Morphological effect of electromagnetic stimulation on the skeleton of fetal or newborn mice

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In vitro *cultures of limbs from fetuses or newborn mice allow for a strict control of the experimental parameters. Moreover the use of controlateral limbs as a control series avoid inter-individual variations and staining artefacts.*

The results show that the electromagnetic signal used produces modifications in the morphology and the metabolism of the different components of the bone. The modifications observed in the stimulated limbs are : a thicker proliferative layer of chondrocytes, a better lining up of the trabecula and a better configuration of the cartilage. These modifications are probably due to a change in the components of the cartilaginous matrix. The histochemical demonstration of mucopolysaccharides would probably bring more clarity to this matter.

I. Introduction

Several years ago, the hypothesis of an enhancement of bone growth and repair by electric stimulation was proposed and investigated.

Recent clinical results have revealed a success rate of 80 % in the therapeutic use of electric stimulation in pseudoarthroses (Bassett, 1979 ; Brighton, 1979).

Nevertheless the translation mechanism of the electric signal by the cell remains unknown.

In his earliest works Becker (1961, 1967, 1970) assumed that a genetic derepression mechanism could be initiated by the action of the electric currents on membranes through an alteration of an electric layer of charged particules in the medium.

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Pilla (1979) carried this hypothesis further by suggesting that electrochemical interactions at cell surfaces could represent key steps in this genetic regulation and he proposed a theoretical analysis of several possible phenomena involved in the processus.

Obvious modifications of cell morphology were obtained by Becker (1961) using amphibian red blood cells stimulated by DC current. He described the transformation of differentiated and specialized cells into a more primitive form with unspecialized functions, followed by a new differentiation with new specific functions. He defined this phenomenon as a « dedifferentiation » process. Using the same amphibian red blood cells stimulated by an electromagnetic field, Hinsenkamp *et al.* (1978) and Chiabrera *et al.* (1979) observed the same « dedifferentiation » process.

Brighton (1979) using rabbits tibia stimulated by a centromedullary cathod showed the replacement of normal hematopoietic cells of the marrow by polymorphic cells like those observed after a single trauma.

In 1979, Ashihara, using a circular cathod around the periosteum of rat femora and tracing the cellular uptake of ^{3}H-thymidine, observed a transformation of the periosteal cells into osteogenitor cells which then proliferate during 24 hours and begin to differentiate into osteoblasts from the second day on.

After a continuous production of osteoid, the osteoblasts differentiate into osteocytes after 11 days while the osteoid matured into cancellous bone.

Different metabolic functions of the cell were investigated. In 1979 Bassett using ^{45}Ca observed that specific electromagnetic signals can increase or decrease calcium uptake in avian chondrocytes *in vitro* after two hours of stimulation.

By radioimmunoassay, Norton (1977) demonstrated an increase in c-AMP in stimulated chick embryo cartilage. This effect was influenced by the state of maturation of the cells and by the orientation of the sample in the electric field.

An increased synthesis of collagen was observed by Bassett (1968) in fibroblast cultures after 14 days stimulation and labelling by ^{3}H-proline. The same observation was reported by Norton (1972) after 6 days stimulation of rat calvaria and labelling by ^{14}C-proline.

Janssen (1978) assessed the amount of bone formation and resorption in cultured calvaria of 19-day rat fetuses by measuring respectively alkaline phosphatase and lactic acid. Both were found increased after electric stimulation by electrods inserted in the culture medium.

These modifications can be either modulated with different concentrations of Ca^{++} or inhibited by ouabain.

In 1972, Norton, after six days stimulation of rat calvaria reported no evidence of an increase in calcified bone using ^{3}H-tétracycline. An increase in cancellous bone matrix labelled by ^{35}S as well as an enhancement ment of ossification (as detected by ^{45}Ca uptake) were observed by Brighton (1976) after 10 days stimulation of rat costochondral junction by an electrostatic field.

More fundamental modifications of RNA and DNA were also studied.

Becker in the above mentioned works using acridinorange found a significant increase in cytoplasmic RNA after 45 hours DC stimulation. He showed that actinomycin D (blocking RNA transcription from DNA) as well as puromycin C (blocking translation by RNA to protein) were able to arrest the morphological changes. By adding tritiated amino-acid to the culture medium, he also found an increased amount of cytoplasmic amino-acid after stimulation.

Norton (1972) also observed an increase in RNA labelled by ^{3}H-glycerine.

After longer stimulation periods, increased amounts of DNA were observed by Bassett (1968) in fibroblasts after 14 days and by Brighton (1976) in rat costochondral junctions after 10 days stimulation and labelling by ^{3}H thymidine. Rodan (1978) noted an enhancement of DNA synthesis in stimulated chick epiphyseal chondrocytes, but not in other non skeletal tissues. Norton (1979) stimulating embryonic rat calvaria cells and adding ^{3}H-thymidine, also observed an increase in DNA synthesis in the area facing the cathode. He noted an improvement of cellular adhesion probably due to a modification of several factors including the amount and composition of the matrix synthesized in culture.

In a common work, Hinsenkamp (1978) and Chiabrera (1979) observed a modification in the spatial configuration of DNA in amphibian erythrocytes stimulated by an electromagnetic field labelled with acridinorange and submitted to cytofluorometric measurements. They advanced the hypothesis of a relationship between DNA modification after electromagnetic exposure and the activity of more functional sites (operons in the DNA molecule. Parallel with DNA modifications, they also observed morphological changes similar to those previously detected by Becker (1961) after DC stimulation and defined as a « dedifferentiation » phenomenon. They noted that this phenomenon can be induced by ionic changes in the medium and then enhanced or inhibited by specific electromagnetic signals. This ionic sensitivity of the cell called « reactivation » was observed by several authors (Cone, 1976 ; Barth, 1969, 1972, 1974).

The importance of the Na^+/K^+ equilibrium in the triggering of the process, as well as the different observations which emphasize the dependence of this reactivation from enzymatic and energetic mechanisms suggest that a probable inducer can be produced by disturbing cell homeostasis or by altering some transport mechanism like the sodium potassium pump. This hypothesis is in agreement with the theory of Jacob and Monod (1961) for regulation of cellular protein synthesis.

Before investigating the modifications underlying the cellular synthesis, the specific morphological action of a given electromagnetic signal have to be defined. The aim of the present work is to describe the morphological modifications induced by an electromagnetic field in bone development by using a highly growing stage with reliable control references. Enzymatic and histochemical modifications will be presented in a following paper (Rooze and Hinsenkamp, 1982).

II. Material and methods

Both right and left limb buds from fetal or newborn Swiss mice were incubated in a semi-synthetic culture medium. The first group was submitted to electric stimulation, and the contralateral untreated group was kept as a control. During the experiment, the various environmental parameters of the culture were kept constant. The incubation period was 4 to 6 days. The stimulation instruments (fig. 1) have been provided by EBI* and the electromagnetic signal used is shown in figure 2 where T is the period equal to 63 msec, the amplitude of the positive peaks H is 16mV and the burs width is P (4.8 msec).

After the incubation period, the samples were prepared either for macroscopic of for microscopic studies of the skeleton.

In toto skeletal staining have been made according to Watson's method (1977) using red alizarin and alcian blue. It respectively stains calcifying tissue red and cartilage blue.

The samples were fixed in Bouin or Serra fixative, embedded in paraffin and serially sectioned (10 µm thick). The sections were stained by the phosphotungstic hematoxylin or by hemalun-safranine. In each technical step, the control and the « stimulated » limbs have been treated strictly together in order to avoid artefacts.

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* Electro Biology Inc. 300 Fairfield Road, Fairfield, New Jersey.

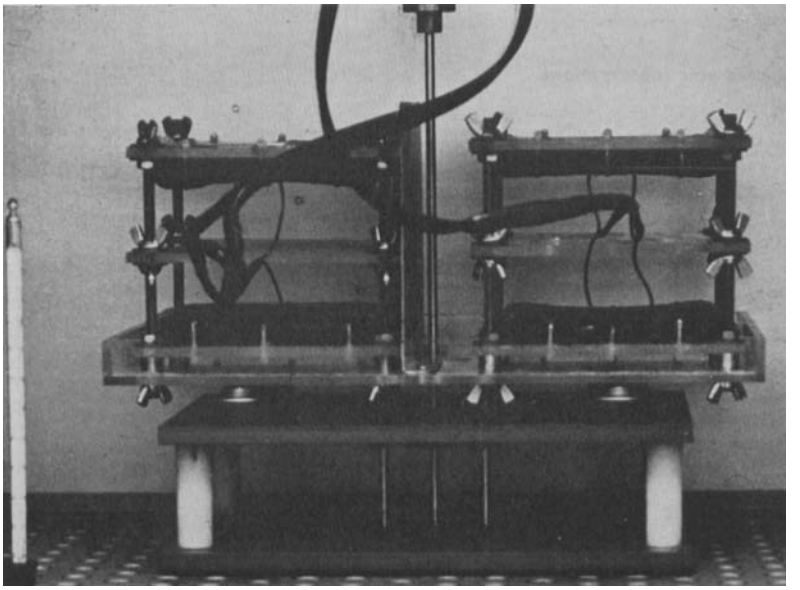

FIG. 1. — The inducing coils in the incubator.

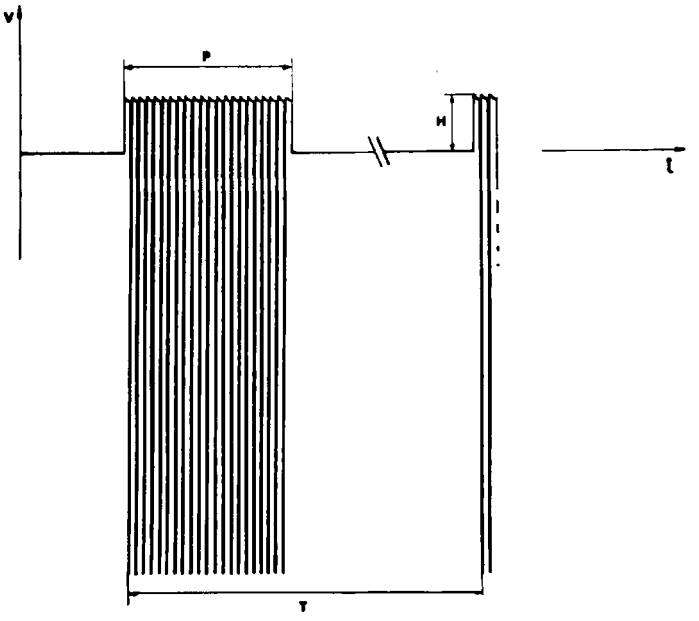

FIG. 2. — Electromagnetic signal used
(period T : 63 msec, H : 16 mV, P : 4.8 msec).

III. Results

1. Macroscopic observations.

In the younger explants where no primary ossification center was observed and where all skeletal primordia are cartilaginous, the activated samples exhibit a higher affinity for alcian blue. This difference is more obvious in the distal skeletal segments.

In older limbs, the diaphysis of the long bones show a red staining due to their early primary ossification. In the activated samples, the primary ossification centers appear thicker and more strongly stained. In control limbs, the metaphyseal plate shows a « double ring » area unstained close to the diaphysis and blue on the epiphyseal side. In activated limbs, this zone shows a larger blue ring and no unstained area (fig. 3). The statistical analysis of these differences is given on table I.

Macroscopical examination allows only a rough evaluation of the morphological differences. The correlation between the double ring area observed in the metaphyseal plate and the histological structure is hazardous. More accurate investigations of the different components of bone are required to bring more precision in this matter.

TABLE I

In toto skeletal double staining

	Total number of pairs	*+ differences*	*No difference*	*Significant*
Head of humerus	7	2	5	NS
Elbow joint	7	2	5	NS
Distal epiphysis of the radius	6	3	3	NS
Distal epiphysis of the ulna	6	3	3	NS
Carpal bones	6	4	2	NS
First phalanx of the finger	6	4	2	NS

2. Histological examination.

The morphological differences described below have been observed in forelimbs as well as in hindlimbs. They appear to be more pronounced in the distal skeletal segments.

a) *Metaphyseal area.*

In control limbs, the germinative and the proliferative areas are composed of an important number of cell layers where the cells are larger transversally and thinner along the proliferative axis ; they are surrounded

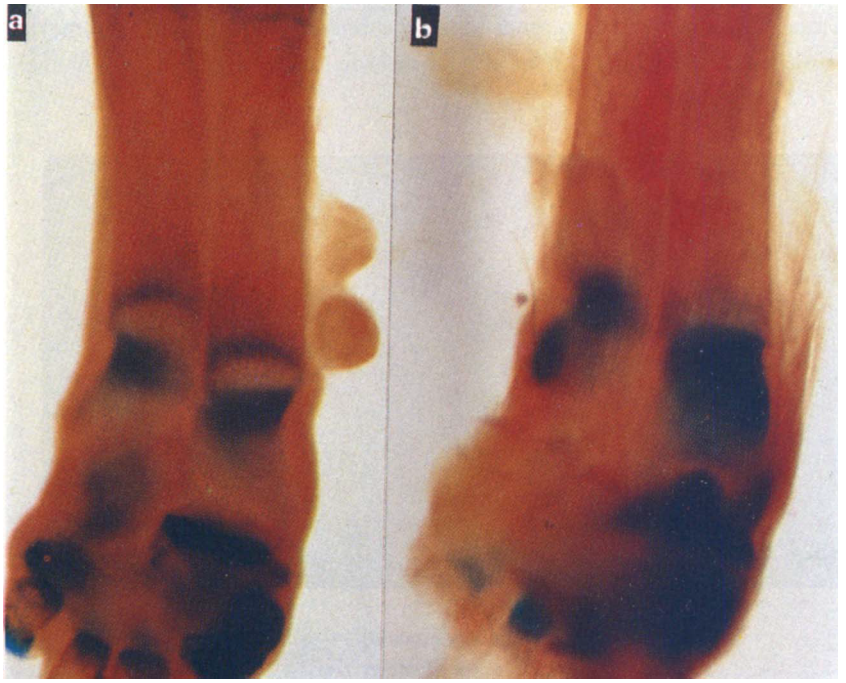

FIG. 3. — In toto skeletal staining (Watson's method), forearm (a : control, b : activated).

Distal epiphysis of ulna and radius : unstained annulus in the control samples and bluer staining in the activated.

by a cartilaginous matrix (fig. 4). This observation suggests a tissular impaction.

In activated samples the proliferative layers are less deformed and the cartilaginous matrix is more darkly stained (fig. 4). The chondrocytes of the activated serial cartilage are more regularly arranged (fig. 4 and 5) and their alveolar space is larger. The cartilaginous matrix surrounding hypertrophic chondrocytes is more weakly stained. These morphological differences are observed both in fore and hind limbs.

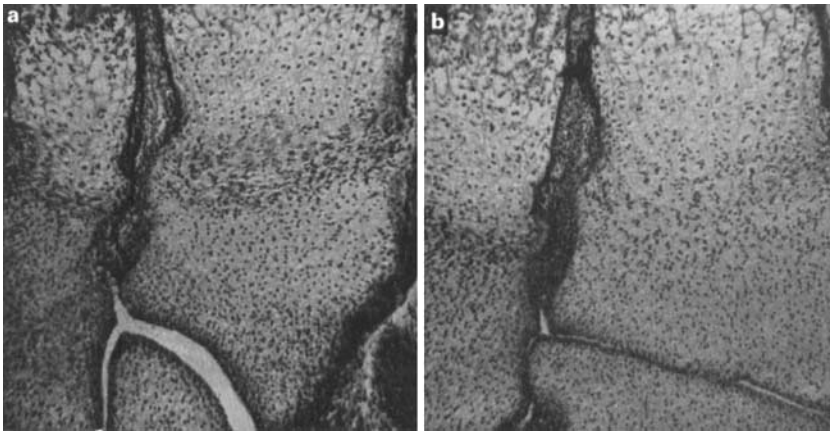

FIG. 4. — Hematoxylin eosin, ankle joint
(a : control, b : activated).

b) *Diaphysis.*

The cortical layer of long bones appear thicker in activated limbs and, as confirmed in whole mount preparations, their whole diaphysis is slighthy increased in size (fig. 5). This observation is correlated with the « in toto » staining. The young bone trabeculae relieving from the serial cartilage are thinner and more intensely stained in the activated bones.

c) *Epiphysis.*

The epiphyseal cartilaginous plate is more regularly shaped in the activated limbs.

These observations are summarized an table II.

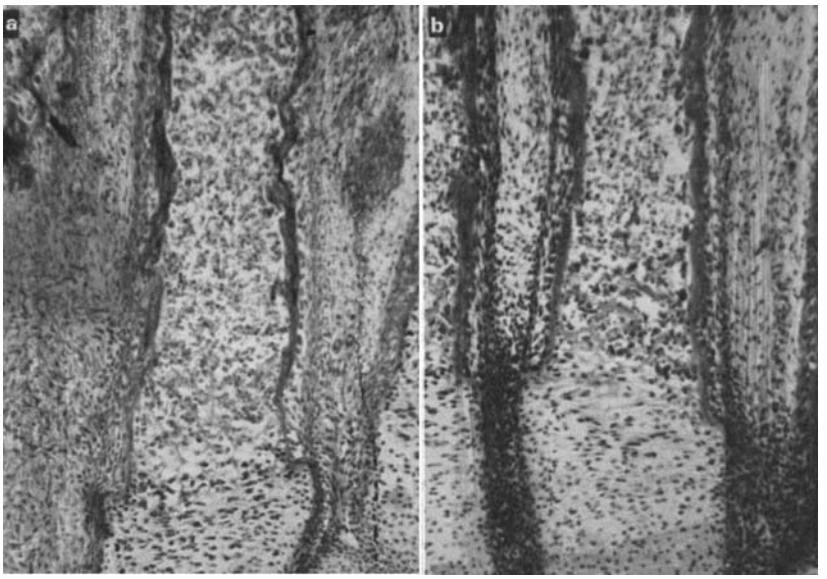

FIG. 5. — Hematoxylin eosin, metatarsal bones (a : control, b : activated).

TABLE II

Hematoxylin eosine

	Total number of pairs	*+ differences*	*No difference*	*Significant*
Tibial metaphysis superior	4	2	2	NS
Tibial diaphysis	4	2	2	NS
Tibial metaphysis inferior	6	4	2	NS
Peroneal metaphysis inferior . . .	6	5	1	NS
Third phalanx metatarsal bone . . .	6	5	1	NS

IV. **Conclusion**

A great number of previous studies (see in introduction) have revealed various changes induced by lectric or electromagnetic stimulation in living tissues treated *in situ* or *in vitro*. The *in vitro* culture of developing limb buds used in the present work has proved a useful experimental model for testing the influence of electromagnetic stimulation on skeletogenesis.

According to the first macroscopic observations, the metaphyseal area seems to be selectively influenced by the stimulation, but the observed

effects are not statistically significant. As seen in table I, however the more sensitive limb parts are the distal ones, i.e. the youngest ones as far as their tissular differentiation is concerned. The gross changes ob served in bulk stained preparations result from differences in the staining intensity by Alcian blue and alizarin. They can be related to more precise modifications observed in histological sections stained with hematoxylin-eosin.

The comparative histological study of stimulated and control samples confirms that selective changes occur in the metaphyseal area. They concern the spatial arrangement of serial cartilage, which appears to be better piled up than in the control limbs, as well as the cartilaginous matrix itself, which is more darkly stained. It seems thus that stimulated tissues grown *in vitro* undergo a better cartilaginous differentiation than the unstimulated control tissues ; the electromagnetic stimulation would thus « normalize » *in vitro* chondrogenesis.

Although they are too few to be statistically conclusive, the data of table II clearly indicate, however, that the differences observed between stimulated and control limbs are more pronounced in the distal than in the proximal limb segments.

A simple and easily controllable experimental system has permitted the demonstration that electromagnetic stimulation can influence the structure and most probably the metabolism of the developing skeleton in mouse limb buds grown *in vitro*. The detailed changes occurring in the diaphyseal plates will require further histochemical and biochemical studies.

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