International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-8, Issue-4, 2017, pp1121-1128
http://www.bipublication.com

Research Article

Histologic Examination of the Effects of Low Level Diode Laser Irradiation and Nano-Hydroxyapatite Grafting Materials on Ossification Process on Rat Calvaria Defects

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ABSTRACT:
Introduction: Periodontitis is one of the most common infectious diseases in humans.aggressive periodontitis causes rapid destruction of the tissues retaining teeth, especially in young people. For this reason, preventing from development and progression is one of the goals of dentists and especially periodontologists. The aim of this study was to evaluate the effects of low level laser therapy (LLLT) on bone formation in critical size defects in ratcalvariawith or without using nano-hydroxyapatite (nHA).

Materials and Methods: This study was performed on 56 adult female Wistar rats weighing 300-250 gr. Animals were anesthetized by intramuscular injection of Xylazine(0.02 ml/kg) and Ketamine Hydrochloride (0.4 ml/kg).A CSD of 5 mm in size was created by surgery. Animals were randomly divided into 4 groups: **1.**control group (filled with blood clot),**2.**nHA group,**3.**LLLT group, and**4.**nHA+LLLT group.Animals treated with LLLT received a laser diode with a wavelength of 980 nm, a power of 300 mW and a dose of 4 J/cm^2in a continuous wave mode.The first radiation was made before the flap was closed,the animals were exposed to radiation every other day for 15 days. 4 and 8 weeks later, 7 rats from each groupwere euthanized, and after decalcification and staining with Hematoxylin and Eosin (H & E), histologic and histomorphometricevaluations were performed and the results were evaluated by one-way ANOVA and Kruskallwallis analysis.

Results: The results of this study did not show any significant differences in the type of newly formed bone, foreign body reaction, and inflammation severity between weeks 4 and 8. Only the inflammation rate in the nHA group was significantly higher than the other groups and in the week 4 ($p < 0.05$). Also, the average mass of newly formed bone in all groups showed a significantly different between weeks 4 and 8 ($P < 0.05$).

Conclusion: The use of LLLT in combination with nHA can stimulate bone regeneration. The freshly bone formed values in LLLT group was similar to using nHA. Therefore, LLLT may be clinically useful in stimulating bone formation in skeletal defects.

Keywords: Low level laser irradiation, Bone formation, Bone grafts, Animal study

INTRODUCTION:
Today, periodontitis is one of the most common infectious diseases in humans. The chronic type of periodontitis affects over 50% of individuals and its aggressive type, which causes rapid

destruction of the tissues retaining teethespecially in young people, affects about 1% of individuals (1). For this reason, preventing from development and progression is one of the goals of dentists and especially periodontologists (2). To date, many methods have been developed to regeneratedegraded periodontal tissues. For this purpose, a variety of absorbable and non-absorbable membranes, as well as grafting materials and biological intermediates, such as growth factors, are used, each of which has its own advantages and disadvantages (3).Materials used in regenerative treatments are divided into four categories:**1.**Autograft, in which graft material is prepared from the person himself, **2.**Allograft,graft material is taken from another person, **3.**Xenograft,in which graft material is taken from other species, and **4.**Alloplast,in which graft material is made industrially (1, 3-7). Numerous calcium phosphate biomaterials have been used since the mid-1970s and are now available for clinical use. These biomaterials have excellent biocompatibility and do not cause inflammation or foreign body reaction (8). There are two types of calcium phosphate:**1.**Hydroxyapatite (HA), which is non-absorbable, and **2.**TricalciumPhosphate (TCP), which is absorbable (1). Among these, Hydroxyapatite, due to its structural similarity to bone components, is used as an alternative to autografts in orthopedic grafts and in dental applications and maxillofacial surgeries(7, 9, 10).

Hydroxyapatite is derived from various materials in nature such as teeth, bones and corals, as well as through industrial processes (9). The size of the hydroxyapatite particles can be as small as micron or nano.The production of hydroxyapatite nanoparticles has been of interest to many researchers in recent years, and it is suggested that these nanometricparticles exhibit better biological and regenerative properties than micrometric particles (8,11). In addition, numerous studies have shown that low level laser has biologic inducing effectsat the cellular level and it can affect bone repair and lesion regenerations in dentistry. The main mechanism is based on the absorption of photons by specific molecular chromophores, which affect the mitochondrial respiratory chain following excitation by specific light wavelengths. Following this induction, metabolism and cell proliferation increase (13-12) and it can increase adenosine triphosphate levels and accelerate the collagen synthesis and angiogenesis(14) and may contribute to increased synthesis of growth factors. Studies have shown the effect of low level laser on stimulating the blood flow, activating osteoblasts, bone formation, and reduced activity of osteoclasts, and it is said that it has anti-inflammatory effects and improves the bone strength during regeneration process (15). However, some studies have not shown the laser efficiency, which can be due to inappropriate selection of radiation parameters (16), and consequently, there is still a controversy in this regard.

Soares et al. (2014) in a review study in Brazil studied the effect of high and low level laser combined with different materials in intra-bone lesions and furcation lesions.The study is conducted on available articles in PubMed, and they concluded that the use of low level laser has a positive effect on regeneration of periodontal tissue, while high level laser does not seem to have a positive effect on improving the EMD properties and lesion regeneration (17).

The aim of the present study was to investigate the histologic and histomorphometric effect of low level diode laser on regeneration process of bone lesions in rat calvarial defect with or without the use of nano-hydroxyapatite, so that a step may be taken in improving the treatment outcome of periodontal lesions.However, it is worth noting that the effects of laser and nano-Hydroxyapatite simultaneously have not been investigated in studies.

MATERIALS AND METHODS:

This is an interventional (semi-experimental) study.The method used for this study included seven steps:

Step 1: Preparation of Nano-Hydroxyapatite powder: In this study, Nano-Hydroxyapatite powder prepared by sol-gel combustion method was used, which was preparedin Department of Physics ofShahidChamran University of Ahvaz and it was previously used in a study conductedon rat skulls.

Step 2: Preparation of Rats: 56 adult female Wistarrats weighing 200 to 250 grams were prepared from Laboratory Animal Reproduction Center of Ahvaz Jundishapur University of Medical Sciences, and they were kept inspecial metal cages (1 head per cage), at appropriate temperature and humidity (21 ° C), and 12 hours of light-dark conditions.

Step 3: Surgery in Rats:General anesthesia was performed by intramuscular injectionof a mixture of 5 mg/kg Xylazine(AlphaZen - Holland) and 75 mg/kg Ketamine (AlphaZen - Holland), and rats were alsoput under prophylaxis using intramuscular injection of Penicillin G (25000U/kg). Surgical site was parietal bone of animals.

Then, animals were randomly divided into four groups of 14:

1. Control group (Control:C): The blood clot only filled the defect.
2. Nano-HydroxyApatite (nHA) group:Nano-Hydroxyapatite prepared in Department of Physics of ShahidChamran University of Ahvaz, which had previously been prepared and tested in a study, was placed in the defect and we avoid squeezing materials in the area (18).
3. Low Level Laser Therapy (LLLT): The area was exposed to laser diode radiation with a wavelength of 980 nm, a power of 300 mW and a dose of 4 J/cm^2 (Quicklase machine, UK).It should be noted that radiation was performed precisely before closing the flap and as non-contact and continuous wave mode.
4. Nano-Hydroxyapatite group with laser irradiation (nHA+LLLT): In this group, the laser was radiated after placing the material and before closing the flap, similar to LLLT group.

Then, the flap was closed as simple interrupted suture with absorbing Catgut Chromic Suture (Supa Medical, Iran),the periosteumwas used as membrane in all the animals (20).

After the surgery, animals were taken to a room with a constant temperature of 21 ° C and kept in separate cages. For controlling the postoperative pain, 0.05 ml of Ketoprofen was given daily to animals for three days (18).

Step 4: Low level irradiation after the surgery: Then, rats in groups 3 and 4 were exposed to laser radiationin defect areas every other day for 15 days (21, 22).

Step 5: Killing therats: Five rats from each group, four weeks after the surgery, and other animals, eight weeks after the surgery (19),first were anesthetized by re-injection of excess Ketamine Hydrochloride and then were killed by cervical vertebrae displacement. Then, the area was revealed by a longitudinal cut, and the surgical area was removed along with a portion of the healthy bone around it usingsurgical mill andwashing with saline.

Step 6: Preparation of the sample for histologic examination: Then, the samples were put under fixationin 10% buffer for 5 days, and in order to prepare histological samples,they were transferred to Pathology Department of the Dental School of Ahvaz University of Medical Sciences.

Step 7: Histologic and histomorphometric examinations:To evaluate the quality and quantity of boneformation,microscopic slides were observed by anexperienced pathologistsand by using an optical microscope (Nika ECLIPSE, E200 POL, Japan). The slides had abbreviated name and the pathologist was unaware of the grouping. The samples were evaluated in terms of inflammation, foreign body reaction, mass and type of newly formed bone (19,23).To make quantitative examination of bone formation, an eye lens grid was used, which would be super imposed on the sample under optical microscope and with a magnification of 160, and the number of houses filled with newly formed bone in the area was counted and calculated as percentage (24).

The inflammation rate was defined and examined based on the frequency of the inflammatory cells at a magnificationof 400,as lack of inflammation (X<25%), mild inflammation (25%<X<50%), moderate inflammation (X<70%), and severe inflammation (X>70%), and they were given zero, one, two, and three rankings, respectively (19).

The type of newly formed bone (spongy, lamellar, or combination of both) was also characterized by a pathologist and based on it, they were divided into three rankings: **1.** Lamellar bone, **2.**Lamellar and spongy bone, **3.** Spongy bone (25), then data were analyzed statistically.

In ethical considerations:

1. In each group, the minimum number of rats were used.
2. Surgical procedure was performed under anesthesia and completely painless.
3. Rats were killed in a completely painless manner.

Finally, one-way ANOVA was used to compare the amount of bone formation between three groups, and Tuckey test statistical analysis was used for pairwise comparisonof the groups. The nonparametric Kruskal-Wallis test was used for comparing the quality parameters (inflammation rate and bone type), and Mann–Whitney analysis was used for pairwise comparison.

FINDINGS:

The mean mass of newly formed bone in the week 4 for groups of control, nano-Hydroxyapatite (nHA), low level laser therapy (LLLT) and nano-Hydroxyapatite with low level laser therapy(nHA+LLLT) were 1±10.9, 28.5±64.74, 22.4±92.15, and 41±4.34, respectively. These numbersin week 8 for the groups obtained as 17.2±14.45, 41.6±14.95, 5±35.17, and 49±6.79, respectively. T-test analysis showed that all groups had significant differences between weeks 4 and 8 (p <0.05) (Table 1).

Table 1. Comparison of bone mass between weeks 4 and 8

Variable	group	P value
Comparison The percentage of fresh bone formed between weeks 4 and 8	Control	0/000*
	NHA	0/002*
	LLLT	0/001*
	NHA+LLLT	0/025*

Table 2. Comparison of bone formationmass between the groups in week 4

timing	(I)modality group	(J) modality group	Mean Difference (I-J)	P value
4weeks	control	NHA	-18.64286*	*.000
		LLLT	-12.92857*	*.000
		NHA+LLLT	-31.00000*	*.000
	NHA	control	18.64286*	*.000
		LLLT	5.71429	.087
		NHA+LLLT	-12.35714*	*.000
	LLLT	control	12.92857*	*.000
		NHA	-5.71429	.087
		NHA+LLLT	-18.07143*	*.000
	NHA+LLLT	control	31.00000*	*.000
		NHA	12.35714*	*.000
		LLLT	18.07143*	*.000

Table 2 shows the comparison of bone formation mass between groups in week 4.Based on the results, in all groups, inflammationrate was decreased from week 4 to week 8, but this difference was only significant in nHA group (P<0.05), and there were no significant differences in other groups. n multi-group comparison, there was a significant difference only in week 4 (P=0.033) and no significant difference was observed in week 8 (P=0.885). Pairwise comparison of the groups in week 4 showed that nHA group had higher rate of inflammation than all other groups and all of these differences are significant (P<0.05), and other groups showed no significant differences in pairwise comparison.

Also, comparison of the type of bones between weeks 4 and 8 showed a significant difference only in nHA group (P<0.05).

DISCUSSION AND CONCLUSION:

The aim of this study was to investigate the effect of LLLT, nHA and their combination on osteogenesis in rat calvaria. The results of this study showed that the highest level of bone formation was observed in nHA+LLLT and after that groups of nHA, LLLT and control, respectively. This difference in week 4 was significant between all groups, and only two groups of nHA and LLLT had no significant different, while in week 8, nHA had no significant difference with groups of laser and nHA+LLLT, but laser group showed a significant difference with nHA+LLLT group. Therefore, the present study suggests that LLLT may have a positive effect on therepair of bone defects, which this result is consistent with many of the previous studies (14, 15, 19, 26), the cause of this effect, according to previous studies, can be due to increased proliferation potential and osteoblast differentiations or increased vascularization and growth factors in the defected area (26).According to the study,nHA can also have a positive effect on bone regeneration process and this finding was also consistent with most of the previous studies (18, 22, 27). On the other hand, the results observed in the LLLT group were similar to that of the nHAgroup,suggesting that laser application with this protocol could be useful in bone regeneration (22). In other studies, the effects of LLLT with nHA have not been compared,but it has been observed that LLLT can have a similar effect on autogenous bone (15), however, in the study of DrRasuliQahrudi et al., it was reported that BIO-0SS has a greater effect than LLLT (19). In this study, it was observed that the combination of nHA material and low level laser irradiation could improve the outcomes. This result is consistent with the study by WEBER, in which they concluded that combination of autogenous bone and laser irradiation can improve the outcomes (15, 28, 29).However, there was no significant difference between the nHA+LLLT group and the nHA group in week 8,which was consistent with the study on autogenous bone (15). But in week 4, this difference was significant, suggesting that addition of laser radiation to the nHA could be effective in the initial regeneration phase and can be used in extensivelesions (26). In other studies, improvement in outcomes in LLLT combination treatments with BMPs or with enamel matrix derivative (EMD) was observed compare to application of laser and BMP alone (30) or laser and EMD alone (17). All of these results suggest that application of combination therapy can be different from single therapy. In the present study, in weeks 4 and 8, the combination therapy was significantly better than laser therapy alone.

However, it should be noted that there is no general agreement on the parameters of laser application (31).Several studies have argued that a very low or very high dose of a laser is completely ineffective, and even large doses of it can have inhibitory effects (32, 33). Peyer et al. (2006) concluded that a laser with a 3-4 J/cm^2dosage has no effect on bone formation (34), while in this study and other studies, a dose of 4$J/cm^2$was effective on bone formation, especially in week 4 (14, 19). Regarding the inflammation rate, the highest rate of inflammation was seen in the nHA group, which showed a significant difference with all four groups in week 4, and this may be partly due to the reaction to this material in comparison with other groups, which did not use the material,or the application of the laser in the nHA+LLLT group may have somewhat diminished its inflammation.Since laser increases OPG and decreases inflammation (19, 26), inflammation rate in the nHA group is consistent with other studies (18),while in the other study, inflammation rate for BIO-OSS was not greater than the control group (19),however, in this study and other studies on BIO-OSS, there was no foreign body reaction to the material (19). It has been well documented in studies that critical size defects model in ratcalvariais suited for investigating potential biomaterialsand new therapeutic techniques for stimulating bone formation (35, 36). However, it has been proven that defect dimensions are a very important parameter for filling the defect by the bone formation of the walls around the defect (37). Therefore, creating a standard sized critical size defect (CSD) is essential for in vitro testing. In previous studies, it has been observed that small defects and about 3 mm in size can be can be spontaneously filled with osteogenesisof animal body (18).Therefore, in this study and according to previous studies, the diameter of the lesion was considered as 5 mm (15). In general, in this laboratory study on rat calvaria defects, it was observed that LLLT with application of laser diode with a wavelength of 980 nm and 4 J/cm^2dosage can stimulate bone regeneration. The most effects were seen in the nHA+LLLT group and after that in the groups of nHA, LLLT and control, respectively.Therefore, the use of LLLT in clinical practice can be useful in bone formation in skeletal defects.

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