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Vital staining of calcifying tissue in animals with Tetracycline, Calcein green and Xylenol orange

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Introduction

In the study of growth, turnover and repair of bone and dentin, fluorescent labels have been used as tissue-time marker for more than thirty years (*Milch et al.* 1957). The labels deposit in mineralizing tissue and the labeled site fluoresce under ultraviolet light.

Treharne & Brighton (1979) claimed that unlabelled bone also will exhibit fluorescence, only not as brightly as newly formed bone. To minimize this phenomenon methods involving double or triple labellings have been developed. However, bone remodelling taking place in the interval between the administration of the various markers cannot be assessed by these methods (*Schwarts & Recker* 1982).

Our purpose was to investigate bone formation in untouched cortical and cancellous bone and in bone, which had been subjected to surgery (i.e. intramedullary reaming and filling). Bone labelling was performed by a constant administration of Tetracycline, and the bone formation rate was quantified by means of Calcein green and Xylenol orange, each administered in two doses, given at weekly intervals.

Material

Vital staining was done with Tetracycline, administered as tablets given orally, and Calcein green and Xylenol orange given intravenously.

Tetracyclines form chalates with calcium ions and other divalente cations and are incorporated into newly formed calcified bone.

Calcein green powder (Sigma no C-0875, St. Louis, USA) was sterilized by irradiation and dissolved in isotonic bicarbonate in a concentration of 8 mg/ml. The solution had a pH of 7.5 and was administered at a rate of 10–20 ml/min. Calcein green contains in fluoran-imino-diacetic acid complex, which appears green in ultraviolet light when combined with calcium.

Xylenol orange powder (Sigma no X-3500, St. Louis, USA) was sterilized by irradiation and dissolved in isotonic bicarbonate to a concentration of 30 mg/ml. The solution had a pH of 7.4 and was given intravenously at a rate of 10–30 ml/min. Xylenol orange is a divalent metal ion. It forms complexes with calcifying tissue and appears orange red in ultraviolet light.

Five adult mongrel dogs (28–38 kg) were used. All received a standard canine diet at least 2 weeks before the surgery and kept in indoors cages till the end of the experiment. One was a male and 4 were female.

Bone surgery was done on the tibia. Intramedullary reaming of the tibial diaphysis was performed and the cavity was filled to prevent endosteal revascularization. As a filling material we used bone-wax, which is considered inert to bone (*Howard & Kelley* 1969, *Rodriques & Carvalho* 1983) and becomes paste-like when heated to a temperature of 42 degrees, thus allowing application with a cement gun. To make the bone-wax radiopaque 15 w/w per cent zirconiumoxide was added.

Method

The operation was performed through the patella ligament. The tibial medullary cavity was reamed with handdriven reamers to a diameter of 8–9 mm, curretted, brushed and flushed with saline. The cavity was filled with bone-wax from the distal end by means of an injection syringe, thin enough to pass all the way down into the medullary cavity.

Postoperative radiographs confirmed adequate filling.
From the first day of the investigation all dogs received a daily dose of 20 mg/kg of Tetracycline orally by a veterinarian.
Calcein green (15 mg/kg) was given intravenously on day 21 and 28 (3 and 4 weeks) respectively and Xylenol orange (80 mg/kg) was given on day 75 and 82 (11 and 12 weeks).
The animals were killed on day 84 (12 weeks).
In order to investigate differences in remodelling of cancellous and cortical bone, biopsies (1×1 cm) were taken from the iliac crest and the radius in three dogs. The bone reaction to intramedullary reaming and obturation was investigated in specimens harvested from the mid-diaphysis of the tibia in all five dogs. The specimens were left immersed in a 10 per cent solution of neutral buffered formaldehyde for three weeks and dehydrated with ethanol (60–99 per cent) with 24 hours between the steps. The undecalcified bone was embedded in epoxy resin. Sectioning and grinding were performed (Exact-Cutting-Grinding system, Germany) producing slides of a thickness of approximately 50 microns (*Jensen et al.* 1991). Three sections from each biopsy were studied under a Leitz Orthoplan microscope (Germany) using a D filter block in an epi-illuminator, and photographed with an Orthomat camera using an Ektachrome 160 film (Kodak, U.S.A.) at a low power field (25 × magnification). To enhance the red fluorescence of Xylenol orange, double exposures with D and N2 filter was done. Four to six photographs were taken of each section. The photographs were projected onto a screen with a grid measuring 100×70 cm with a total number of 7000 intersections. In cancellous bone the percentage of the relative labelling of surfaces of the cancellous bone was calculated as the length of labelled surface of the trabecular as measured with a map measure in relation to the total surface lenght of the trabecular. In cortical bone the percentage of intersections corresponding with Tetracycline labelled bone was calculated in relation to the total cortical bone being intersected.
Qualitative evaluations of bone labelled with Calcein green and Xylenol orange were based on images at 100 magnification. The maximal distance between the double labelling bands was measured and recorded as the bone formation rate at 4 and 12 weeks (*Melsen & Mosekilde* 1978). The value was uncorrected for oblique cutting.

Results

No adverse effects were observed following intravenously infusion of Calcein green, but one dog vomited after a rapid infusion of Xylenol orange.
The administration of Xylenol orange entailed a purple discolorations of the gingivae and conjunctivae, which lasted for 24–36 hours and within half an hour the urine become dark purple.

Cancellous bone

A high bone turnover was encountered in cancellous bone from the iliac crest as respectively 38, 40 and 43 per cent of the grid points intersected with Tetracycline labelled trabeculars.
Eighty-eight per cent of the surface lenght of of the trabecular was labelled during the 12 weeks labelling period. Double labelling with Calcein green was located within the Tetracycline labelled bone and corresponded well with the time of administration. A similar labelling result was encountered with Xylenol orange, but the bands were not quite as bright. The maximum bone formation was ninety microns per week, when measured as the distance between two Calcein green lines or two Xylenol orange lines.

Cortical bone

Cortical bone from the radius showed only minor bone turnover with labelling of only 0.4, 0.8 and 1.8 per cent of the intersections. The labelled osteons were all located in the periphery of the bone.

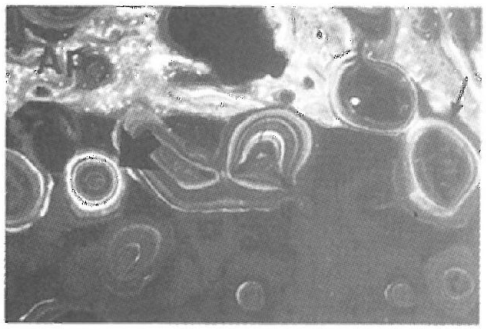

Figure 1. Section from border between the cortex and its apposition, labelled with daily Tetracycline in 12 weeks, Calcein green infusion at 3 and 4 weeks (short, thick arrow) and Xylenol orange infusion at (long, thin arrow). Areas marked with a C illustrated unremodelled cortex, AP is newly formed bone apposition. × 100.

Calcein green and Xylenol orange double labelling were all located within the Tetracycline labellings. The maximum rate of bone formation was sixty microns per week.

Intramedullary reaming and filling with bone-wax

In the diaphysis of the tibial bone a median of 26 per cent (range 21–28) of the cortex was labelled with Tetracycline after 12 weeks (Figure 1).

Double Calcein green bands were located in the outer one third of the cortex, whereas single bands were observed in the central third, indicating that the new bone formation had started peripherally at 3 weeks, but delayed to between the third and fourth week in the central part of the cortex. Double Xylenol orange bands were seen in both the middle and the outer parts of the cortex, but were rarely seen in the inner part, indicating that the inner third of the cortex started bone formation at approximately 12 weeks.

The maximum bone formation rate was 130 microns per week, assessed at both 3 to 4 and 11 to 12 weeks.

Discussion

If bone remodelling were in equilibrium, i.e. bone resorption equalled new bone formation in the time interval under investigation, a method with double labelling might be adequate (*Frost* 1969). Imbalance between bone resorption and bone formation will, however, make the assessment of bone formation difficult. A method involving a daily administration of one marker (Tetracycline) and periodic infusions of other markers (Calcein green and Xylenol orange) entails the labelling of all newly formed bone by Tetracycline, whereas the other markers can be seen as bands within the Tetracycline labelled bone.

Several markers have been used for the assessments of bone formation.

Tetracycline has been used in vital bone staining for more than 20 years (*Harris et al.* 1968). However, some authors (*Treharne & Brighton* 1979) claim that even dead bone takes up Tetracycline and fluorescence, and despite the fact that the intensity of dead bone is minimal compared with that of newly calcified bone, the phenomenon is likely to render assessments ambiguous. By our method bands of the infused dyes will be visible within the bone labelled with the constantly administrated dye, thus facilitating the evaluation of Tetracycline. Both Calcein green and Xylenol orange are well tolerated by various animals (*Suzuki & Matthews 1966, Olerud & Lorenzi* 1970, *Rahn & Perren* 1972), although cases of temporary hyperventilation and muscular hypertonia have been reported after rapid infusion of Xylenol orange (*Rahn & Perren* 1972).

Rahn & Perren (1972) reported that the fluorescence of Xylenolorange fades less easily than Calcein blue, hematoporphyrin, Tetracyclines and fluoresceins, but we found the intensity of Xylenol orange to be less pronounced than that of Calcein green. This could be due to the simultaneous administration of Tetracycline both competing for calcium ions bindings.

In our experiment the intensity of Calcein green was found to be high, and its binding capacity may differ from Tetracycline and Xylenol orange.

Other agents, such as different kinds of Te-

tracycline or Calcein blue may be used for staining. Alizarin red and Alizarin Complexon have been widely used, but these dyes are toxic and may inhibit bone formation (*Harris et al.* 1964).

Our findings of remodelling in untouched cortical bone are in accordance with those of other authors (*Harris et al.* 1968), who report annual growth rates of 3 to 14 per cent with regard to the radius.

We found that the constant administration of Tetracycline combined with labelling bands of Calcein green and Xylenol orange was a valuable method of assessing bone formation patterns, particularly in tubular bone exposed to a surgical trauma. The error of autofluorescence (*Treharne & Brighton* 1979) of bone is eliminated, as the Tetracycline band contains the bands originating from the periodically administrated labels.

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Summary

Vital staining of calcifying tissue has been reported in previous studies, but such investigations have involved either a constant labelling with one dye, or double or triple labellings with one or more dyes. In the present study five adult dogs had a constant oral administration of Tetracyclines and two infusions of Calcein green and Xylenol orange, each administered twice after 3 and 4 weeks and 11 and 12 weeks, respectively. This method caused all newly formed bone to be marked with Tetracycline. Qualitative information can be obtained by observing the location of the Calcein green bands and the Xylenol orange bands. The bone formation rate can be determined by measuring the distance between labelling bands of Calcein green and Xylenol orange. We found this method of combined labelling to be of value in the investigation of bone remodelling, particularly in cases of surgical trauma.

Resume

Vital farvning af kalkholdigt væv er tidligere rapporteret, men er gjort med enten konstant farvning med et farvestof eller med dobbelt eller tripel farvning med et eller flere farvestoffer. I dette arbejde har vi anvendt en metode, hvor Tetracycline gives daglig per os og derudover gives der infusioner med Calcein green og Xylenol orange. Denne metode vil mærke alt nydannet knogle med Tetracycline. Kvalitative informationer kan bedømmes ved lokalisation af Calcein green og Xylenol orange. Knogledannelseshastigheden kan bedømmes ved hjælp af afstanden mellem de farvede linier af Calcein green og Xylenol orange. Fem voksne hunde indgik i forsøget og vi finder, at denne metode med kombination af farvninger er af værdi, specielt efter kirurgiske indgreb.

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