Histochemical modifications induced « in vitro » by electromagnetic stimulation of growing bone tissues

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The histochemical modifications induced on a mouse limb bud culture system by electromagnetic stimulation have been investigated using mucopolysaccharide (MPS) staining methods.

An increase in the amount of MPS with cartilage has been observed. This difference is more obvious on the more distal segments of the limb, i.e. on the youngest structures.

These observations are correlated with some modifications observed in the enzymatic activities of acid phosphatase and of β glucuronidase.

I. Introduction

Previous experiments on the effects of electromagnetic stimulation on *in vitro* cultivated limbs have revealed some morphological modification such a thicker proliferative layer of chondrocytes, a better alignment of the trabeculae and a better configuration of the joint cartilage (Rooze M., Hinsenkamp, 1979, 1982).

As such, morphological changes are most probably related to metabolic changes, some histochemical features of the cartilaginous tissue have been investigated.

Two different groups of techniques have been comparatively applied to normal and stimulated tissues, demonstrating respectively mucopolysaccharides and two acid hydrolases.

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II. Material and methods

The fore- and hindlimb buds of mouse embryos obtained by Caesarian sections have been cultivated in a semisynthetic culture medium during 4 to 6 days. The procedure of sampling and culture is the same as in our previous experiments. The samples have been fixed in the Serra's fixative (95° alcohol, formalin, acetic acid, 6 : 3 : 1). Some have been fixed 10 minutes in Baker's fixative ($CaCl_2$ 1 % in Locke's physiological solution), embedded into OCT medium and frozen at — 20°C. The first type of fixation allows the histochemical demonstration of mucopolysaccharides in paraffin sections ; the second allows enzymatic detection in cryostat sections.

Three different staining methods were used for the demonstration of mucopolysaccharides : the metachromatic staining of toluidine blue at pH 3.8, the Rhinehart and Abul-Haj (1951) modification of Hale's method with colloidal iron and the Alcian blue staining at pH 1 and 2.5.

The enzymatic activity of β-glucuronidase and acid phosphatase have been demonstrated according to the methods of Burnstone.

III. Results

1. *Toluidine blue.*

The purple metachromasia of toluidine. Blue at pH 3.8 characterize the MPS having strong carboxylic groups or having sulfuric groups partially free. The different joints of the same forelimbs were studied. The purple metachromasia is not yet very strong at the considered developmental stages (14 day embryos). There is no difference in the amount and quality of MPS present in the head of the humerus. Even at high magnifications, little modification can be found and they have no statistical significance.

The radio humeral joint in the control limbs shows a decrease in the metachromasia as well as a thinner germinative zone in the metaphyseal plate, confirming our previous morphological observations. The ulna compartment of the elbow shows the same differences as the radial one. For both joints of the elbow the shape of the cartilage looks more normal in the stimulated limbs. There is a well formed joint cavity. The same features can be found in the more distal joints of the forelimbs. The distal segments of the limb reveal more variability in the intensity of

the staining. It is important to note that we have found the same proximodistal gradient as that observed in the structural changes. This is specially well illustrated in the distal extremity of the radius (fig. 1), the carpal bones (fig. 2) and the third phalanx (fig. 3).

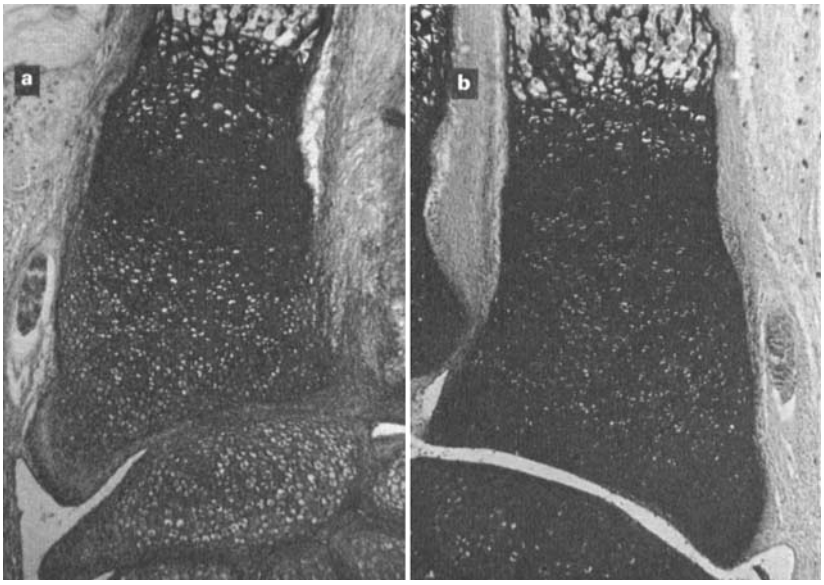


FIG. 1. — Toluidine blue, distal epiphysis of the radius (a : control, b : activated). Differences of staining between the two samples and better shaped joint surfaces in the activated limb.

TABLE I

Toluidine blue

	Total number of pairs	*+ differences*	*No difference*	*Significant*
Head of humerus	9	1	8	NS
Elbow joint	9	3	6	NS
Distal epiphysis of the radius .	9	7	2	+ ($\alpha = 0.05$)
Distal epiphysis of the ulna . .	9	5	4	NS
Carpal bones	9	7	2	+ ($\alpha = 0.05$)
First phalanx of the finger . .	7	6	1	+ ($\alpha = 0.05$)

In conclusion, the observations reveal a proximodistal gradient of affinity for the metachromatic dye (revealed by the toluidine blue). The difference of staining between control and stimulated limbs suggests

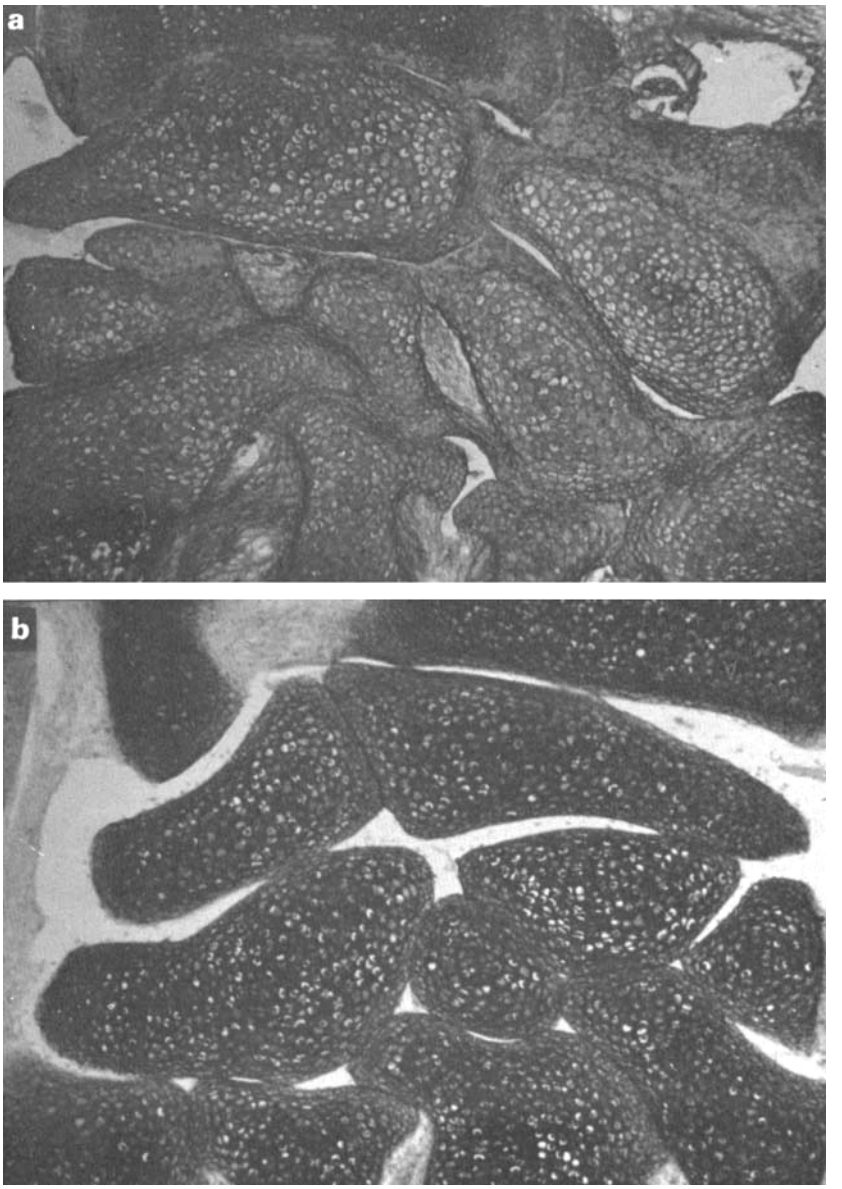


FIG. 2. — Toludine blue, the carpal bones (a : control, b : activated). Same observations as in figure 1 but the differences are more pronounced.

that the glycoproteins containing carboxylic or sulfuric groups are in lower amount than in the control cartilages. Table I gives the statistical analysis of these observations.

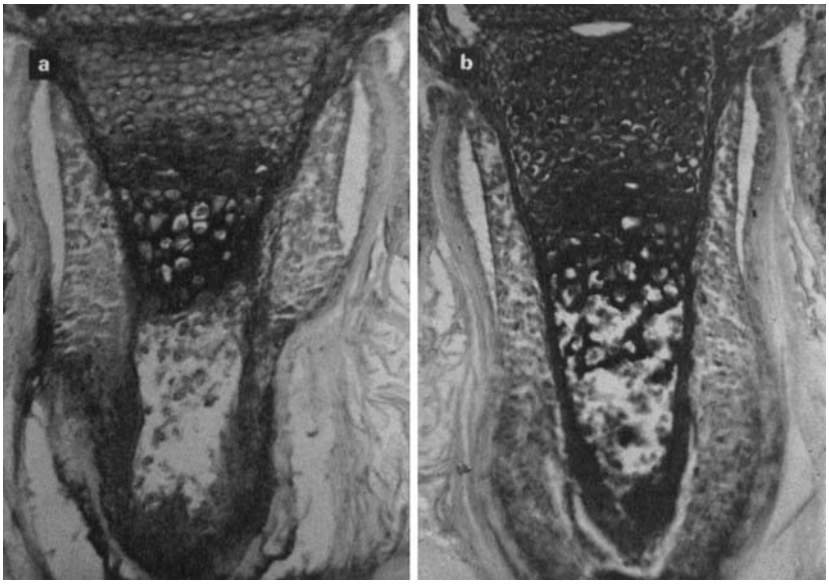


FIG. 3. — Toluidine blue, third phalanx (a : control, b : activated). Same remarks as in figure 2.

2. **Hale's method.**

The blue staining obtained by the colloïdal iron method is observed in areas containing all sorts of acid MPS. A section through the head of the humerus of stimulated samples shows an increase in the blue staining. This increase is mainly observed near the metaphyseal plate and at the periphery of the head of the humerus (fig. 4).

In the centrum of the humeral head of control limbs, hypertrophic chondrocytes announcing the approaching epiphyseal ossification are surrounded by deeply stained matrix.

The same difference have been found still more obvious in the other joints of the limb according to a proximodistal gradient (fig. 4 and 5). They all confirm the increase in acid MPS in the stimulated samples. The difference observed after the Hale's method between stimulated and control limbs appears to be more obvious than after staining with toluidine blue.

Table II gives the statistical analysis of these observations.

TABLE II

Colloidal iron

	Total number of pairs	*+ differences*	*No difference*	*Significant*
Head of humerus	8	4	4	NS
Elbow joint	8	5	3	NS
Distal epiphysis of the radius .	10	9	1	+ ($\alpha = 0.01$)
Distal epiphysis of the ulna . .	10	7	3	+ ($\alpha = 0.05$)
Carpal bones	10	9	1	+ ($\alpha = 0.01$)
First phalanx of the finger . .	9	8	1	+ ($\alpha = 0.02$)

3. **Alcian blue method.**

This method using the staining of the acid MPS by the Alcian Blue at two different pH values is more specific. At pH 1 only the MPS having sulfuric groups are revealed. At pH 2.5, the MPS having carboxylic groups are stained in blue. The application of these methods is interesting to precise if the relative decrease in acid MPS in the control limbs results in a loosening or an absence of synthese of sulfated or non sulfated MPS.

The two techniques have revealed a lower intensity in the staining of the control limbs but the differences are not very important. However at pH 1 the amount of sulfated MPS in both samples is very low.

It means that the decrease of staining is due more to a quantitative decrease in MPS acid than to a specific decrease in the amount of sulfated or carboxylated MPS.

IV. **Histoenzymatic results**

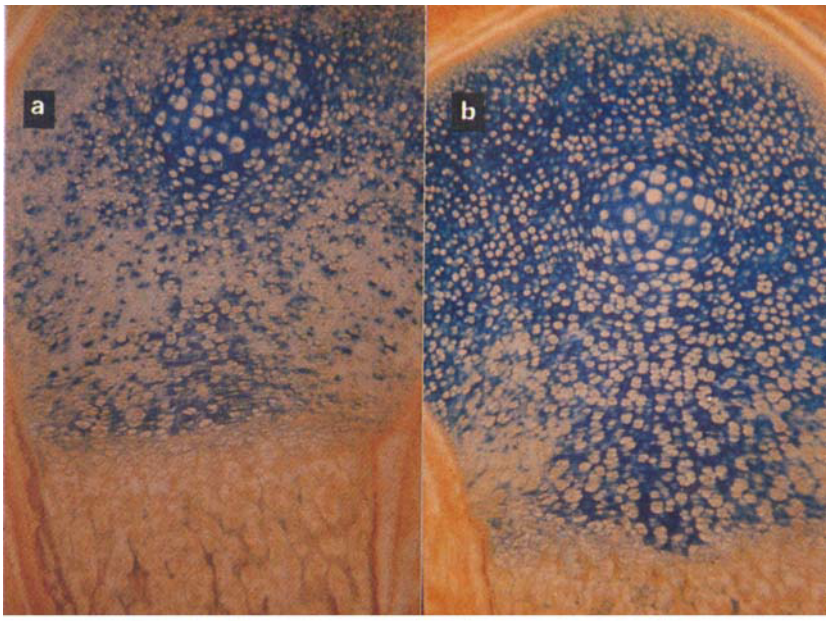
The histochemical results obtained let us presume that the qualitative and quantitative differences observed between the stimulated and the control samples are a reflection of modifications of the metabolisms leading to bone differentiation.

The following observations are only our preliminary results and no statistical analysis has been made.

No difference has been observed between stimulated and control samples in the activity of alkaline phosphatase, AMPase, ATPase and leucinaminopepidase

However the reactions of the β-glucorunidase and of the acid phosphatase have revealed some differences.

FIG. 5.



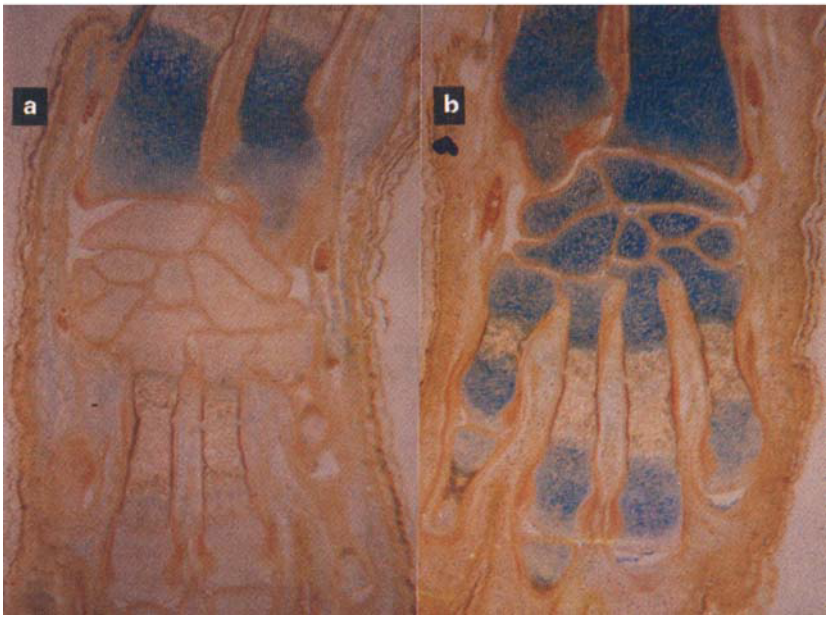


FIG. 4.

FIG. 4. — Hale's method (colloidal iron), caput humeri (a : control, b : activated). Differences of staining between a and b (see text). The serial cartilage is better formed in the stimulated samples. The hypertrophic chondrocytes are well stained in both samples.

FIG. 5. — Hale's method (colloidal iron), distal epiphysises of the forearm, carpal and metacarpal bones (a : control, b : activated). More pronounced differences between the samples on the more distal skeletal segments of the limb.

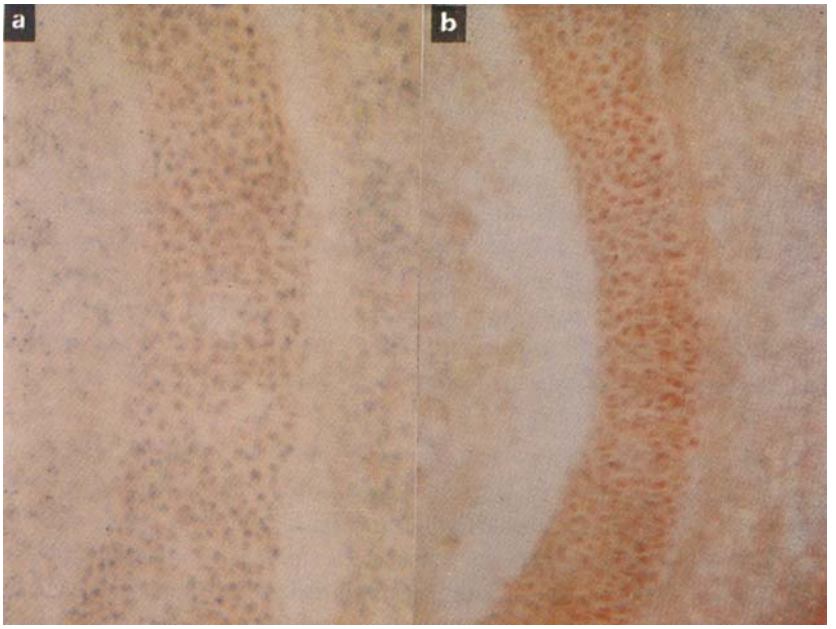


FIG. 6. — β glucuronidase revealed on frozen sections of the clavicula. Increased activity in the stimulated bone (a : control, b : activated).

The activity of the β-glucorunidase is increased in the stimulated limbs (fig. 6).

The acid phosphatase activity is lower in the stimulated series. An increased activity is observed in the middle portion of the cartilaginous blastema.

However, these results are just some « indices » of histochemical modifications of the cellular metabolism due to the influence of the electromagnetic stimulation.

V. **Conclusion**

It was previously shown (Rooze, 1979 ; Hinsenkamp, 1982) that electromagnetic stimulation of developing mouse limb buds grown *in vitro* induces macro-and microscopic changes in skeletal tissues. Even though they were not statistically conclusive, the results obtained showed that the treatment obviously influences the *in vitro* behaviour of differentiating tissues. The present work investigates comparatively the histochemical content of treated and control tissues in mucopolysaccharides (MPS).

Typical differences were demonstrated and they appear to be increasingly obvious as more distal segmental skeletal pieces are observed. Whatever the technique used (toluidine blue, colloidal iron, alcian blue), the epiphyseal content in carboxylated or sulphated acid MPS is higher in the stimulated limbs than in the control group. A more obvious difference, however, was observed after staining with the modified Hale's method using colloidal iron.

Two different hypotheses can be suggested to explain the observed modification. It may first be assumed that the culturing conditions would somehow be responsible for a partial loss of MPS synthesized *in vitro* in the control limb buds, a phenomenon which would be prevented by electric stimulation in the treated limbs. According to the second hypothesis, the electric treatment would modify the metabolism of chondrocytes in such a way that the matrix content in acid MPS would be higher or qualitatively different.

Histoenzymatic investigations have been planned in order to try to decide between these two interpretations ; preliminary observation which need confirmation have revealed some differences in the intensity of acid phosphatase and β-glucorunidase reactions between treated and control limb buds. It may thus be suggested that electromagnetic stimulation induces some unknown cellular change which results in a modification of the matrix content in acid MPS. The cellular changes might somehow

affect the membrane permeability or the biochemical mechanism of transmembranous transfer.

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