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Study of bone cells by confocal microscopy in fractures stimulated by ultrasound

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Abstract

The ultrasound mechanism of action in fracture healing is still unknown. The mechanism that controls the compartment of each cell during healing process derives, possibly, from the microenvironment of said cells; studies in the field show differences of 55 to 84% between cross-sectional areas, depending on the sectioning mode. In order to assess the impact of ultrasound application to bone cells, we have examined bone tissue by help of Nikon E-600 microscope; the three-dimensional images have been obtained by Zeiss LSM 510 laser confocal microscope, from the Riken Physico-Chemical Analysis Laboratory, Japan. Morphometry analysis was made by Lucia Computer Program. An increase by 10% in the surface of cavities containing osteocytes has been noticed in the batch stimulated by ultrasound as compared to control batch, with individual values of 21.86 to 98.139 in both batches. The changes that appear in stimulated bone, from both morphometrical point of view and that of osteonal positioning, as compared to normal bone tissue, are the final result of such remodeling, in which the bone did not take its initial form, but the one that could best perform its function.

***Keywords*:** osteoplast, stimulation, laser confocal microscopy.

Introduction

The need of developing more complex and efficient diagnostic and therapeutic methods has made biologists and physicians research the manner in which ultrasound stimulation devices could be used in establishing new medical techniques or to replace old conventional ones. Many research work has been published until to date, the majority reporting positive beneficial effects of ultrasound on bone fractures but there are also many others that criticize the method, therefore it is not unanimously accepted in therapy. The present study aims at understanding and disclosing ultrasound action on bone tissue.

The mechanism that controls the compartment of each cell during healing process derives, possibly, from the microenvironment of those cells; studies in the field show differences of 55 to 84% between cross-sectional areas, depending on the sectioning mode [1, 2]. Compression or the absence of tension inhibits the formation of fibrous connective tissue [3]. The variations in oxygen pressure lead to formation of bone or cartilage; the latter forms in areas where oxygen pressure is relatively low, assuming that this is due to the distance between cells and capillaries [4]. The so-formed cartilage is eventually resorbed through an indistinct process of endochondral bone formation. Bone will be formed by those cells that receive enough oxygen and which are correctly stimulated by mechanical means [5].

Materials and Methods

Twenty-four male adult rabbits, weighing 3 kg, have been used in the study. They were divided into two batches; osteotomy was performed in both batches on day D0 and plaster bandage applied subsequently. As of day four, D4, pulsed ultrasound therapy was applied to rabbits in batch one, 20 minutes/day for 20 days, until D24. Rabbits were sacrificed on day 45 (D45). Bone tissue was harvested from contralateral limbs. Samples were selected and prepared by grinding. All experimental activities were in accordance with the *Code of Practice for Animal Experimentation and with the Universal Declaration of Animal Rights – Article 8* (UNESCO, 1978). Ethical consent for legal consideration was seeked for the laboratory experiments.

In order to assess the impact of ultrasound application to bone cells, we have examined bone tissue by help of Nikon E-600 microscope; the three-dimensional images have been obtained by Zeiss LSM 510 laser confocal microscope, from the Riken Physico-Chemical Analysis Laboratory, Japan. Morphometry analysis was made by Lucia Computer Program. Source image was decomposed in frames arranged as a frame pile along a well-defined sense of direction, such as *Oy* axis. These successive images represent different views from plans parallel to *xOy* plan. Thus, the depth exploration of newly-formed bone, due to ultrasounds, was made possible.

The act of exploring images along *Oy* axis, concomitantly with changes in *xOy* plan, updates the images from the other two plans of the Cartesian coordinate system. Thus, the investigator knows exactly which point inside the bone volume is being assessed. The inside of the cortical bone could thus be explored entirely; the 3D representation being reconstructed from the source-frames.

Results

By examining sample slides in light microscopy, both batches exhibit osteoplasts, cavities carved in ground substance, which harbor osteocytes, adult bone cells, destroyed by grinding. Osteoplasts are located between the bone lamellae that encircle the Haversian canal; the dimensions of osteocytes and Robin osteoplasts depend on the size of osteoblasts from which they derive (Figure 1).

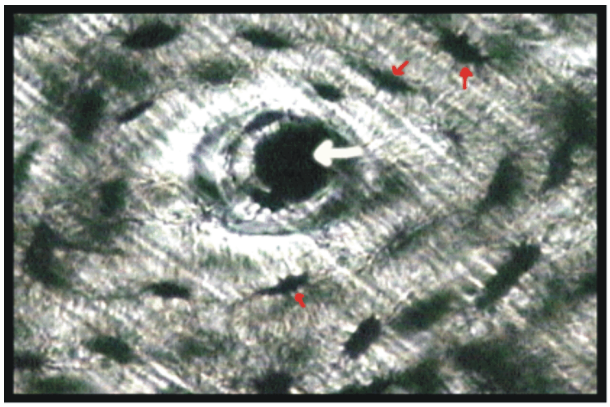

Figure 1 – ***Part of Haversian system of newly-formed bone. The white arrow shows the Havers canal in the center of the osteon; the red arrows point to the osteoplasts located parallel to the bone lamellae. Canaliculi emerge from the osteoplasts toward the Haversian canal. Ground bone, 40×, stimulated batch.***

The aspect of osteoplasts changes depending on the section plan. In longitudinal sections, where collagen fibers are sectioned longitudinally (being refringent in polarized light), osteoplasts appear narrow, elongated, and elliptical; in cross-sections they appear more rounded (Figure 2).

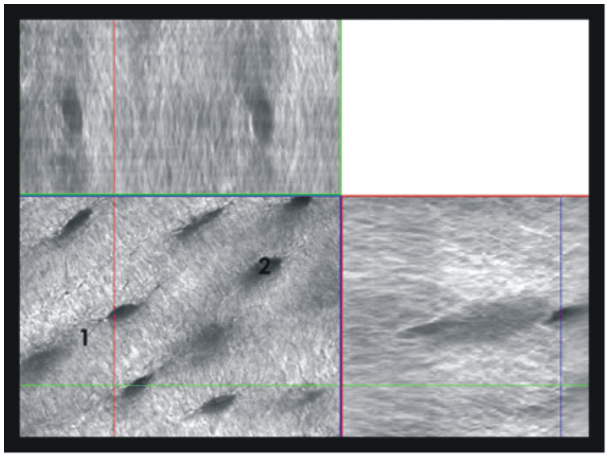

Figure 2 – ***Ground bone, laser confocal microscopy, 140×, stimulated batch. Robin osteoplasts and inter-connecting canaliculi, disposed in three plans. In longitudinal sections Oy, where collagen fibers are sectioned longitudinally, osteoplasts are elliptical; in cross-sections, they appear more rounded.***

An intense remodeling process takes place in stimulated bone, characterized by big osteoplasts, similar to osteocytes harbored inside.

The number of osteoplasts is 20% higher in the stimulated batch, the mean values being 3.4 in the first lamella, 5.7 in the second lamella and 8.1 in the third lamella. In the control batch, the mean values of the number of osteoplasts were 2.8 in the first lamella, 5.5 in the second lamella and 5.57 in the third lamella.

The quantitative assessment of metabolically activity of osteocytes was made by morphometrical analysis of the surfaces of osteocytic lacunae. An increase by 10% of the surface of osteoplasts was noticed in the stimulated batch as compared to the control batch, the individual values ranging between 21.86 and 98.139 for both batches: $P(T \leq t)=0.006$ (Figure 3).

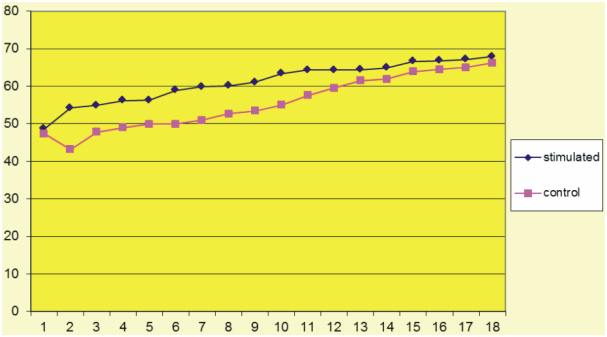

Figure 3 – ***Graphic representation of the dynamics of mean area of osteoplasts per microscopic field, in the control and stimulated batches.***

This increase in osteoplasts surface shows a good development of the cellular component, needed for tissue recovery, presently knowing that osteocytes are no longer considered inactive adult cells. Osteocytes take part in the neoformation of bone tissue, secreting fragments of collagen fibers inside the osteoplast that subsequently mineralize. This process is similar to that of fibrous osteoid production in ossification processes.

The increase in osteoplasts surface in the stimulated batch is due to the increase in the number of osteoplasts.

Within the framework of osteocytic mini-remodeling, periosteocytic osteolysis is not noticed, the osteoplast edges in both batches being smooth and regular. The bone in the area adjacent to osteotomy of the central cortical section displays large lacunae, whereas in the more recently formed bone, these are smaller on the outer surface.

The images obtained by means of the confocal laser microscope demonstrate highly intense local activity. The interosteoplastic canaliculi are well developed, thus expanding the exchange surface between the tissular and cellular compartment of the bony tissue, by means of intraosseous liquid, mobilized by contractions and dilations of osteocytes. The extensions of osteoplasts in the periphery of osteon describe a loop to return to the peripheral lamella.

Collagen fibers may already have acquired typical structure, as they are parallel among themselves in the same bony lamella (Figure 4).

The presence of intraosseous canaliculi-lacunar systems has been well known for over a century. They play a significant part in the transfer of nutrient elements of blood vessels to the adult osseous cell. The link between osteocytic canaliculi and blood vessels in the bone under stimulation is extensive. The growth of canalicular grid in young, newly formed osteons point to the assembling of intercellular connections and to those between osteocytes and Havers canal, these being mandatory to bone maintenance and regeneration. The excellent

evenness of osteoplastic canaliculi (in terms of disposition, length, width and frequency) is to be noted in the stimulated batch.

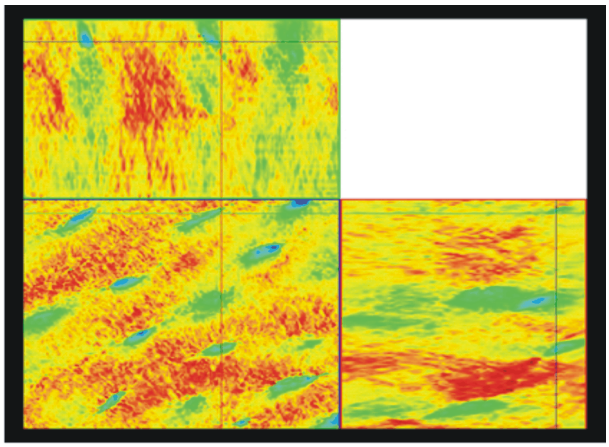

Figure 4 – ***Display of osteoplasts, canalicular network and of collagen fibers in ultrasound stimulated bone, prepared by grinding. Confocal laser microscopy, 140×.***

Osteocytic canaliculi interfere with homeostasis of phosphocalcic metabolism, they being highly sensitive to pressure exerted on the bone. The cytoplasmic extensions may lengthen, as they discharge their function in activating liquids rich in metabolites, from the level of canaliculi and osteoplasts towards the bony surface.

A growth in calcium absorption occurs at the level of bony cells along with the modulation of adenylate cyclase performance, as well as the adjustment and the synthesis of β-growth factors. *In vitro*, the effects of ultrasound trigger differentiation within osteoblast cultures.

Ultrasound-stimulated osteoblasts lead to structuring more numerous Haversian systems, almost uniform in shape and of greater dimensions in the stimulated callus as against those in the non-stimulated callus.

An increase in osteoblasts area demonstrate good development of cellular component, needed in tissue recovery, as currently osteocytes are not considered anymore as inactive mature bone cells.

Osteocytes take part in the neoformation of bone tissue, secreting fragments of collagen fibers inside the osteoplast that subsequently mineralize. This process is similar to that of fibrous osteoid production in ossification processes.

Changes appearing in stimulated bone, from both morphometrical point of view and that of osteonal positioning, as compared to normal bone tissue, are the final result of such remodeling, in which the bone did not take its initial form, but the one that could best perform its function.

Discussion

After fracture, the bone itself is damaged, the soft tissue envelope including the periosteum and the surrounding muscles are destroyed, and many blood vessels that cross the fracture line are broken. Hematoma occurs in the spinal canal, between fracture ends and subperiosteal. Effect of this vascular disaster is crucial. [1, 6]. Osteocytes lack source of nutrition and die on junction side channels. Osteocytes that survive do not take part in the repair process they are destroyed during resumption. However, most cells directly involved in fracture healing enter the fracture together with granular tissue that invades region-surrounding vessels [2, 3]. If these cells with repair role came directly from the endothelium, are migratory cells or generate from pre-reticulocite, seems less important than the fact that repair is related to penetration of vascular buds [4, 7]. In our experiment, periosteum was interrupted during osteotomy, destroying the local blood circulation and for appreciation of his recovery, we evaluated the external fundamental system that arises mainly from periosteum, thanks its internal, osteogenic layer.

Proinflammatory effects of ultrasound accelerate the onset of inflammatory phase, rapidly filling the exudate in the interstitial fluid. Ultrasound irradiation generates adhesion of leukocyte cells and endothelial cells. During fracture repair, the process facilitates leukocytes migration from blood to the injured compartment, increasing efficiency of the elimination of tissue derbies and pathogens agents from the wound [5]. They stimulate the release of growth factors of fibroblasts by macrophages. It is thus encouraged the proliferation of fibroblasts, facilitating collagen synthesis and angio-genesis [6–8].

Thus, fibroblast growth factors have a role in cartilage growing and help to build precursor cells of bone formation, leading to an early onset of bone repair process.

The mechanism that controls every cell behavior in the repairer probably is derived from the micro-environment in which each cell found [9, 10]. Compression or lack of tension discourages the formation of fibrous tissue [11]. Undoubtedly, oxygen pressure variation lead to the formation of bone or cartilage, cartilage forming in areas where oxygen pressure is relatively low, assuming that it is a result of the distance between the cell and capillary [12]. Thus, formed cartilage is eventually reabsorbed by an indistinguishable process of endochondral bone formation, excepting his lack of organization. In unstimulated group can be observed two changes that arise consecutive hypoxia: in perilesional area, osteons closing occurs in the lesion – the presence of cartilaginous tissue. Bone is formed by those cells that receive enough oxygen and are properly stimulated mechanically [13].

Direct influence of ultrasound on osteoblasts is obvious, all subjects belonging to ultrasound-stimulated lot of our study developed in the fractured area a bone tissue having the structure of a normal bone; in terms of macroscopic, tibial cortex was completely rebuilt. Increased calcium absorption occurs in bone cells and some modulation in the activity of adenylate cyclase [14], transformation and synthesis of β-growth factors. *In vitro*, effects of ultrasound would be differentiation of the osteoblast cultures compared with the electric field, which does not directly stimulate osteogenesis but through fibrochondrocytes calcification any obstruction by soft tissue ossification is eliminated [5, 8, 15].

Conclusions

The direct influence of ultrasounds on osteoblasts is evident; all subjects in the ultrasound-stimulated batch

developed, in the fractured area, a variant of bony tissue macroscopically similar in structure to normal bony tissue. Pulsed ultrasound ensures necessary conditions to bone tissue recovery: it provides the vascularization needed in the differentiation of bony cells, it also prevents infections and, by "internal tissular massage", it provides the physical conditions needed in stimulating bony structuring. Callus derived by stimulation with pulsed ultrasound is of high quality, fact also confirmed by the histomorphometrical study carried out by us.

Contribution Note

All the authors have equal contribution to the paper.

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