

Effects of mellitic acid (MA) and sodium fluoride (NaF) on the histological appearance of murine fetal tibiae cultured *in vitro**

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Summary. The aim of this study was to develop a standardized image analysis method for localization and quantitative measurement of calcified structures of murine fetal tibiae cultured *in vitro* as a completion and verification of previous biochemical studies. The calcified structures of bone stained by von Kossa silver technique and the epiphyseal cartilages showing intensive metachromasia with toluidine-blue staining were converted with grey-value window programs and afterwards the areas of the selected structures were measured. The histomorphological investigations showed that the murine tibiae, incubated for a period of 6 days in a medium with addition of 5 nmol mellitic acid, showed both a significant reduction of calcium deposits and an increase of epiphyseal intercellular cartilage matrix. The tibiae incubated in a medium with addition of 0.5 mmol sodium fluoride significantly showed an increase of calcium deposits in the thickened lamellae of the compacta. These histomorphological results confirm previous biochemical studies.

Key words: Mellitic acid (MA) - Sodium fluoride (NaF) - Murine fetal tibiae - Histological appearance - Image analysis

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Introduction

Histochemical techniques in combination with microphotometry are commonly used for the localization and quantitative measurements of calcified structures of bone. However, in the past, investigators have predominantly used stereological methods to collect data of bone resorption and mineralization (Schenk, 1967). Our interest was to develop a low cost image analysis system and a standardized method for quantitation of bone matrix as completion and verification of previous biochemical studies (Krisinger et al., 1985).

Materials and methods

Freshly excised murine tibiae on the 19th of pregnancy, tibiae incubated for a period of 6 days in the incubation medium described by Krisinger (1985) with the addition of beta-glycerophosphate serving as controls, and tibiae incubated for 6 days in the medium with the addition of 5 mmol MA or 0.5 mmol NaF respectively were histomorphologically and morphometrically investigated.

For histological investigations, the non-decalcified murine embryonic tibiae of mice were fixed in buffered formalin solution according to Lillie (1954). Thereafter, the bone tissue was embedded in Technovit 7100 (2-hydroxyethyl-methacrylat). Sections of the preparation were cut in 4 μ with a Leitz microtome 1401. The following dyes were used: Giemsa's stain and von Kossa's stain (silver impregnation, to stain calcium deposits) in combination with toluidine-blue counterstaining.

Image analysis system: Leitz-microscope Orthoplan, Apple II microcomputer with video-interface, Hitachi-videocamera VKC 2000E, two floppy-drives, Epson MX-80 printer and graphic-tablet (Fig. 1).

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Two video-scan modes were used:

1) 2-grey-value mode (280*192 pixels) to measure the calcified areas of trabeculae, total area of bone (after the use of the "fill"-function) and the metachromatic epiphyseal cartilage areas (after subtraction of diaphysis).

2) 16-grey-value mode (140*96 pixels) to differentiate between percentual grey value distribution of bone components. The image-analysis system was aligned for the best brightness and contrast. Optimal alignment was assessed when the digitized picture contained 14 out of 16 possible grey-values including the grey-value 0 and 15 (the colours black and white) which were displayed by histogram of percentual distribution.

Basic and machine-language programs were developed to count areas, to determine percentual grey-value distribution (grey level histogram) and to set the threshold (grey-value windows) of the selected bone component to be investigated. The digitized pictures were stored on diskettes. Hardcopies of digitized pictures were made to compare with microphotographs.

Statistics

The median of the counted total area of bone, of cartilages and the area of mineralization and the percentual difference of reduction or increase of areas were calculated. The results were tested for significance using the Mann-Whitney U-Test.

Results

1. Murine embryonic tibiae freshly excised on the 19th day of gestation

The epiphyseal cartilage was found to show an increased proliferation of cartilage matrix, which had an intensive metachromatic staining with toluidine-blue. The chondrocytes of the epiphysis had a partially vacuolated, partially foamy or also slightly shrunken cytoplasm. A relatively well-developed bone cuff surrounded the diaphyseal shaft. The mineralization of the collagenous bone matrix showed slight variation. In the primary zone of calcification (zona aperta) cell degenerative processes existed as important signs of transformation. In the sinusoidal capillaries of the primary medullous space, neutrophilic granulocytes, erythrocytes, mononucleated cells and unidentifiable mesenchymal cells could be observed. The length of tibiae was at this time 3.6 to 4.1 mm.

2. Murine fetal tibiae incubated for a period of 6 days in a medium with the addition of beta-glycerophosphate (controls, Figs. 2,5,6)

The epiphyseal cartilage showed in comparison to unincubated tibiae a continued proliferation of epiphyseal intercellular cartilage matrix which showed intensive metachromasia. In the primary medullous space only a few neutrophilic granulocytes and no erythrocytes could be demonstrated. The number of fibroblasts, mononuclear

cells and cells with karyopycnosis in the primary medullous space increased. The number of unidentifiable mesenchymal cells seemed to be unchanged.

3. Murine fetal tibiae incubated for a period of 6 days in a medium with the addition of 5 mmol mellitic acid (Figs. 3,7,8).

The epiphyseal cartilage showed in comparison to incubated tibiae a significantly increased proliferation of epiphyseal intercellular cartilage matrix which showed intensive metachromasia. Neither neutrophilic granulocytes nor erythrocytes could be demonstrated in the primary medullous space. The number of fibroblasts and mononuclear cells in the primary medullous space decreased. The number of unidentifiable mesenchymal cells seemed to be unchanged. The calcified lamellae of the compacta, reduced in number, were thinned. Under the influence of 5 mmol MA in the medium a significant increase ($\alpha=0.05$) of cartilage matrix of 29% in comparison to controls could be demonstrated. Furthermore MA induced a significant reduction of 23% ($\alpha=0.05$) of calcium deposits in bone.

4. Murine fetal tibiae incubated for a period of 6 days in a medium with the addition of 0.5 mmol sodium fluoride (Fig. 4)

The epiphyseal cartilage showed in comparison to incubated tibiae a slight but not significant decrease of area. Neither neutrophilic granulocytes nor erythrocytes could be detected in the primary medullous space. The number of unidentifiable mesenchymal cells seemed to be reduced. The murine tibiae showed a marked increase of calcium deposits in the thickened lamellae of the compacta. The total bone area was not significantly affected by NaF.

Table 1. Influence of mellitic acid and sodium fluoride on the morphological parameters of murine embryonic tibiae cultured in vitro.

Group	length of tibiae (mm)	% area of epiphyseal cartilages	% area of calcification
Controls	4.9-5.2	59	17
5 mmol Mellitic Acid	4.9-5.6	72	11
0.5 mmol Sodium Fluoride	4.7-5.1	57	29

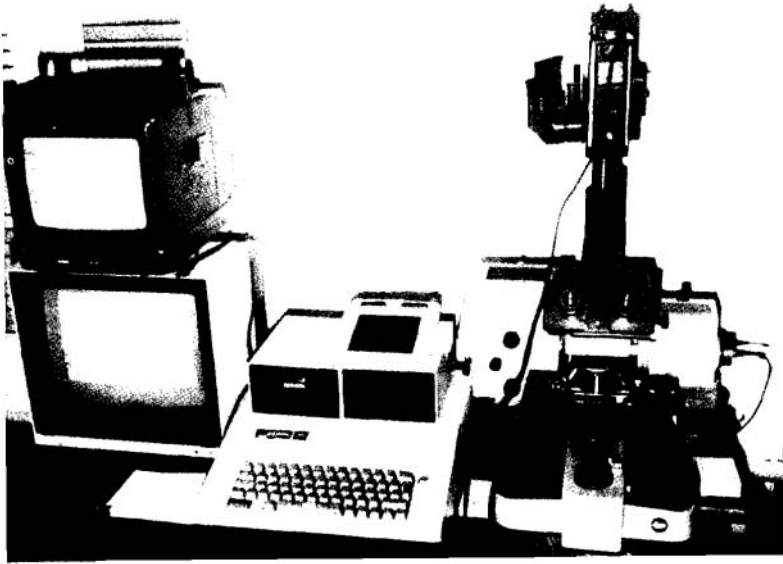


Fig. 1. Image-Analysis-System: Apple IIe microcomputer with video-interface, 2 floppy drives, graphic tablet and videocamera (Hitachi VKC2000E).

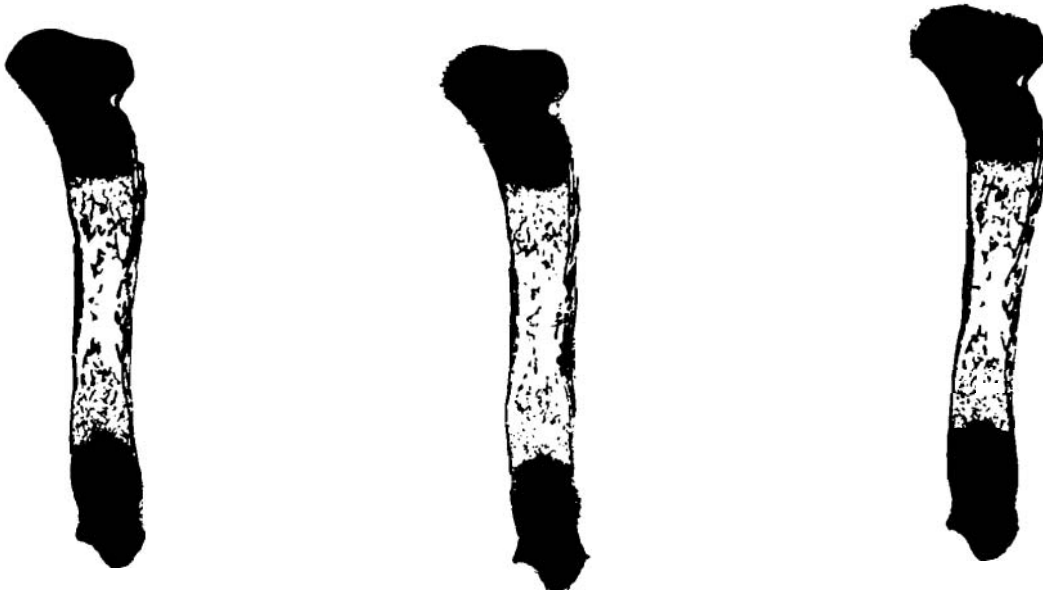


Fig. 2. Murine tibia cultured in vitro, 4 μ serial sections, control group, von KOSSA's silver impregnation with Toluidine-blue counterstaining.



Fig. 3. Murine tibia cultured in vitro, 4 μ serial sections, Mellitic acid (5mmol) incubated, von KOSSA's silver impregnation with Toluidine-blue counterstaining.



Fig. 4. Murine tibia cultured in vitro, 4 μ section, Sodium Fluoride (0.5mmol) incubated. von Kossa's silver impregnation with Toluidine blue counterstaining.



Fig. 5. Murine tibia cultured in vitro, control group, digitized section, scan mode 1 (280*192 pixels).



Fig. 6. Murine tibia cultured in vitro, control group, digitized section, scan mode 1 (280*192 pixels) after subtraction of diaphysis, containing only the part of the epiphyseal cartilages.



Fig. 7. Murine tibia cultured in vitro, Mellitic acid (5mmol) incubated, digitized section, scan mode 1 (280*192 pixels).



Fig. 8. Murine tibia cultured in vitro, Mellitic acid (5mmol) incubated, digitized section, scan mode 1 (280*192 pixels) after subtraction of diaphysis, containing only the part of the epiphyseal cartilages.

Discussion

These studies confirm that computer-based image analysis is favourable to quantitate the histochemical labeling of bone components. The Basic and machine-language programs used for the counting of areas and grey level windows require only a few seconds to run. The calcified structure in bone stained by von Kossa silver technique and the epiphyseal cartilages showing intensive metachromasia with the toluidine-blue staining could be counted in the 2-grey level mode with a resolution of

280*192 pixels. This resolution seemed to be sufficient for estimation of the calcification index of bone, as the comparison of original microphotographs and digitized pictures showed a good resemblance of tissue structure. The results obtained with the 16-grey level mode showed a good correlation to the results of the 2-grey level mode.

The histomorphological investigations showed that the incubation medium containing beta-glycerophosphate was sufficient for investigation of the growth of cartilage matrix and of mineralization, as there were neither signs of necrosis nor destruction in the osteoid or in epiphyseal cartilages

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in comparison to freshly excised tibiae. In correlation with biochemical results the murine tibiae, incubated for a period of 6 days in a medium with addition of 5 mmol **MA**, showed both a significant reduction of calcium deposits and an increase of epiphyseal intercellular cartilage matrix. The total bone area of tibiae incubated with the addition of **MA** was significantly increased which only was the result of an increase of epiphyseal intercellular cartilage matrix, while the area of diaphysis remained unchanged (see Table 1). The reduction of calcium deposits in **MA** incubated tibiae was accompanied by a reduction and thinning of lammellae of the compacta. The tibiae incubated in a medium with addition of 0.5 mmol **NaF** significantly showed an increase of calcium deposits in the thickened lammellae of the compacta. However, the total bone area and area of epiphyseal cartilages were not affected by **NaF**. These histomorphological results confirm previous biochemical studies of Krisinger et al. (1985).

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