent or reversible.

METHOD AND MATERIAL

The skulls of Vervet monkeys and of Wistar strain rats were used for the *in vitro* experiments; the femurs of the rats were used for the *in vivo* investigations.

A. *In Vitro* SERIES

Rectangular sections were removed from the flat bones of the skull of the monkeys and rats. The sections were removed in pairs from similar sites on opposite sides of the skull and across a suture; one of each pair served as a control while the other was subjected to pressure. All specimens were taken during the morning on which the animal was sacrificed; they were cut out with a slowly rotating disc which was kept cool with a fine spray of water, and to which a minimum of pressure was applied.

The small notches in the bones on either side of the suture were cut to localize accurately the elastic bands and hence the direction of the force due to their tension.

After the rubber bands had been placed in position around the specimens, they, with their controls, were immersed in a normal saline solution. Stressed specimens with their controls were then removed after successive intervals of five minutes, half an hour, and 24 hours. Each specimen and its control were then fixed in five per cent formo-saline solution and later decalcified in five per cent nitric acid. Some sections were cut perpendicular to the external surface of the bone and others parallel to it. The sections were subsequently stained with several of the conventional stains as well as with Masson's Triple stain. Only the specimens stained with Masson's Triple stain are reported on in this communication.

B. *In Vivo* SERIES

In these experiments on the hind legs of Wistar rats attempts were made (*a*) to reduce and (*b*) to try and stop function of the leg. The following methods were adopted:

1. *Immobilization of the Limb*

In 20 male and female rats, four to six weeks old, one hind leg was fixed in the extended position by means of a plaster bandage extending from the toes to a collar round the abdomen. These animals were sacrificed at half-day intervals from the time of operation up to three days.

2. *Open Tenotomy of the Tendo Achillis of one Hind Leg*

The tendo achillis was sectioned in one hind leg of 20 male and female rats four to five weeks old. The animals were sacrificed at daily intervals from one to seven days after the operation.

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3. *Section of Portion of the Nerve Supply to the Leg*

The sciatic nerve was sectioned through an incision immediately posterior to the greater trochanter of the femur in 18 animals. These animals were sacrificed at daily intervals from one to 15 days.

In a further group of 20 rats one leg was completely denervated. These animals were sacrificed at daily intervals for the first week, and then at weekly intervals for two to three weeks after the operation.

4. *Fracture of the Femur*

This bone was exposed by an incision over its lateral aspect. In four rats the femurs were cut with scissors while in 23 others the diaphysis of the bone was divided by means of a rotating disc. In nine rats the fractured femurs were immobilized with intramedullary polytetrafluorethylene pins, in two with vitallium pins, while those of the remainder were not immobilized.

After the animals were sacrificed they were skinned and the hindquarters removed. To avoid applying stress during removal of the femurs the hindquarters were fixed in their entirety prior to removal of the bones. Subsequent treatment of the specimens was as described for the *in vitro* series.

RESULTS

A. *In Vitro* SERIES

The sections of the control specimens which were stained with Masson's stain have a characteristic staining pattern. The predominant colour of the section is green, but there are isolated areas which stain brown. The brown stained tissue has no uniform pattern but is more frequently found in the outer cortical plates.

The birefringence of the section varies according to the staining properties of the bone. The areas which stain green are non-birefringent, while those areas which stain brown are birefringent.

The pattern of staining is completely changed in the sections of the stressed specimens. The basic green stain remains as in the control specimens but the areas adjacent to and below the notch, which were subjected to the pressure of the rubber band, stain brown and are birefringent. These alterations in the staining properties and birefringence of the bone occurred after it had been stressed for only five minutes. Similar changes occurred in the specimens which were stressed for longer periods up to 24 hours.

B. *In Vivo* SERIES

The pattern of staining with Masson's stain is much less predictable in the femur than in the flat bones of the skull. The sections of the control femurs as well as others taken from untreated normal rats show marked variations in the brown and green staining areas.

The attempts to reduce function uniformly were not achieved. Some rats immobilized with plaster made no attempt at all to move around; others again subjected the immobilized leg to a great deal of stress in trying to rid it of the plaster or in using it to move about. The animals which had the tendo achillis sectioned started to use the leg two or three days after the operation and soon used the limb in an apparently normal way.

Although no uniform histological change is observed in the sections of limbs which were immobilized or on which a tenotomy was performed, some similarity in the staining patterns can be seen. The cross-section of the femurs of the animals which were sacrificed within the first two days show an increase and often a predominance of green staining bone. The sections of the femurs of animals sacrificed longer than two days after operation tend to show a predominance of brown staining bone. The overall changes are more marked in the series which had tenotomies than in those which were immobilized.

Section of the sciatic nerve caused some disuse of the limb but most animals soon regained the use of the leg. The results of these experiments were inconclusive but section of the entire nerve supply to one leg, together with immobilization to prevent trauma to the leg, did however alter the pattern of staining more frequently. The sections of femurs so obtained show a predominance of green staining bone when they are compared with similar sections of femurs taken from untreated rats. However, in this series the control specimen also shows an increase in the green staining tissue, sometimes equal to that of the

unstressed femur. These changes are evident after one week and are first seen in the perivascular bone and that portion of the bone nearest to the osteocytes. After two weeks these areas become confluent as the relative amount of green staining bone increases. The amount of green staining bone, however, seldom reaches the proportions commonly found in bones of the skull.

The change in the staining properties and birefringence of sections taken from the site of fracture of a femur is similar to those described before but takes longer to occur. The relative amounts of existing brown and green staining bone is often similar to that of the control groups during the first week but all the newly formed bone stains green and is non-birefringent. After the first week the existing bone tends to alter its staining characteristics and to stain predominantly green.

As union occurs and the animal regains the use of the limb, sections of the shaft at the site of healing stain predominantly brown. Sections of the newly formed bone, all of which at first stain green, stain increasingly brown as union proceeds between the two fragments.

DISCUSSION AND CONCLUSIONS

The results of these experiments show that the alteration in staining properties of bone due to applied stress occurs rapidly, and also that it takes place in the decalcified portion of the bone. In another series of experiments similar results were obtained when stainless steel wires were used instead of rubber bands to supply the force to the bone. It must therefore be concluded that the change in staining properties of the bone is not due to any chemical action of the rubber bands. MacConaill[2] suggests that these differences in the staining properties of bone are due to the stage of development of the collagen fibres. The mature fibres, he maintains, stain green while immature fibres stain brown. However, the *in vivo* experiments indicate that the staining properties of bone vary according to the functional activity of the bone. Thus if MacConaill's supposition is correct a conversion of mature into immature forms of collagen and *vice versa* takes place continuously in living bone.

The inconclusive results obtained after attempts to reduce activity in a limb seem to be due to a variety of factors, for the marked variation in the staining properties of the bones occurred not only in different animals but also in the right and left legs of the same animal. Certainly one factor responsible for the different staining patterns of the sections is the almost impossible task of controlling the degree of reduced function in a limb. However, when these colour changes in the sections did occur during these experiments, it was observed that it took from one to several days for the brown stain of the stressed bone to change to the green of unstressed bone.

Complete denervation and splintage of a limb alters its bone so that sections of it stain predominantly green. Similar changes, however, also occur in the bone of the control leg. The normal leg was used by these animals after the operation but never as actively as by those of the other reduced function groups. These results indicate that the nerve supply to a hind limb of a rat does not play a major part in the changes which may occur to the organic matrix of the bone of such a limb.

The changes in the staining properties and birefringence of sections cut through a healing fracture are the most consistent of the *in vivo* series. All newly formed bone stains green with Masson's stain and is non-birefringent. Sections of the shaft of a normal femur of the rat as well as sections taken immediately after fracture of the femur usually stain predominantly brown. Two to three weeks after the fracture the sections stain predominantly green. After the third week the sections begin to revert to the normal staining pattern. Similar changes in staining are seen in sections of the new bone of the callous as union takes place between the fragments. The change to the green stain in sections of the femur takes longer to occur after a fracture than it does after the other *in vivo* experiments on that bone. This delay in the change of staining properties of sections of the bone may be due to a change in the blood supply to the limb as a result of the operation performed on it or due to the stress applied to the bone when it was fractured.

From these experiments it may be concluded that pressure to bone inside, as well as outside, the living body, alters its physical properties.

The relationship between the morphological pattern of a bone and its functional activity has been fully discussed by Murray[3], Weinmann and Sicher[6] and others, while the response of the bone in a tooth socket to physiological and other forces has been described by Reitan [4, 5]. It appears that if the general metabolism of the animal is normal, decreased functional activity of a bodily part will tend to reduce the bone of the affected part, while increased functional activity will tend to stimulate new bone formation in the affected bone.

The manner in which functional activity produces osteogenic stimuli is still obscure; vascular and nervous factors play a part in the process but they are not the only ones. The results of the experiments described above may shed some light on this problem, for it is clear from them that pressure to vital or non-vital bone alters the organic matrix. This alteration from the green to the brown staining matrix may be an "activation" process which is the result of physical changes which accompany functional activity. The experiments also show that the changes which occur in bone as a result of stress take place rapidly. Short, but often repeated functional stimuli, which may not be of sufficient duration to produce a cellular response, may thus in effect be stored in the organic matrix as longer acting stimuli which are necessary for the cellular changes which accompany osteogenesis. The conversion of "activated" matrix to the "resting" form takes days to occur, and since it is first seen in the bone immediately around the osteocytes and blood vessels it may be associated with a metabolic process.

The matrix of newly formed bone is always in the resting form. This in itself could act as a limiting mechanism for further osteogenesis as the stimuli from the "activated" matrix become more and more removed from the periosteum and endosteum as new bone is formed.

The matrix of bone which has been stressed, gradually changes to the "resting" form when the stress is removed. The bone which stains green with Masson's stain does not stimulate further osteoblastic response and activity. This portion of the bone will show no visible change or will slowly be removed.

This mechanism for the stimulation of local osteogenesis does explain how the gross internal structure of a bone becomes orientated to withstand the forces normally applied to it. The limitation of periosteal bone growth may also be associated with the direct pressure exerted on this part of the bone by the muscles and other overlying soft tissue.

SUMMARY

1. It has been shown that the birefringence and staining properties of the organic matrix of fresh undecalcified bone alters when direct pressure is applied to it. *In vitro* experiments have shown that the changes occur within minutes after the application of the force.

2. Reversible changes have been demonstrated in the femur or rats after immobilization, tenotomy, denervation and fracture of the hind leg.

3. The possible role of these changes in the organic matrix in relation to osteogenesis is discussed.

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