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Usage of Low Level Laser Biostimulation and Platelet Rich Fibrin in Bone Healing: Experimental Study

Zastosowanie lasera biostymulacyjnego z osoczem bogatopłytkowym w gojeniu się kości

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Abstract

Background. Experimental studies have shown that low level laser therapy (LLLT) has a positive local biostimulative effect in the early stage of bone healing. Platelet rich fibrin (PRF) also has been shown to be effective in the treatment of intrabony periodontal defects.

Objectives. The objective of our experimental study was to demonstrate the combined effects of LLLT and PRF on bone healing.

Material and Methods. Our experimental study was done over 80 bony cavities in 20 adult male rabbits, aged 12 months. An incision was made for exposure of the femur bone of all rabbits. Then, by using a large, round surgical bur, a perforated hole was made in the femur. The cavities induced in these rabbits were divided into 4 groups: The control group which was neither subjected to any laser irradiation nor filled with any bone substitute (group I); The bony defects were filled with PRF (group II); The cavities were subjected to low level laser (LLL) for biostimulation (group III); The cavities were subjected to LLL for biostimulation then were filled with PRF (group IV). Histological assessments of the four groups were done using a hematoxylin and eosin stain. Statistical analysis was done using ANOVA and Bonferroni tests for comparisons between the four groups.

Results. The area percentage of the newly formed bone in group IV was significantly higher than the other three groups. The area percentage of the newly formed bone in group III is significantly higher than group II.

Conclusions. LLLT could induce bone formation in the bone defect at a faster rate than PRF. However, a combination of both LLLT and PRF as treatment modalities could induce bone formation in the bone defect more than that of LLLT or PRF alone (**Dent. Med. Probl. 2016, 53, 3, 338–344**).

Key words: low level laser, biostimulation, experimental study, bone healing, platelet rich fibrin.

Słowa kluczowe: laser niskoenerygetyczny, biostymulacja, badanie eksperymentalne, gojenie się kości, osocze bogatopłytkowe.

Low level laser therapy (LLLT) is defined as supplying direct biostimulative light energy to the cells. The wound healing process could be enhanced using low-level semiconductor diode lasers [1]. It has

been reported that absorbed laser energy stimulates the molecules and atoms of the body's cells [2].

While several studies have demonstrated that LLLT has stimulating effects on stem cells of the

bone and could accelerate the bone repair process [3–6], others have reported delayed fracture healing or no effects after low-intensity laser irradiation [7, 8]. A narrative review has shown that low intensity laser therapy could accelerate bone healing in extraction sites and bone fracture defects [9].

Platelet rich fibrin (PRF) is a fibrin matrix in which platelet cytokines, growth factors and cells are confined [10]. Choukroun and his collaborators were amongst the pioneers for using a PRF protocol in oral and maxillofacial surgery to improve bone healing in implant dentistry [11].

Recent reviews have reported that the healing benefits of platelet-rich preparations along with the low risk and availability of simple preparation procedures should encourage more clinicians to incorporate platelet-rich products in their practice to accelerate healing and reduce adverse events, especially in oral and maxillofacial surgery [12–14].

Many of the published experimental studies have assessed the effects of either LLLT or PRF on bone healing; however, an evaluation of the combination of these modalities (LLLT and PRF) has not yet been considered.

The objective of our experimental study was to demonstrate the combined effects of LLLT and PRF on bone healing.

Material and Methods

This study was done over 80 bony cavities in 20 adult male rabbits, aged 12 months and with an average body weight of 3 kg. Before the procedures, all the rabbits were separated from each other, then they were acclimatized in the laboratory environment for 5 days. They were fed by a special, pelleted commercial diet.

The animals were anesthetized using general anesthesia with intra-muscular injections of ketamine and barbiturate.

Five milliliter blood samples were collected for each rabbit using capillary tubes from the inner canthus of the eye into syringes without anti-coagulants. The balancers were prepared at the same weight by using test tubes with equal amounts of water. The tubes were placed in a centrifuge on opposite sides. The program of centrifugation was programmed at 30,000 RPM for 14 min. Three components were noted and were taken out of the syringes. The hard gel (as little as 1 mL), which was platelet rich fibrin (PRF), was picked up with forceps. It included an increased amount of cytokines and fibrin.

The surgical field was prepared for the surgical intervention by being shaved carefully, then

was sterilized using spirits and hibitane. An incision was made for exposure of the femur bone of all rabbits. Two holes in each femur, with a diameter of 2 mm, were prepared by using a round surgical bur revolving at low speed (25000 rpm) with copious physiological saline irrigation. The cavities induced in these rabbits were divided into 4 groups. Each group contained 20 cavities in the same 20 rabbits.

The first group (group I): This was the control group which was neither subjected to any laser irradiation nor filled with any bone substitute.

The second group (group II): The induced bony defects were filled with PRF as a natural and satisfactory alternative to a bone substitute. It appears as a bioactive surgical additive to regulate inflammation and increase the speed of the healing process [15].

The third group (group III): The induced cavities were subjected to Low Level Laser (LLL) for biostimulation using a single application of a 100 mW diode laser (Ga As) continuously for 1 m, intra-operatively. A gallium aluminum arsenic (GaAlAs) diode laser was then applied at a continuous wavelength of 808 nm, at a power output of 0.1 w (100 mW), for 120 s (2 min). A dose of 4 J/cm² was applied to the defect [16].

The fourth group (group IV): The cavities were subjected to LLL for biostimulation using a single application of the 100 mW diode laser (Ga As) continuously for 1 min intra-operatively, then were filled with PRF as natural bone substitute.

The flaps were sutured immediately for all samples. The rabbits were given long-acting benzathine penicillin (400000IU) intra-muscularly as a single dose injection immediately after suturing the flaps. The rabbits were inspected and examined regularly to be sure that they had a normal appearance, weight and activity. The rabbits were sacrificed at 1 month after the surgery, because callus formation takes a minimum of ten days and an uncomplicated fracture may heal by 6 weeks [17]. Healing was expected to be faster than this, when using various treatment modalities that initiate bone healing such as laser and PRF.

Histo-pathological evaluations of the bone filling of the induced cavities were performed. The femurs were fixed in 10% formol calcium for 48 hours then washed and soaked for 8 weeks in 10% ethylene diamine tetraacetic acid (EDTA) for decalcification. After that, the decalcified tibias were rinsed in distilled water, dehydrated in ascending grades of alcohol then embedded in paraffin. Sections of 5 µm were obtained and subjected to hematoxylin and eosin (H&E) staining for routine histological examination.

A histo-morphometric analysis was carried out by one investigator and the slides were coded so that the investigator was blind to the test groups. The histo-morphometric data was obtained using a Leica QWin® 500 image analyzer computer system using Leica QWin 500 software (Leica Microsystems, Wetzlar, Germany). The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The area and area percentage of the newly formed bone trabeculae were measured using an objective lens with a magnification of $\times 20$. Ten fields were measured for each specimen. Using the color detector, newly formed bone areas were masked by a blue binary color. The bone area percentage (bone area %) was calculated in relation to a standard measuring frame with an area of $118476.6 \mu\text{m}^2$.

A data entry screen was built using Microsoft Excel. The data was checked, coded and entered on the computer. Double data checking was done. First, the following descriptive analysis was done: mean and standard deviation. Thereafter, general comparisons were done using ANOVA and Bonferroni tests for comparisons between the four groups. The level of significance was set at $p < 0.05$. The analysis of the data was done using SPSS (Statistical Package for Social Science) software version 15.0.

Results

Histological Results

Group I (control group): The histological evaluation of the decalcified specimens from group I (defects left without treatment) revealed patchy filling of the large-sized defect with newly ossified areas and predominantly fibrotic tissue. On the surface of the defect, there was an area of regenerated cortical bone. On the sides of the defect, the newly formed bone showed thicker bone trabeculation compared to that on the surface of the defect (Fig. 1). In most of the specimens, the newly-formed bone appeared basophilic, indicating less mineral content. Large spaces containing normal-looking fatty bone marrow were observed. Some areas of the bone marrow showed fibrosis and scattered areas of chronic inflammatory cell infiltration. The osteocytes showed an increased quantity, indicating that the newly-formed bone trabeculae were immature (Fig. 2C, D). There were many reversal lines, indicating post-osteoclastic activity and bone remodeling in the defect area (Fig. 2A–D). No haversian systems were detected in the newly-formed bone in the defect area.

Fig. 1. A photomicrograph of group I showing: A) the defect mostly filled with fibrous tissue (asterisks) and patches of newly-formed bone (arrows); B) higher magnification showing the osteoid tissue (arrow heads); C) areas of regenerated cortical bone (arrows) observed on the defect surface and sides; D) the newly-formed bone (arrows) appeared basophilic with large marrow cavities (mc). Original magnification: (A) $\times 40$, (B) $\times 100$, (C) $\times 40$, (D) $\times 100$

Fig. 2. A photomicrograph of group I showing: newly formed bone trabeculae (arrows) on the surface and sides of the defect (A, C & D). Reversal lines (arrow heads) between newly-formed bone (arrows) and old bone (ob). Original magnification: (A) $\times 40$, (B) $\times 100$, (C) $\times 40$, (D) $\times 100$

Group II (PRF group): The histological examination of the decalcified specimens from group II (defects treated with PRF) revealed a decreased defect size which was filled with newly ossified areas of thickened bone trabeculae. On the surface of the defect, there was thicker regenerated cortical bone compared to group I. On the sides of the defect, the newly-formed bone showed thick bone trabeculation resembling the old bone.

Fig. 3. A photomicrograph of group II showing: thicker bone trabeculae (arrows) on the surface and sides of the defect. Reversal lines (arrow heads) viewed between the newly-formed bone (arrows) and old bone (ob) (A and B). Cartilage cells (cc) and endochondral bone formation (eb) were observed (C & D). Original magnification: (A) $\times 40$, (B) $\times 100$, (C) $\times 40$, (D) $\times 100$

In most of the specimens, the newly-formed bone appeared more acidophilic in comparison to the control group, indicating greater mineral content (Fig. 3A, B). The marrow cavities appeared of smaller size in comparison to the control group (Fig. 3B). The bone marrow showed large, dilated blood vessels and fewer areas of fibrosis when compared to those in the control group. The osteocytes were fewer in number and were more regularly arranged, indicating more mature bone trabeculae. Regular osteons were detected in most of the specimens. In other specimens, cartilage cells and endochondral bone formation were observed (Fig. 3).

Group III (laser group): The histological examination of the decalcified specimens from group III (defects treated with laser) revealed that the defect was almost filled with irregular bone trabeculation. In some specimens, the surface and the sides of the defect showed thick bone trabeculation resembling the old bone. The newly-formed bone lamellae were differentiated from old bone lamellae as the former were oriented perpendicular to the long axis of the shaft of the long bone while the old lamellae are parallel to the outer circumference of the long bone. Also, the presence of many reversal lines that separate the new bone and the old bone were noted, which indicated bone remodeling activity.

The marrow cavities appeared to be of smaller size. In some specimens, the osteocytes were of regular arrangement, indicating mature bone trabeculae. Well-formed osteons were detected

Fig. 4. A photomicrograph of group III showing: thick irregular new bone trabeculae (nb) filling the defect. Reversal lines (arrow heads) between old bone (ob) and new bone (nb) are obvious. Regular osteons were detected (os) (A, B, C and D). Original magnification: (A) $\times 40$, (B) $\times 100$, (C) $\times 40$, (D) $\times 100$

in some specimens. There were many reversal lines, indicating post-osteoclastic activity and bone remodeling in the defect area (Fig. 4).

Group IV (laser and PRF group): The histological examination of the decalcified specimens from group IV (defects treated with laser and PRF) revealed that the defect was almost filled with well-formed bone trabeculation. On the surface of the defect and on the sides, there were thick bone trabeculations resembling the old bone. In most of the specimens, the newly-formed bone appeared acidophilic, indicating maturation of the formed bone. The only difference between the old and the new bone was the direction of the bone trabeculae, as in old bone, the lamellae are parallel to the outer circumference of the long bone. However, the new bone trabeculae appeared perpendicular to old bone trabeculae (Fig. 5A–D). The marrow cavities appeared to be of very small area in comparison to the control group (Fig. 5A–D). The osteocytes had regular arrangement, indicating mature bone trabeculae. A large number of well-formed osteons was detected in most of the specimens. Normal haversian systems, haversian canals and interstitial lamellae were observed in most of the specimens (Fig. 5).

The software of the morphometric analysis enabled us to outline the area of the newly-formed bone in the defect site, masked by a blue binary color, and also exclude other areas of the bone marrow cavity, old bone on the periphery of the defect or any other spaces not included in the area of the defect.

Fig. 5. A photomicrograph of group IV showing well-formed new bone trabeculae (nb) resembling old bone (ob), small marrow cavities (mc) and regular osteons (os) (A–D). Original magnification: (A) $\times 40$, (B) $\times 100$, (C) $\times 40$, (D) $\times 100$

Statistical results

Table 1 shows the comparative analysis regarding area % between the four groups. A statistically significant difference was noticed between the four groups, according to the ANOVA test.

Discussion

Our experimental study evaluated the effects of LLLT, PRF and a combination of both on bone healing. We aimed to compare these 3 modalities of treatment with a control placebo group. To our knowledge, there is no experimental study that has evaluated the combined effect of LLLT and PRF on bone healing.

Before reaching conclusions based on the present results, it is necessary to consider a number of potential limitations. Although our methodology can be applied in different settings, these results pertain solely to animal experimental studies and cannot be considered generalizable. Our study was conducted on 80 rabbits and response to LLL bio-

stimulation and/or PRF in humans could be different. However, our findings were consistent and coherent, strongly indicating the external validity of the study.

Development of the bioactive surgical additives used to regulate inflammation and increase the speed of the healing process is one of the challenges of clinical and experimental research.

PRF consists of an autologous leukocyte-platelet-rich fibrin matrix, composed of a tetramolecular structure, with cytokines, platelets, and stem cells within it, which acts as a biodegradable scaffold that favors the development of microvascularization and is able to guide epithelial cell migration to its surface [15].

PRF has also been shown to be an effective treatment for intrabony periodontal defects. In a split-mouth study design, 20 patients were treated with nanocrystalline hydroxyapatite (NcHA) alone or NcHA with PRF. The results showed that NcHA bone graft in combination with PRF had clinical advantages superior to that achieved by the NcHA alone [18].

The use of PRF in the present investigation as one of the treatment modalities in bony surgical defect showed stimulation of bone formation greater than that formed in the control group, with statistically significant difference ($p < 0.000$).

The application of LLLT has shown to increase mitochondrial activity, DNA/RNA synthesis in osteoblasts, cell viability and alkaline phosphatase [19].

A recent experimental study showed that LLLT had a positive local biostimulative effect in the early stages of bone healing [20]. In concordance, our results showed better bone healing after LLLT compared to the control group, with statistically significant difference ($p < 0.000$).

The use of LLLT has been used to accelerate healing in large bone defects [21, 22]. It is considered a noninvasive, safe technique to stimulate osteogenesis [23, 24]. Several previous studies have shown that LLLT could increase both osteoblast and osteoclast activities, together with stimulating the formation of callus and new bone matrix [25–28].

Table 1. Comparative analysis regarding area % between the four groups. A statistically significant difference was noticed between the four groups, according to the ANOVA test

Groups	Number	Mean	Standard Deviation	95% Confidence Interval
Group I	20	28.76	8.48	24.79–32.73
Group II	20	45.99	10.82	40.93–51.05
Group III	20	43.89	8.76	39.79–47.98
Group IV	20	66.48	6.33	63.52–69.44

ANOVA = 62.8; P-value < 0.001 (very highly significant).

Of note, LLLT alone as a treatment modality in surgical defects enhanced faster and a greater area of bone formation compared to the PRF treatment modality.

Bone healing is a complex and lengthy process of inflammation, bone formation, and bone remodeling. The mechanisms of action of LLLT and PRF in accelerating the bone healing process are different. The synergic effect of both LLLT and PRF should be superior to using them separately. To date, no study has evaluated that possible synergic effect.

In this experiment, the combined effect of

LLLT with PRF as a treatment modality in bony surgical defects showed the greatest amount of bone formation with the best quality of the newly-formed bone. Histological examination and statistical analysis confirmed the superiority of combining the bioactive surgical additive PRF and LLLT in bone repair.

In conclusion, LLLT could induce bone formation in bone defects at a faster rate than PRF. However, the combination of both LLLT and PRF as treatment modalities could induce bone formation in bone defects more than that of LLLT or PRF alone.

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