

Effects of low-level laser therapy on bone formed after distraction osteogenesis

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Abstract This study evaluated the effect of low-level laser therapy (LLLT) on the chemical composition, crystallinity and crystalline structure of bone at the site of distraction osteogenesis. Five rabbits were subjected to distraction osteogenesis (latency = 3 days; rate and frequency = 0.7 mm/day for 7 days; consolidation = 10 days), and three were given LLLT with arsenide–gallium–aluminum (AsGaAl; 830 nm, 40 mW): 10 J/cm² dose per spot, applied directly to the distraction osteogenesis site during the consolidation stage at 48 h intervals. Samples were harvested at the end of the consolidation stage. X-ray fluorescence and X-ray diffraction were used to analyze chemical composition, crystallinity and crystalline structure of bone at the distraction osteogenesis site. The analysis of chemical composition and calcium (Ca) and phosphorus (P) ratios revealed greater mineralization in the LLLT group. Diffractograms showed that the crystalline structure of the samples was similar to that of hydroxyapatites. Crystallinity percentages were greater in rabbits that were given LLLT. Crystallinity (41.14% to 54.57%) and the chemical composition of the bone at the distraction osteogenesis site were

similar to the that of the control group (42.37% to 49.29%). The results showed that LLLT had a positive effect on the biomodulation of newly formed bone.

Keywords Osteogenesis · Distraction · Lasers · X-ray diffraction

Introduction

In the past two decades distraction osteogenesis has become an effective therapeutic alternative to the use of bone grafts in the treatment of several congenital or acquired dentofacial deformities. Distraction osteogenesis consists of the use of distraction devices implanted in adjacent and independent bone sites to promote their separation and the consequent osteogenesis between them. After the time necessary for separation and defect repair, the distraction device is held in place but is no longer activated. It should be removed only after the interposed osteoid tissue has mineralized. Because of the time required for bone maturation and for removal of the distractor, distraction osteogenesis may generate discomfort, which has led some authors to study solutions to accelerate new bone formation [1–7].

Distraction osteogenesis involves metabolic activities that can be modulated by physical and chemical agents. Therefore, the use of low-level laser therapy (LLLT) may reduce total treatment time and provide more comfort to patients. The use of LLLT has been proven to be beneficial to soft tissue and bone repair [8–13].

Bone may be evaluated by several types of tests that differ in their accuracy and complexity. These tests should determine the quantitative and qualitative differences in bone tissue between study groups. The analysis of how operator-independent the test is may also be relevant,

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because of the influence that operators may have on their object of study.

Histological analysis after hematoxylin–eosin (HE) staining is widely used to evaluate bone changes. Other stains are also used with the same purpose, and their performance and analysis are similar. Although relatively easy to perform, they have the disadvantage of being operator-dependent, which requires us to use a greater number of animals or specimens to obtain a statistical sample with a good level of significance.

X-ray fluorescence (XRF) and X-ray diffraction (XRD) are physical analyses requiring high-technology equipment that can be used for the study of bone and to generate data about its mineral characteristics, such as type of crystals (crystalline structure), the perfection of those crystals (crystallinity), and their mineral content (amount of calcium, phosphorus, and other elements). In these tests, which are operator-independent, the so-called counting statistics indicate that the measurement of each point has the accuracy of 1 to 100 parts per million (ppm), which rules out some biases associated with data collection [14].

These mineral characteristics of bone at the distraction osteogenesis site have not been documented in the literature. As distraction osteogenesis promotes bone formation from the initial stages to maturity, the mineral characteristics of bone may change. Therefore, physical analyses may be useful in the identification of possible changes and in the development of innovative and accurate procedures for the analysis of changes in bone metabolism. This study evaluated the action of LLLT on the crystalline structure, chemical composition and crystallinity of bone at the distraction osteogenesis site.

Materials and methods

All experimental procedures were approved by the Ethics Research Committee of Pontificia Universidade Católica do Rio Grande do Sul (PUCRS), Brazil. Five adult male New Zealand rabbits (*Oryctolagus cuniculus*) were used, but the data from one was excluded from analysis because of early union of fracture fragments. Rabbit 1 was subjected to distraction osteogenesis and LLLT; rabbit 2, distraction osteogenesis, but no LLLT; rabbit 3, no distraction osteogenesis, but LLLT; and rabbit 4, neither distraction osteogenesis nor LLLT.

The animals that underwent distraction osteogenesis (rabbits 1 and 2) were anesthetized with 2% xylazine hydrochloride 1 mg/kg (Anacedan®) and zolazepam hydrochloride plus tiletamine hydrochloride at 3 mg/kg (Zoletil®). The right submandibular area was shaved and cleaned with 4% chlorhexidine. Sterile fields were used to isolate the operation area. Antibiotic prophylaxis consisted of 50 mg enrofloxacin administered 1 h before the procedure and for the next 3 days.

After 0.9 ml lidocaine and 2% epinephrine had been infiltrated, a 3 cm incision was made on the skin of the lower border of the right hemi-mandible. Careful subperiosteal dissection was performed and the mandible was exposed. A corticotomy was performed using drills and osteotomes, and the lower alveolar nerve was preserved. The distractor (PROMM®, Porto Alegre, Brazil) was fixed to the mandible with four 1.5 mm×5 mm screws at a plane perpendicular to the corticotomy. The wound was irrigated with saline solution and sutured in layers. Distraction osteogenesis followed a latency stage of 3 days, one daily 0.7 mm distractor activation for 7 days, and a consolidation time of 10 days.

Rabbits 1 and 3 were given LLLT at 10 J/cm² per spot every 48 h during consolidation, at a total dose of 50 J/cm². LLLT was applied with 830 nm and 40 mW gallium-aluminum-arsenide diodes. At the end of the consolidation stage, the animals were killed in a carbon dioxide chamber, according to the recommendations of the Brazilian Council for Animal Experimentation.

Physical examinations were conducted at the Laboratory of Materials and Nanosciences [Laboratório de Materiais e Nanociências (LMN)] of the School of Physics, Center of Research and Development in Physics, Technological Center, PUCRS.

X-ray fluorescence spectroscopy

Samples were prepared for XRF in three stages:

- The four rabbit hemi-mandibles were dissected, removed and stored in 2% glutaraldehyde
- Immediately after that, the specimens were embedded in acrylic resin for sanding and polishing
- For manual polishing, sequential grits (180 to 4,000) of sandpaper were used; the sandpaper was fixed to a glass block and was used under irrigation with running water

The purpose of this preparation was to obtain a polished surface at the distraction osteogenesis site and adjacent areas for the direct incidence of X-rays in the spectrometer.

The acrylic resin blocks containing the distraction osteogenesis site were placed directly in the spectrometer (XRF-1800, sequential X-ray spectrometer, Shimadzu) for the analysis of calcium (Ca) and phosphorus (P). The samples were measured using rhodium K α (Rh, X-ray source) at 40 kV and 95 mA. To measure the Ca and P, we used lithium fluoride (LiF) and germanium (Ge) diffraction crystals, which specifically detect the fluorescence of each of these elements (Ca and P). Procedures were performed in vacuum at a pressure of 25 Pa, scanning speed of 8°/min, in steps of 0.1°, and 0.75 s per step.

Measurements were made at different points along the distraction osteogenesis vector using a millimeter grid

(template) and along the axis of distraction in the mandibles, starting at the point most proximal to the border of the produced fracture (P1) and extending to the most medial point in the newly formed bone at the osteogenesis distraction site (P3–P5). The purpose of multiple measurements was to evaluate the fluctuation of Ca and P content at the distraction osteogenesis site. Several stages of bone maturity were expected, because of the characteristic gradual stretching of the distraction osteogenesis technique. Only three to five points were measured at the distraction osteogenesis site because small bone irregularities (medullary spaces), found even after polishing, severely distorted the results. Therefore, when differences between results were too large, the point where the measurement was made was examined under the microscope, and, if it was located in a topographically irregular area, those values were immediately discarded.

The values obtained for Ca and P at each point were used to calculate Ca-to-P ratios, which were analyzed statistically and organized in tables.

X-ray diffraction spectroscopy

After XRF data had been obtained, the specimens were cut transversally in a microtome, and the anterior and middle portions of the distraction osteogenesis site were separated. A slice of newly formed bone containing cortical and medullary portions was ground to a powder with a ceramic mortar and pestle. For XRD analysis, the material (powder) should be pressed in specific equipment devices. However, because of the very small amount of material obtained, it was necessary to produce glass plate wells that measured 6 mm in diameter and 1 mm deep. As glass plates do not have X-ray diffraction peaks, the material obtained from each specimen was placed in the well and pressed satisfactorily there.

Each glass plate containing powder was analyzed by the X-ray diffractometer (XRD Maxima 7000, Shimadzu). Samples were measured using copper (Cu) $k\alpha$ ($\lambda=1.5406 \text{ \AA}$) at 40 kV, and 30 mA. Bragg–Brentano geometry ($\theta-2\theta$) was used for measurements, at 30–45° scanning, 2°/min speed, 0.02° steps, and 6 s per step. Angle scanning covered only the region between 30° and 45° because this is the only region where hydroxyapatite (HA) diffraction peaks are found. Total measurement time for each sample was 4,500 s, which generated excellent statistical data.

A diffractogram was obtained for each sample; peaks, ratio of signal amplitude, and noise were identified and compared with those of known hydroxyapatites. Similarities were sought using the equipment software.

The Shimadzu software uses Lorenz adjustment for the measurement of sample crystallinity. Crystallinity is calculated according to the American Society for Testing and

Materials (ASTM) D5357 and D5758 norms, which are used for XRD calculations of the crystallinity of zeolites, a large group of minerals with a porous structure.

At an initial stage of the process of new bone formation, after the reabsorption of tissues, the organism has to supply the region with:

- (a) the necessary amounts of minerals to form inorganic tissue (Ca-to-P ratio)
- (b) cell organization, to differentiate tissues (mineral deposition)
- (c) specialization of tissues, to support expected loads (crystallinity)

The more advanced the process of bone maturation, the greater the degree of bone organization, and, therefore, the greater its crystallinity. Therefore, based on these assumptions, crystallinity indicates the degree of bone maturity.

Results

X-ray fluorescence spectroscopy

The presence of calcium and phosphorus in the samples was determined according to their characteristic X-ray emission peaks (k_{α} and k_{β}) detected by the spectrometer. Figure 1a shows newly formed bone in distraction osteogenesis sites, and Fig. 1b shows areas of newly formed bone in pixels squared to the total area of distraction osteogenesis obtained in the HE analyses. Figure 2 shows Ca-to-P ratios according to X-ray fluorescence measurements for each rabbit (Table 1).

Figure 2 clearly shows that the Ca-to-P ratios were almost constant in the rabbits that had not been subjected to distraction osteogenesis (rabbits 3 and 4) and are within the usual statistical variation at a Ca-to-P ratio of 0.82, as found in bone biology studies in the literature. In the mandibles of rabbits that underwent distraction osteogenesis, however, the action of LLLT is clear. Rabbit 2, which was not given LLLT, showed great fluctuation of the composition of bone matrix and evidence of a still immature phase of osteogenesis. With the use of LLLT (rabbit 1), there was greater homogeneity, and the mineral composition of newly formed bone after distraction osteogenesis was variable and changed from the area of greatest bone maturation (P1) to the area of most recently formed bone (P3), even inverting the Ca-to-P ratio.

X-ray diffraction spectroscopy

The diffractograms of all bone samples showed a similar diffraction pattern, as shown in Fig. 3. The (002), (211), (112) and (300) hydroxyapatite diffraction peaks were

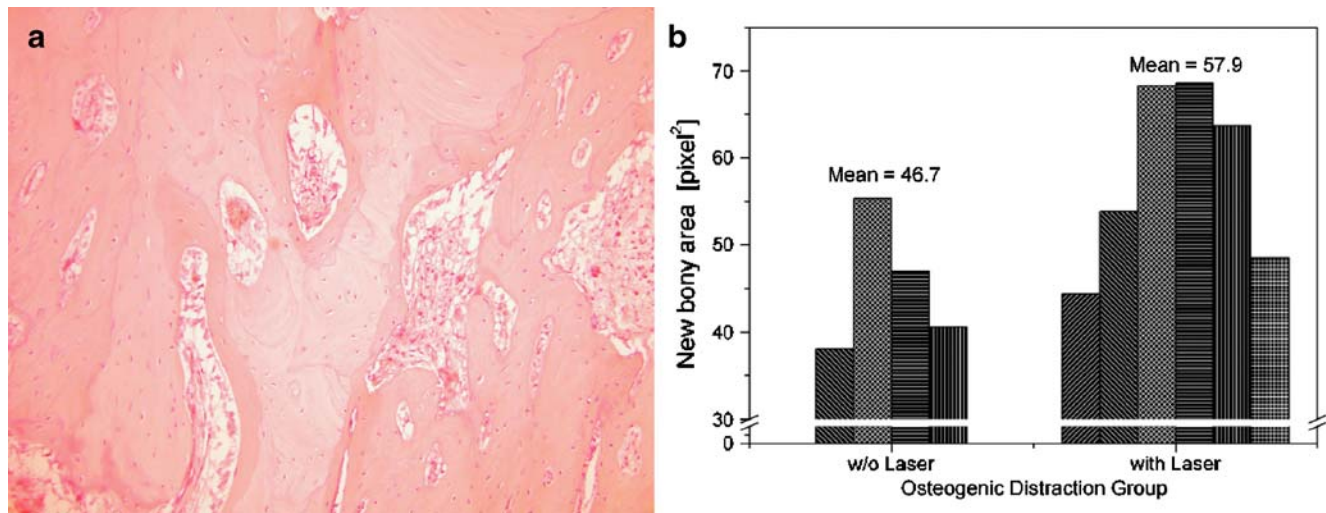


Fig. 1 a Histological analysis of newly formed bone in distraction osteogenesis site of a rabbit that was given LLLT (study group). HE, ×100. b Area of newly formed bone in pixels squared to total area obtained after distraction osteogenesis according to HE analyses (w/o without)

detected at the 2θ angles of 25.9°, 31.8°, 32.1° and 32.9°. Those peaks were found in all samples and quantified the bone crystallinity, as described in the Methods section. Figure 4 shows the crystallinity values for all samples.

Discussion

Few studies in the literature have investigated distraction osteogenesis by XRD and XRF to evaluate the quality of newly formed bone. The results of X-ray fluorescence spectroscopy in this study suggested a trend towards greater mineralization in the groups that were given LLLT. The Ca-to-P ratios of all samples were lower than expected for known pure hydroxyapatite, but they were similar to those expected for bone [15], which demonstrated, therefore, that newly formed bone was detected according to its mineral and organic portions.

The comparison of Ca-to-P ratios in rabbits 1 and 3, both in the LLLT group, suggested that LLLT affected the initial

stages of osteogenesis, which is consistent with findings reported in the literature [16, 17].

Ca and P are the basic components of hydroxyapatite and are found in bone in the form of small crystals that may contain impurities, such as carbonates and magnesium. Such imperfections make bone hydroxyapatite more soluble and promote the ion exchanges that are necessary for homeostasis. The mineral portion of bone contains 96% of the calcium and 85% of the phosphorus found in the human body. The ratios between these elements determine the mechanical properties of bone and are evidence of its organization and maturity [18, 19].

The presence of (002), (211), (112) and (300) diffraction peaks (Fig. 3) in all diffractograms showed that the samples analyzed were hydroxyapatite. Noise is common in bone measurement and may be assigned to the small amount of material available for analysis, which precludes the preparation of a powder with minimally homogeneous granulation. The use of larger animals, such as sheep or pigs, may be a solution for such limitation. This problem may be overcome

Fig. 2 Ca-to-P ratios calculated by X-ray fluorescence measurements for each rabbit, as shown in Table 1

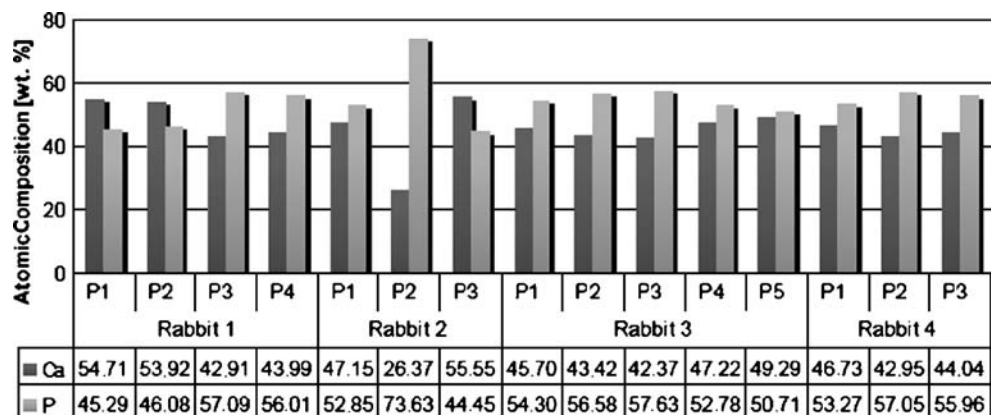


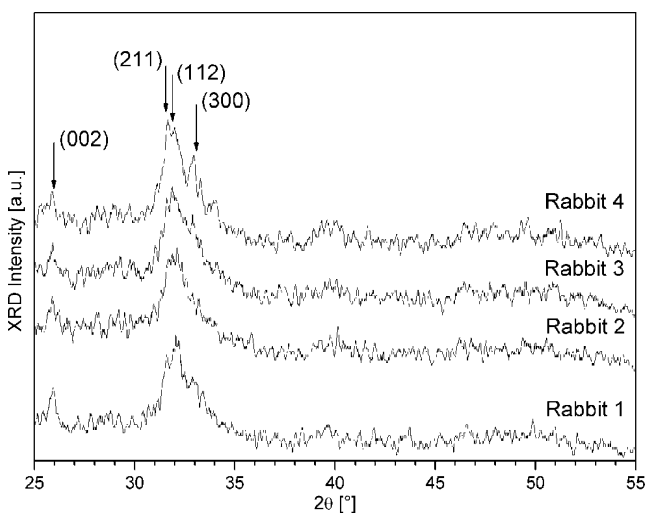
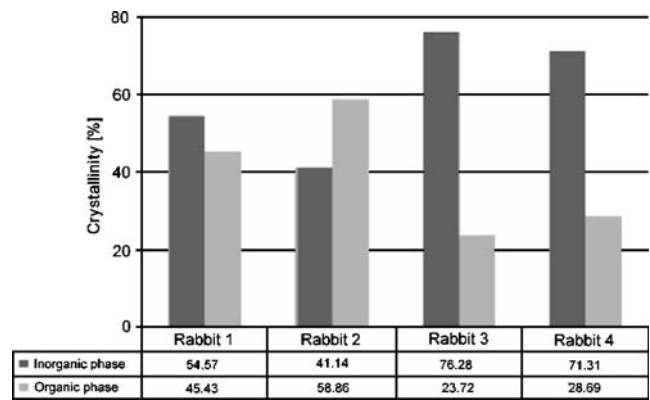
Table 1 Mean Ca-to-P ratios according to rabbit ($n=4$) (SD standard deviation)

Rabbit no.	Mean	SD	Minimum	Maximum
Rabbit 1	0.98	0.24	0.75	1.21
Rabbit 2	0.83	0.44	0.36	1.25
Rabbit 3	0.84	0.09	0.74	0.97
Rabbit 4	0.81	0.06	0.75	0.88

by using mathematical calculations for adjustments, such as the Lorentz calculations, similar to those used for crystallinity. The shape of an XRD peak indicates the crystallinity at a specific diffraction plane: the narrower the peak, the more crystalline the sample.

The crystallinity percentages accurately indicated a biomodulatory effect of LLLT. The comparison of bone with the increase in the crystallinity percentage in rabbits with and without distraction osteogenesis showed that bone crystals were superior in size, order and perfection. The results showed that the percentage of crystallinity of the samples from the rabbits that had been subjected to distraction osteogenesis and had received LLLT was close to that of rabbits that had not undergone distraction osteogenesis, which points to faster bone regeneration with the use of LLLT.

Physical analyses, as previously described, were easily performed and did not require any special sample preparation, such as that required for acid attack for demineralization, precise sectioning for slice preparation, or readings that may be operator dependent. Therefore, the use of crystallinity to measure the level of bone maturity is a rapid and accurate measurement that provides a quantitative value of the variable of interest.

**Fig. 3** X-ray diffractograms for all samples studied (*a.u.* arbitrary units)**Fig. 4** Crystallinity of newly formed, distracted, portion of bone of all samples

Similar crystallinity values were found for rabbits 3 and 4, which had not been subjected to distraction osteogenesis. This finding suggested that the action of LLLT on normal, mature, bone is not significant. The bone of the rabbit that had undergone distraction osteogenesis and had received LLLT was expected to progress faster to crystallinity, as in the rabbit that had not undergone distraction osteogenesis at later bone consolidation times.

Miller et al. [20] found an inverse association between the number of phosphate ions (PO_4^{3-}) and crystallinity. The XRF finding that P content (presence of PO_4^{3-}) was lower in irradiated rabbits may indicate a more mature bone, which is in agreement with the superior crystallinity found in these animals and the irradiated group analyzed by means of percentage of new bone formation. No other studies using XRD analysis of bone samples were found in the literature.

Other studies used XRD to describe the following materials: Zyman et al. [21], ceramic calcium phosphate implants; Hayakawa et al. [22], hydroxyapatite on the surface of titanium implants coated with calcium phosphate; Lobato et al. [23], α - and β -tricalcium phosphate implants; Zhou et al. [24], titanium implant surfaces. However, those studies used the XRD technique only to describe characteristics of commercial materials, differently from what our study focused on: the use of an accurate test for quantitative and qualitative analyses of bone without the influence of operator subjectivity on the results.

Increased crystallinity of the samples in this study, as well as the histological analysis after HE staining, indicated a better quality of newly formed bone. These findings suggest that more mature bone may also have greater mineral organization, which is described by the XRD crystallinity results. The agreement between results of histological analysis after HE staining and crystallinity suggest the possibility of the use of XRD to measure bone quality.

Diversified analysis techniques for the same animal samples were used to maximize the results of studies using animals, and they were in agreement with the current influence of ethics concerns on research committees. Our use of animals that were subjected to distraction osteogenesis without LLLT as control groups was justified by the systemic effect assigned to the independent variable used in this study. Miloro et al. [25] did not take this effect into consideration when comparing the effect of LLLT applied to one side of the animals that underwent bilateral mandibular distraction osteogenesis. The other side was used as a control, but that would limit the inference of the results, even if positive effects of LLLT were found.

Physical analysis does not pose the risk of examiner influence on results, but their interpretation demands knowledge and experience in the use of specific techniques.

The evaluation of fluorescence spectra brings accurate information about the proportion of Ca-to-P in bone and indicates whether the environment is adequate for the formation of quality bone. The evaluation of X-ray diffractograms indicates whether the phases formed are the ones that correspond to hydroxyapatite, and crystallinity shows the maturation stage of newly formed bone.

The results of this study showed that LLLT had a positive effect on the percentage of newly formed bone, on the chemical composition according to the Ca-to-P ratios, and on the crystallinity and crystalline structure according to the detection of hydroxyapatite phases in the distraction osteogenesis sites.

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