UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

LUCAS MONTEIRO DE VASCONCELOS ALVES DE SOUZA

Effect of low level laser on the healing of critical defects filled with or without autogenous bone at 30 and 60 postoperative days. *In vivo study*

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Effect of low level laser on the healing of critical defects filled with or without autogenous bone at 30 and 60 postoperative days. *In vivo study*

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Orientador: Profª Drª Ana Lúcia Pompéia Fraga de Almeida

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Dedico este trabalho à minha mãe Maria Luzia e meu pai Valter, responsáveis por construir meu conhecimento e guiar meus passos para a odontologia.

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E a todos que direta ou indiretamente fizeram parte da minha formação.

“A primeira glória é a reparação dos erros”

Machado de Assis

RESUMO

Efeito do laser de baixa intensidade na cicatrização de defeitos críticos preenchidos com osso autógeno aos 30 e 60 dias pós-operatórios. Estudo *in vivo*

O sucesso de diversas intervenções odontológicas depende de um eficiente processo de reparo tecidual. Em muitos casos de reabilitação oral com implantes e reparo de defeitos ósseos periodontais, há necessidade de procedimentos de enxertia. O osso autógeno continua sendo o material de enxerto padrão ouro segundo a literatura por apresentar as características de osteogênese, osteocondução e osteoindução. Com intuito de aprimorar o conhecimento a respeito do processo de reparo ósseo com osso autógeno e estudar os efeitos do laser de baixa intensidade na cicatrização óssea, este estudo tem por objetivo avaliar o efeito do laser de baixa intensidade na cicatrização óssea de defeito crítico tratados ou não com osso autógeno (AB) aos 30 e 60 dias pós-operatório. Foram utilizados 80 ratos machos adultos (*Rattus norvegicus, albinus*, Wistar), pesando entre 250 e 300 g. Um defeito ósseo de tamanho crítico com 5 mm de diâmetro foi criado na calvária de cada animal. Os animais foram divididos aleatoriamente em 4 grupos (n= 20) de tratamento: 1) Grupo C (controle preenchido somente com coágulo sanguíneo), 2) Grupo LLL (laser de baixa intensidade – GaAlAs, 780 nm, 100mW, 210J/cm^2, 6J), 3) Grupo AB (enxerto autógeno) e 4) Grupo ABL (enxerto autógeno + laser de baixa intensidade) eutanasiados aos 30 e 60 dias. A quantidade de novo osso (NBF) foi calculada como porcentagem da área do defeito original. Os dados foram submetidos à análise estatística (ANOVA, Teste de Tukey, $p<0,05$). Os grupos AB, LLL e ABL, em ambos os períodos, não apresentaram diferença estatisticamente significante de NBF entre si ($p > 0,05$). Houve diferença estatisticamente significativa ($p < 0,05$) quando AB, LLL e ABL foram comparados aos grupos C aos 30 dias e o grupo AB comparado com C aos 60 dias. Dentro dos limites deste estudo, o LLL não apresentou capacidade biomudolatória no aumento da NBF quando associado ou não ao enxerto autógeno. Além disso, não foi observada diferença estatisticamente significativa quando se comparou a mesma técnica em tempos de 30 e 60 dias de cicatrização. O grupo LLL mostrou maior formação óssea em relação ao grupo C somente em análise de 30 dias, sugerindo capacidade apenas de acelerar a formação óssea.

Palavras-chave: Transplante ósseo, Lasers, Ratos

ABSTRACT

Effect of low level laser on the healing of critical defects filled with or wihout autogenous bone at 30 and 60 postoperative days. In vivo study

The success of various dental interventions depends on an efficient tissue repair process. In many cases of oral rehabilitation with implants and repair of periodontal bone defects, there is a need for grafting procedures. Autogenous bone remains the gold standard grafting material according to the literature because it presents the characteristics of osteogenesis, osteoconduction and osteoinduction. In order to improve the knowledge about the process of bone repair with autogenous bone and to study the effects of low intensity laser on bone healing, this study aims to evaluate the effect of low intensity laser on critical defect bone healing treated with Autogenous bone (AB) at 30 and 60 postoperative days. 80 adult male rats (*Rattus norvegicus, albinus*, Wistar) weighing between 250 and 300 g were used. A critical-size bone defect with a diameter of 5 mm was created in the calvaria of each animal. The animals were randomly divided into 4 groups (n = 20) of treatment: 1) Group C (filled with blood clot), 2) LLL group (low intensity laser - GaAlAs, 780 nm, 100mW, 210J/cm^2, 6J), 3) Group AB (autogenous graft), 4) Group ABL (autogenous graft + laser of low intensity) euthanized at 30 and 60 days. An amount of new bone (NBF) was calculated as a percentage of the original defect area. Data were submitted to statistical analysis (ANOVA, Tukey test, $p < 0.05$). Groups AB, LLL and ABL in both periods do not present statistically significant difference of NBF between their self ($p > 0.05$). There was only statistically significant difference ($p < 0.05$) when AB, LL and ABL was compared to C group at 30 days and group AB was compared to C at 60 days. In conclusion, the LLL did not present biomudolatory capacity in the increase of NBF. Also, no statistically significant difference was observed when comparing the same technique at times of 30 and 60 days of healing. The LLL group showed greater bone formation compared with C group only in 30-days analysis, suggesting capacity of accelerate the bone formation.

Key words: Bone transplantation, Lasers, Rats.

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ABBREVIATION LIST

LLL	Low-level laser
CSD	Critical-size defect
C Group	Control Group
LLL Group	Low-level laser Group
AB Group	Autogenous Bone Group
ABL Group	Autogenous bone + Low-level laser Group
NBF	Percentage of New Bone Formed
TA	Total Area
TI	30 days
TII	60 days
ATP	Adenosine triphosphate

SUMMARY

1 INTRODUCTION

1 **INTRODUCTION**

The tooth loss is followed by the adjacent alveolar bone repair due by the particular ability to regenerate. However, in some situations due to the defect size, the bone tissue is not regenerated completely, turning the aesthetic and functional rehabilitation a challenge. (FARDIN et al, 2010).

Bone quality used in surgery and their physical and biological ability to form new bone are determining factors of success of bone grafting techniques for recovery the volume lost. Among the types of grafts known as homologous, xenogenous and autogenous, the patient's own origin is on the literature which can gather closer to the ideal characteristics. (FARDIN et al, 2010). In addition to the volume, the time needed to bone repair is very important to determine the period required to develop an implant dental Installation in areas of bone graft.

With interest in evolving knowledge and improve the quality of bone repair in grafts, studies have been developed in experimental defects created in rat calvaria. The choice of these animals takes into account the phylogenetic scale, the possibility of genetic and phenotypic standardization and reliable results obtained in scientific studies that evaluated bone repair in this species. (GOMES & FERNANDES, 2011; VAJGEL et al, 2014; JORDAN, 1971)

Evaluations are preferably practiced in critical size defects (CSD), which have sufficient size to not spontaneously repair during the lifetime of these animals. This process allows to evaluate the osteogenic potential of different techniques and types of grafts reliably. (SCHMITZ; HOLLINGER, 1986)

The dimensions most commonly used in calvarial defects are 5 or 8 mm. Defects with 5 mm can already be regarded as critical as in 22 studies conducted defects of this size, there was complete closure in any of the defects made in the center of the calvaria (VAJGEL et al., 2014). Use 5 mm defects are an advantage because being smaller diameter have a lower risk of injury to the sagittal sinus and operative bleeding. (BOSCH et al., 1998)

The evaluation period of the study is also an important factor to consider in choosing the experimental design, and there are reports that the greatest amount of bone formation is found in 4 weeks, decreasing gradually to 24 weeks. (HONMA et al., 2008; VAGJEL et al., 2013) Therefore, it is recommended that the evaluation of bone formation in specimens is carried out within this period.

To stimulate the process of bone formation in autografts, studies have evaluated the potential benefits of low-level laser (LLL) in this type of technique (CUNHA et al 2014). The principle of laser use in biological tissues is based on the photochemical theory provides more speed healing and repair, accelerating the neovascularization increase in ATP synthesis, reduction of intracellular pH, changes in cell proliferation and motility, among other important biological features for repair. (KITCHEN; PARTRIDGE, 1991; CARVALHO, 2011)

The application of LLL has the ability to increase new bone formation by the release of endocrine factors, which express osteoblastic differentiation markers that enhances osteogenesis ability of the fabric. (MANZANO-MORENO et al., 2015; FEKRAZAD et al., 2015; GARCIA et al., 2014)

Despite the known biological potential of LLL in the repair of soft and hard tissues, there is still approaching the universal parameter ideal protocol for use in bone regeneration. Thus, studies undertaken on this subject contribute to the future development of systemic protocols as the different ways of application and applicability of LLL.

2 ARTICLE

2 ARTICLE

ARTICLE

The article presented in this dissertation was written according to instructions and guidelines for article submission presented in **Journal of Periodontology**.

Title: Effect of low-level laser (LLL) on the healing of critical defects with or without autogenous bone at 30 and 60 postoperative days. In vivo study.

2.1 Abstract

Background: The involvement of bone grafts in oral rehabilitation is important in the volumetric recovery of reduced alveolar ridges. The scientific understanding of its healing has collaborated in the improvement of its surgical techniques, as well as in the application of supporting methods in the objective to increase the bone volume conquered. Low-level laser (LLL) is one such method and the objective of this study is to evaluate the effects of LLL on the healing of critical defects filled with autogenous bone at 30 (TI) and 60 (TII) postoperative days.

Methods: Eighty male rats (*Rattus norvegicus, albinus*, Wistar) weighing 250 to 300 g. were divided in 4 groups (n = 20): 1) C Group (control) - 2) LLL Group – Low-level laser (GaAlAs - 780nm, 100mW, 6J, 210J / cm^2), 3) AB Group (autogenous bone), 4) ABL Group (autogenous bone + LLL). Each group was subdivided into two groups of animals sacrificed at 30 (TI) and 60 (TII) days. The quantity of newly formed bone was calculated as percentages. The parametric test ANOVA followed by Tukey's test was used ($p<0.05$).

Results: Groups AB, LLL and ABL in both periods do not present statistically significant difference of BNF between their self ($p > 0.05$). There was only statistically significant difference when AB, LLL and ABL was compared to C group at 30 days and group AB was compared to C at 60 days ($p < 0.05$).

Conclusion: The LLL did not present biomudolatory capacity in the increase of NBF when applied with wavelength of 780 nm, energy density of 210J/cm^2, 6 J per point in times of 60 seconds each application, in single session, in critical size defects created in calvaria of rats grafted with particulate autogenous bone. Also, no

statistically significant difference was observed when comparing the same technique at times of 30 and 60 days of healing. The LLL group showed greater bone formation compared with C group only in 30-days analysis, suggesting capacity of accelerate the bone formation.

Key words: Alveolar Bone Grafting, Low-Level Light Therapy, Osteogenesis.

2.2 Introduction

The tooth loss is followed by the adjacent alveolar bone repair due by the particular ability to regenerate. However, in some situations due to the defect size, the bone tissue is not regenerated completely, making the aesthetic and functional rehabilitation a challenge.[1]

Bone quality used in surgery and their physical and biological ability to form new bone are determining factors of success of bone grafting techniques for recovery the volume lost. Among the types of grafts known as homologous, xenogenous and autogenous, the patient's own origin is on the literature which can gather closer to the ideal characteristics.[1] In addition to the volume, the time needed to bone repair is very important to determine the period required to develop a implant dental Installation in areas of bone graft.

With interest in evolving knowledge and improve the quality of bone repair in grafts, studies have been developed in experimental defects created in rat calvaria. The choice of these animals takes into account the phylogenetic scale, the possibility of genetic and phenotypic standardization and reliable results obtained in scientific studies that evaluated bone repair in this species.[2,3,4] Evaluations are practiced in critical-size defects (CSD), which have sufficient size to not spontaneously repair during the lifetime of these animals. This process allows evaluating the osteogenic potential of different techniques and types of grafts reliably. The dimensions most commonly used in calvarial defects are 5 or 8 mm. Defects with 5 mm can already be regarded as critical as in 22 studies conducted defects of this size, there was complete closure in any of the defects made in the center of the calvaria.[3] Use 5mm defects is an advantage because being smaller diameter have a lower risk of injury to the sagittal sinus and operative bleeding.[6]

The evaluation period of the study is also an important factor to consider in choosing the experimental design, and there are reports that the greatest amount of bone formation is found in 4 weeks, decreasing gradually to 24 weeks.[3,7] Therefore, it is recommended that the evaluation of bone formation in specimens is carried out within this period.

Many studies have evaluated the potential benefits of LLL to stimulate the process of bone formation in autografts. The principle of laser use in biological tissues is based on the photochemical theory provides more speed healing and repair, accelerating the neovascularization increase in ATP synthesis, reduction of intracellular pH, changes in cell proliferation and motility, among other important biological features for repair.[8,9]

The application of LLL has the ability to increase NBF by the release of endocrine factors, which express osteoblastic differentiation markers that enhances osteogenesis ability of the fabric.[10,11,12] Despite the known biological potential of LLL in the repair of soft and hard tissues, there is still approaching the universal parameter ideal protocol for use in bone regeneration.

Thus, studies undertaken on this subject contribute to the future development of systemic protocols as the different ways of application and applicability of LLL.

The objective of this study will be to evaluate the effect of LLL on critical bone healing treated with autogenous bone (AB) at 30 and 60 postoperative days.

2.3 Methods

2.3.1 Experimental Model

The experimental protocol was approved by the Ethics Committee on Animal Education and Research of the University of São Paulo - USP, School of Dentistry of Bauru (CEEPA PROC Nº 018/2016).

Eighty male rats (*Rattus norvegicus*, *albinus*, Wistar) weighing between 250 and 300 g were used (University of São Paulo - USP, Central Animal Laboratory of the School of Dentistry of Bauru). During the whole experimental period the animals were kept in an environment with 12 hours light cycle per day and temperature between 22 and 24ºC, consuming selected solid feed and water ad libitum. The animals were divided equally and randomly into 4 experimental groups (n = 20): 1)

Group C (control): defect filled with blood clot; 2) LLL group: bone defect filled with blood clot and application of LLL - (GaAlAs, 780nm, 100mW, 6J, 210J/cm^2, TheraLase DMC®, São Carlos, São Paulo, Brazil); 3) Group AB: bone defect filled with autogenous bone graft; 4) Group ABL: defect filled with autogenous bone graft and LLL application.

2.3.2 Surgical procedure

The animals were anesthetized by intramuscular injection of xylazine (Vetbrands, Paulinia, Brazil) (0.02 ml/kg) and ketamine hydrochloride (Vetbrands, Paulinia, Brazil) (0.4ml/kg). Antissepsis was performed with PVPI on the dorsal region of the skull of each animal.

The access to the bone tissue was made from a semilunar incision. A trephine drill bit was used to create a CSD of 5 mm diameter under continuous irrigation with sterile physiological saline solution (0.9%). The dura mater was preserved during removal of the bone tissue (parietal bone) to keep the integrity of the brain.

An "L" shaped marking was performed 2 mm anterior and 2 mm posterior to the margins of the surgical defect with diamond drill in the 3069 (Microdont Micro Precision Machining Ltda., São Paulo, Brazil), under continuous irrigation with sterile saline solution and with the aid of a pre-surgical guide made of acrylic resin (Jet, Campo Limpo Paulista, São Paulo, Brazil) after performing the animal defect used in the pilot study. Small cavities were made in the markings region, and filled with amalgam. These markers served to aid in histological and histometric processing, identification of the center of the surgical defect, and location of pre-surgical bone margins.

In Group C, the surgical defects were filled only with blood clot. In the LLL Group the defects were filled with clot and irradiated with the LLL. In Group AB the defect was filled with autogenous bone. In the ABL group, they were filled with autogenous bone and submitted to LLL.

At the end of the procedure, the flap was repositioned and sutured. Each animal received an intramuscular injection of 24,000 units of Penicillin G-benzathine (Pentabiotic Small Porcine Veterinary, Fort Dodge® Animal Health Ltda., Campinas, São Paulo Brazil) and an intramuscular dose of 0.3 mg/kg Ketoprofen (Teuto®, Anapolis, Goiás, Brazil). The operator was a member of the team responsible for

applying the LLL. At the moment of the surgical defect the operator was unaware of the treatment to be performed. The animal was randomly selected after the surgical defect to receive the treatment of one of the groups.

2.3.3 Protocol of application of low-level laser

The laser used was TheraLase DMC®, with active gallium aluminum-arsenide (GaAlAs) in the infrared spectrum, with wavelength of 780 nm, energy density of 210J / cm^2, using 6J per point in 60 second times each application. In total, 5 application points were performed, one center and four located on the margins of the defect at equal distances between them following the "clock" positions (12h, 3h, 6h, 9h) beyond the central point.[13]

2.3.4 Tissue processing

Half of the animals in each group (n = 10) underwent euthanasia at 30 days postoperatively and the other half at 60 days, with 5 mg/ml of the combination of ketamine hydrochloride and xylazine. A solution of 4% Ethylenediamine tetraacetic acid (EDTA) (Dinâmica® Química Contemporânea Ltda., Diadema, Brazil) was used to decalcify the blocks removed from the region containing defects and surrounding areas. The specimens were cut and divided into two halves guided by the amalgam "L" channels. [13]

The specimens were included in paraffin and longitudinal sections 6 µm thick from the center of the original surgical defect were made and subsequently stained by the Hematoxylin and Eosin (HE) techniques, and the first two cuts from each half of the piece from the center for the histometric and histological histological analyzes in light microscopy. [13]

2.3.5 Histomorphometric analysis

The images of the histological sections were captured with Dino-Lite Edge (AM4115TW, 1.3M pixels, 10x~50x) in magnification of 35x and saved in a computer. Two histological sections were selected for the histomorfometric analyzes, representing the central area of the original surgical defect.

Linear measurements were used to delineate the area (in mm^2) of the defect originally created, corresponding to the Total Area (TA), and the area of the bone using Dino-Lite software (Software DinoCapture 2.0, version 1.5.18, Windows 10) Neoformed (NBF). All 5 mm CSDs were performed with a surgical guide standardizing the 2 mm distance from the defect margin to the amalgam channels created anteriorly and posteriorly. Thus, in the histological analysis of the laminas, to determine the total area (TA), the evaluator subtracted 2 mm from each side of the image, resulting in 5 mm of the defect. All bone tissue visualized within this area was detached and calculated as the sum to obtain the area of NBF. [13]

2.3.6 Statistical analysis

The normality and the homoscedasticity of the data were analyzed and they were submitted to the normality test. Thereafter, it was found that TA and NBF data followed the normal distribution. The ANOVA parametric test was used, followed by the Tukey test to verify the differences between the groups. The significance level adopted was 5%.

2.4 Results

In the 30-day period, the laboratory procedure made unviable 3 specimens of the LLL group, 2 specimens of the ABL group, one specimen of C and one specimen of AB. In the period of 60 days, one specimen of the LLL, one specimen of the ABL and 3 specimens of the AB were unviable.

2.4.1 Analysis of histological quality

During histological analysis of 30 and 60 days, the histological characteristics were very similar in both time healings. Inflammatory infiltrate formation was not observed in any of the study groups. In group C, which was filled by clot and was not submitted to LLL, the presence of collagen fibers was observed, forming fibrous connective tissue occupying the area of the critical defect. A small amount of NBF occurred in this group only on the margins of the defect, and the center remained filled with a thin layer of connective tissue (Figure 1).

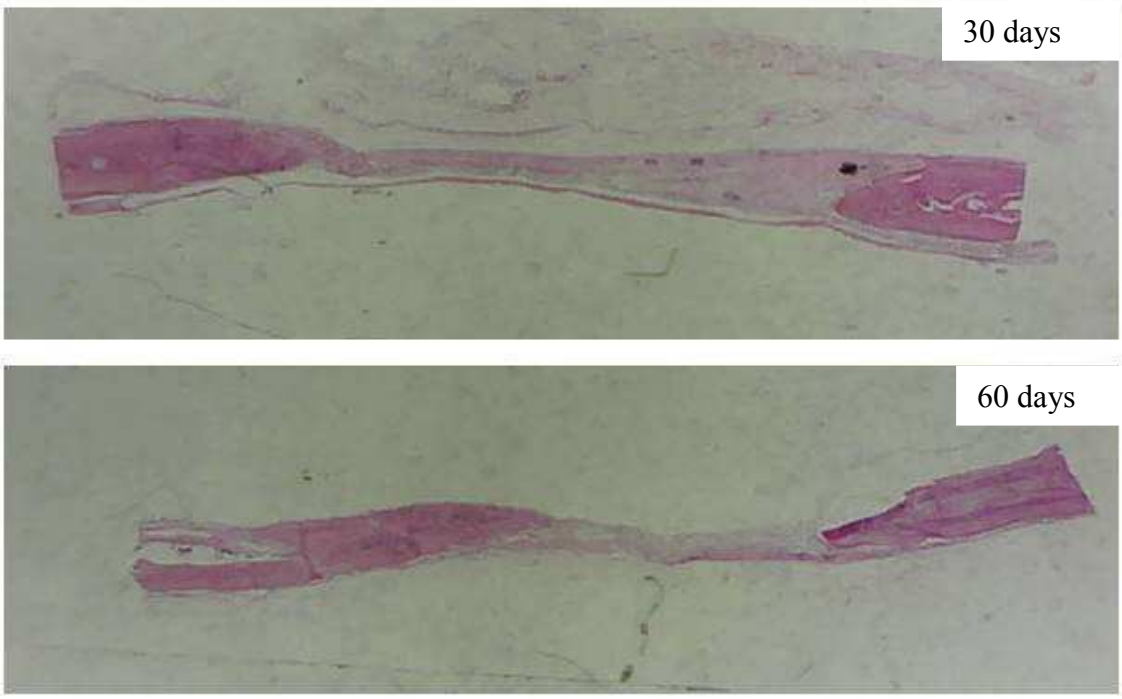


Figure 1 - Microscopic photograph of histological slide group C (30 and 60 days), stained with hematoxylin and eosin (35x), showing small bone formation from the margin of the defect.

In the AB group, which the defect was filled with the animal bone itself, not only the bone formation was observed from the margins of the defect, but also around the graft particles, creating in some slides several osteoid matrix nucleus (Figure 2). Connective tissue with collagen fibers surrounds the nucleus with fibers parallel to each other. Like the previous group described, the ABL group showed very similar characteristics, but with an apparently larger amount of collagenous tissue.

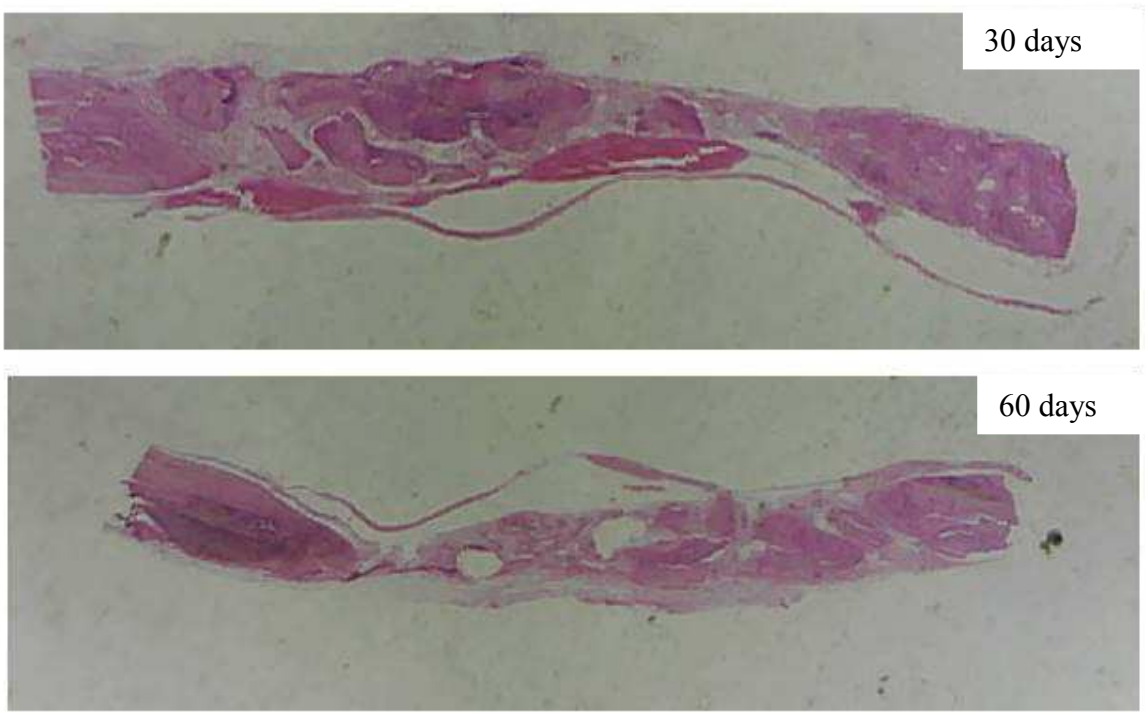


Figure 2: Microscopic photograph of histological slide AB group (30 and 60 days), stained with hematoxylin and eosin (35x), showing bone formation on the margins of the defect and osteoid nuclei involved by collagen fibers.

The LLL group showed characteristics close to the control group, differentiating in the greater presence of collagenous tissue and in the greater amount of NBF in the margin of the defect. Unlike AB and ABL group (Figure 3), no NBF was observed at the center of the defect (Figure 4).

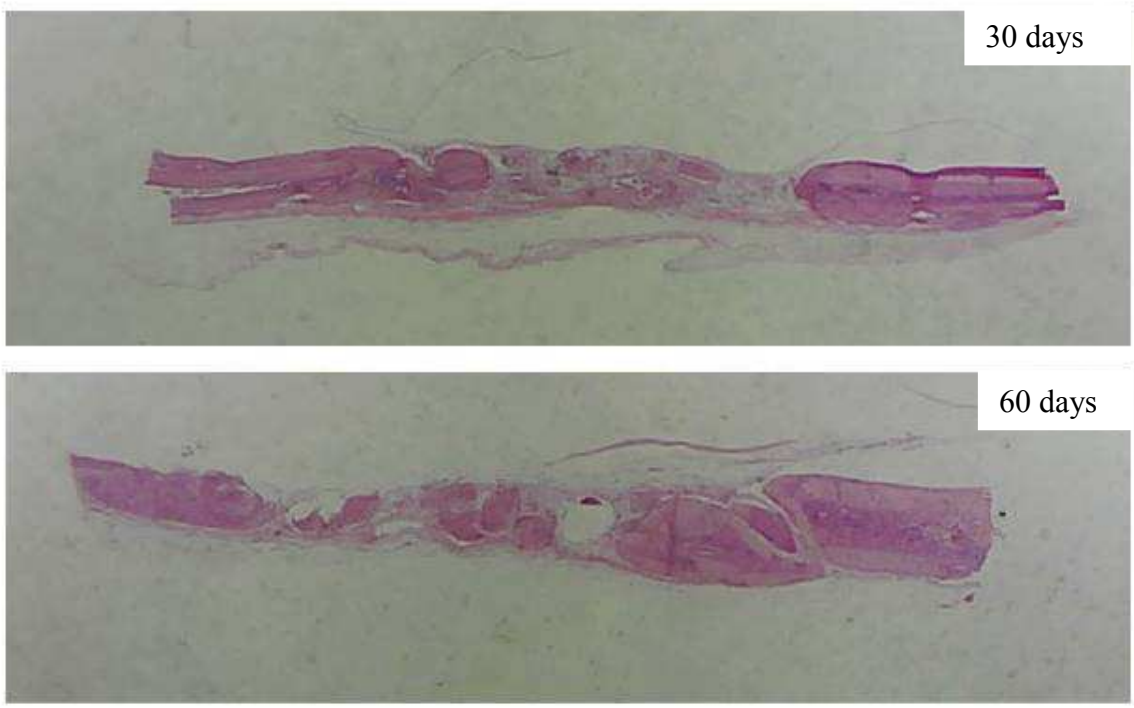


Figure 3: Microscopic photograph of a histological slide ABL group (30 and 60 days), stained with hematoxylin and eosin (35x), which similarly to AB group, shows bone formation in the margins of the defect and osteoid nuclei involved by collagen fibers.

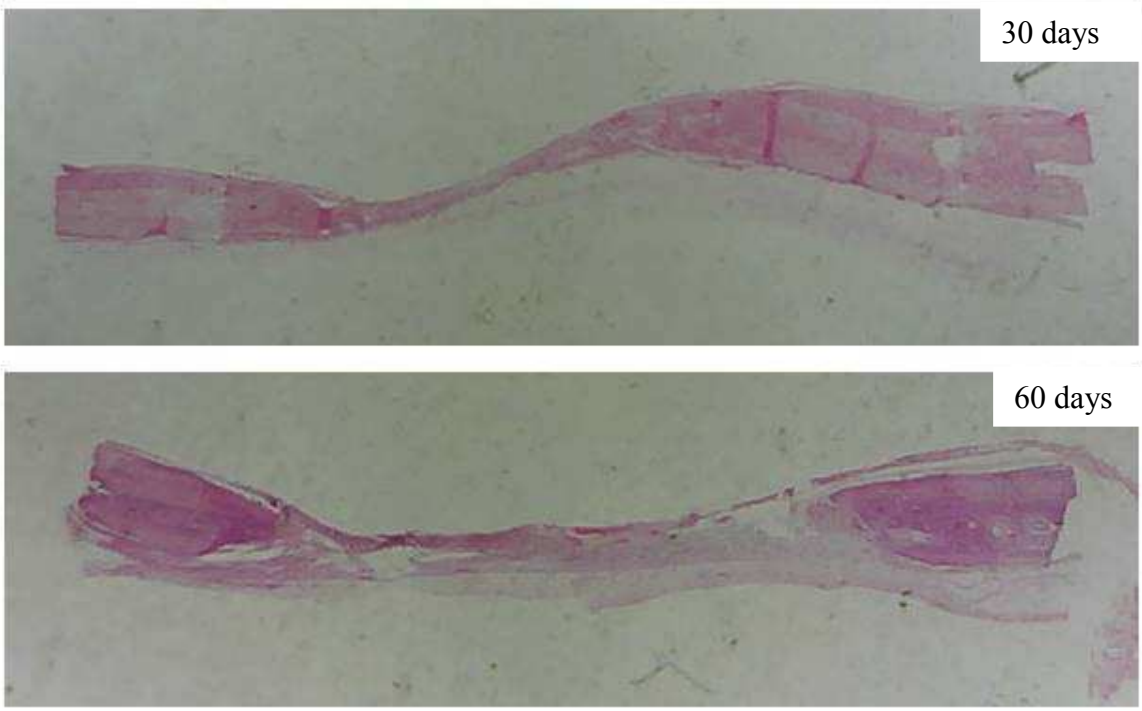
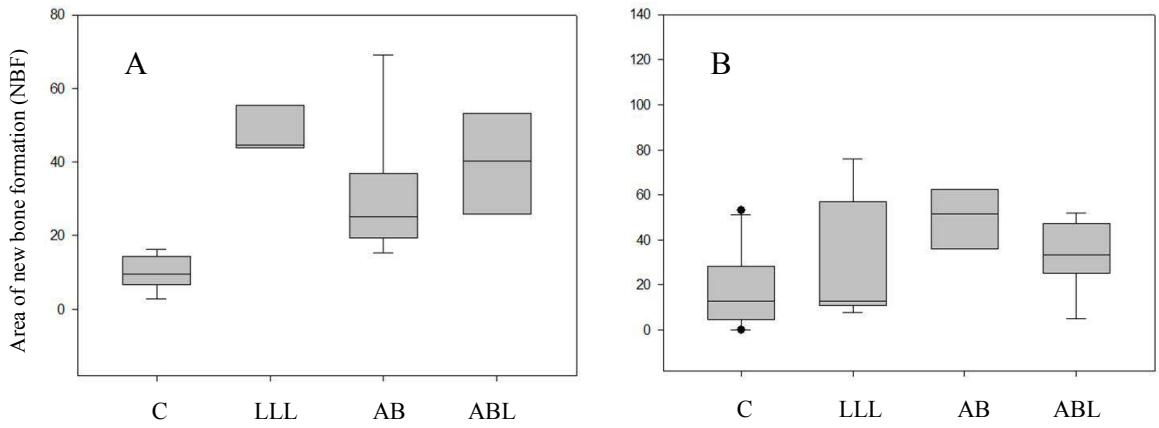


Figure 4: Microscopic photograph of histological slide LLL group (30 and 60 days), stained with hematoxylin and eosin (35x), showing discrete bone formation at the margins of the defect and strict range of collagen fibers in the center.

2.4.2 Histomorphometric and statistical analysis

When comparing the groups in relation to the healing time (Graph 1), the mean amount of NBF increased in 60 days when compared whit 30 days, which osteoid matrix was present in 9.9% and 16.8% of the total area respectively. The same could be observed between the AB groups, as mean NBF of 30.9% in the 30 days and 51.1% in the 60 days. On the other hand, the increase in bone formation in relation to time was not observed in the other groups. The 60-day LLL group (28.9%) presented mean of NBF less than 30 days (47.6%) and the ABL group 60 days (34.1%) also in relation to the 30 days (39.1%). However, these differences are not statistically significant (p > 0,05).



Graph 1 - Distribution of new bone formation area of the groups of 30 days (A) and 60 days (B)

There was a statistical difference in relation to time (Table 1 and 2) between groups (p<0.05) C (TI) x AB (TII); C (TI) x ABL (TII); LLL (TI) x C (TII); AB (TI) x C (TII); AB (TI) x AB (TII); AB (TI) x EA/LB (TII); ABL (TI) x C (TII).

In the 30 days period (Table 1), the groups LLL (47.6%), AB (30.9%) and ABL (39.1%) presented a higher NBF statistically significant (p <0.05) Group C (9.9%). However, these groups did not present significant statistical difference between them. In the 60 days study (Table 2), the only statistically significant difference (p <0.05) occurred between AB (54.3%) and C (17.3%). Figure 5 shows the distribution of NBF area of the 30 days and 60 days groups.

Table 1: Mean and standard deviation of the amount of new bone formation in 30 days

Groups	*N*	Mean	Sd	Q25	Median	Q75
C	9	9,96	4,49	7,61	9,52	13,82
LLL	7	47,67	8,66	43,96	44,58	53,25
AB	9	30,98	16,59	20,16	25,10	36,53
ABL	8	39,15	16,72	26,89	40,41	53,31

Table 2: Mean and standard deviation of the amount of new bone formation at 60 days

Groups	*N*	Mean	Sd	Q25	Median	Q75
C	10	17,38	16,352	6,12	12,96	26,65
LLL	9	28,83	27,643	11,15	13,01	49,09
AB	7	54,39	35,189	37,07	51,59	60,32
ABL	9	33,99	14,952	26,20	33,22	45,19

2.5 Discussion

Low-level laser has become increasingly popular and frequently used in clinical routine as a supporting in different dental treatments. The reconstructions of reduced borders and defects from periodontal diseases, through bone grafts, are part of the dental procedures that the LLL could collaborate with.

Various combinations of density, power and wavelength have been employed in studies of bone repair involving LLL.[14] The protocol used in this study was a single application during the surgical procedure with wavelength specifications of 780 nm, energy density and each point received an energy dose of 6 J (210 J/cm^2 of energy density) delivered on the system, 60 s per point.

The process of formation of new bone in grafted regions involves numerous cellular and biochemical events. The current hypothesis is that LLL can collaborate in the process of osteogenesis by having properties that stimulate cell proliferation, cell differentiation and blood flow. Among the cells known to be stimulated by low doses of LLL are osteoblasts[15] and endothelial cells[16] that are protagonists in the process of

bone regeneration. In addition, the anti-inflammatory characteristics of LLL could improve the postoperative period and contribute to tissue healing.

Despite the literature reports that these cells are stimulated by the LLL, the present study did not find statistically significant difference when comparing grafted regions with autogenous bone submitted to the application of LLL with grafted regions not submitted to irradiation (LLL x ABL). This difference was not observed in the two 30 and 60 day histological evaluation periods. Observing this result, it is noted that the laser did not demonstrate the ability to accelerate and/or increase in volume the formation of new autogenous graft bone. The bone formation in the 30 and 60 days ABL group apparently originated from the osteoinduction, osteoconduction and osteogenesis properties of the autogenous graft, since the 30 and 60 days ABL groups did not present a statistically significant difference in relation to the AB of 30 and 60 days.

In spite of this, it can't be affirmed that the LLL did not present biostimulatory abilities in this work, since in 30 days, the group in which the laser was applied in a region filled only by clot (LLL group) presented a statistically significant difference when compared to the Group C of the same period. In this case, it is suggested that the LLL, without the graft association, that is, in a single application, is capable of stimulating bone production at the initial healing time of the CSD created in rat's calvaria.

However, no statistically significant difference was observed between the LLL and C groups in the 60 days healing period. Considering that the 60-day C group had a mean NBF greater than the 30 days C group and that the 60 days LLL group did not present a statistically significant higher NBF in relation to the 30 days LLL group, it may be suggested that the laser Has the ability to accelerate the formation of new bone, that is, shorten the healing time. The unique application of the laser at the surgical moment may also be the reason for the positive result to have occurred only in 30 days.

Another important observation to be made and regarding is about a smaller quantity of new bone formed in days of 60 days compared to 30 days in the irradiated groups compared to the non-irradiated groups. In view of this observation it can be suggested that LLL get a harmful role in bone formation when applying the methodology of this study. In the literature it has been reported that the properties of modulating the inflammatory response and promoting cellular biostimulation cause a

decrease in the healing time of some soft tissues.[17,18,19,20] Studies have also found that the use of LLL laser can also reduce healing time in bone defects, such as the alveolus of extraction. When the LLL is applied to grafted alveoli, bone formation can be observed around 60 days similar to that found in 120 days in non-irradiated grafted areas. Findings such as these corroborate the results found in the present study when comparing the LLL and C groups at periods of 30 and 60 days.[20] However, this finding does not corroborate the results found in this study when comparing the ABL group with the AB group in both the 30 day period and the 60 day period, in which no statistically significant difference was observed. The absence of statistical difference between the groups reaffirms the gold standard quality of the autogenous graft, which may prevent the benefit of the laser from being detected in the statistical analysis.

In addition, this discrepant result of the literature may be due to the LLL application protocol. In the present study, LLL was applied in a single session during the surgical procedure. In studies in which bone formation was faster, the application of LLL was continued postoperatively. In the study in extraction alveolus,[21] which the healing time of the grafted and irradiated region was halved, laser application was daily for 21 consecutive days.

In a study conducted in rat's femur, the LLL application protocol in grafted regions consisted of 7 sessions with 48 hours intervals. The results showed that the laser was capable of forming a greater amount of new bone than in the non-irradiated region in the same period of time.[22] In addition to the number of laser therapy sessions, the studies that show positive results from the laser have different intensity, wavelength and power specifications from the present study.[22,23]

Thus, it is suggested that the positive result found between the 30 days LLL and C groups was not observed between the AB and ABL groups probably due to masking over the laser effect that the osteogenic properties of the graft cause. The lack of beneficial laser results between AB and 60 days ABL groups can also be explained by the lack of continuity of the laser therapy sessions.

The results obtained in this work indicate that the application of the laser in a single session during procedure does not stimulate the formation of new bone in grafted regions. The benefit found in relation to the ungrafted region was restricted to 30 days of healing, and it was not observed in 60 days.

2.6 Conclusion

The LLL did not present biomudolatory capacity in the increase of NBF when applied with wavelength of 780 nm, energy density of 210J/cm^2, 6 J per point in times of 60 seconds each application, in single session, in critical size defects created in calvaria of rats grafted with particulate autogenous bone. Also, no statistically significant difference was observed when comparing the same technique at times of 30 and 60 days of healing. The LLL group showed greater bone formation compared with C group only in 30-days analysis, suggesting capacity of accelerate the bone formation. However, LLL may present a harmful effect when observed smaller amounts of bone formed in irradiated defects compared to non-irradiated in 60 days.

2.7 References

1. Fardin AC, Jardim ECG, Pereira FC, Guskuma MH, Aranega AM, Garcia Júnior IR. Bone graft in dentistry: review of literature *Innov Implant J, Biomater Esthet* 2010;5(3):48-52.
2. Gomes PS, Fernandes MH. Rodent models in bone-related research: the relevance of calvarial defects in the assessment of bone regeneration strategies. *Lab Anim* 2011;45(1):14-24.
3. Vajgel A, Mardas N, Farias BC, Petrie A, Cimões R, Donos N. A systematic review on the critical size defect model. *Clin Oral Implants Res* 2014;25(8):879-93.
4. Jordan HV. Rodent model systems in periodontal disease research. *J Dent Res* 197;50(2):236-42.
5. Schmitz J, Hollinger J. The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clin Orthop Relat Res* 1986;(205):299-308.
6. Bosch C, Melsen B, Vargervik K. Importance of critical-size bone defect in testing bone-regenerating materials. *J Craniofac Surg* 1998 Jul;9(4):310-6.
7. Honma T, Itagaki T, Nakamura M, et al. Bone formation in rat calvaria ceases within a limited period regardless of completion of defect repair. *Oral Dis* 2008;14(5):457-64.

8. Kitchen SS, Partridge CJ. A review of low level laser therapy. *Physiotherapy* 1991;77(3): 161–168.
9. Carvalho FB, Aciole GTS, Aciole JMS, Silveira L, Santos JN, Pinheiro ALB. Assessment of bone healing on tibial fractures treated with wire osteosynthesis ssociated or not with infrared laser light and biphasic ceramic bone graft (HATCP) and guided bone regeneration (GBR): Raman spectroscopic study. *Proceedings of SPIE* 2011;(7887):1-6.
10. Manzano-Moreno FJ, Medina-Huertas R, Ramos-Torrecillas J, García-Martínez O, Ruiz C. The effect of low-level diode laser therapy on early differentiation of osteoblast via BMP-2/TGF-β1 and its receptors. *J Craniomaxillofac Surg* 2015;43(9):1926-32.
11. Fekrazad R, Sadeghi Ghuchani M, Eslaminejad MB. The effects of combined low level laser therapy and mesenchymal stem cells on bone regeneration in rabbit calvarial defects. *J Photochem Photobiol B* 2015;151(11):180-5.
12. Garcia VG, Sahyon AS, Longo M. Effect of LLLT on autogenous bone grafts in the repair of critical size defects in the calvaria of immunosuppressed rats. *J Craniomaxillofac Surg* 2014;42(7):1196-202.
13. Cunha MJ, Esper LA, Sbrana MC, de Oliveira PG, do Valle AL, de Almeida AL. Effect of low-level laser on bone defects treated with bovine or autogenous bone grafts: in vivo study in rat calvaria. *Biomed Res Int*. 2014;(2014):104230.
14. Prados-Frutos JC, Rodríguez-Molinero J, Prados-Privado M, Torres JH, Rojo R. Lack of clinical evidence on low-level laser therapy (LLLT) on dental titanium implant: a systematic review. *Lasers Med Sci*. 2016;31(2):383-92.
15. Stein A, Benayahu D, Maltz L, Oron U. Low-level laser irradiation promotes proliferation and differentiation of human osteoblasts in vitro. *Photomed Laser Surg.* 2005;23(2):161-6.
16. Moore P, Ridgway TD, Higbee RG, Howard EW, Lucroy MD. Effect of wavelength on low-intensity laser irradiation stimulated cell Proliferation in vitro. *Lasers Surg Med* 2005;36(1):8–12.
17. Vale FA, Moreira MS, de Almeida FC, Ramalho KM. Low-level laser therapy in the treatment of recurrent aphthous ulcers: A systematic review. *Scientific World Journal*. 2015;(2015):150412.

18. Aggarwal H, Singh MP, Nahar P, Mathur H, Gv S. Efficacy of low-level laser therapy in treatment of recurrent aphthous ulcers - a sham controlled, split mouth follow up study. *J Clin Diagn Res*. 2014;8(2):218-21.
19. Karu TI. Yearly review: effect of visible radiation on cultured cells. *Photochem Photobiol* 1990;52(6):1089-98.
20. Garavello I, Baranauskas V, da Cruz-Höfling MA. The effects of low level laser irradiation angiogenesis in injured rat tibia. *Histol Histopathol.* 2004;19(1):43-8.
21. Monea A, Beresescu G, Boeriu S, Tibor M, Popsor S, Antonescu DM. Bone healing after low-level laser application in extraction sockets grafted with allograft material and covered with a resorbable collagen dressing: a pilot histological evaluation. *BMC Oral Health.* 2015;29(15):134.
22. Pinheiro AL, Limeira Júnior Fde A, Gerbi ME, Ramalho LM, Marzola C, Ponzi EA. Effect of low level laser therapy on the repair of bone defects grafted with inorganic bovine bone. *Braz Dent J.* 2003;14(3):177-81.
23. Rasouli Ghahroudi AA, Rokn AR, Kalhori KA, Khorsand A, Pournabi A, Pinheiro AL, Fekrazad R. Effect of low-level laser therapy irradiation and Bio-Oss graft material on the osteogenesis process in rabbit calvarium defects: a double blind experimental study. *Lasers Med Sci* 2014;29(3):925-32.

3 DISCUSSION

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Low-level laser (LLL) has become increasingly popular and frequently used in clinical routine as a supporting in different dental treatments. The reconstructions of reduced borders and defects from periodontal diseases, through bone grafts, are part of the dental procedures that the LLL could collaborate with.

The process of formation of new bone in grafted regions involves numerous cellular and biochemical events. The current hypothesis is that LLL can collaborate in the process of osteogenesis by having properties that stimulate cell proliferation, cell differentiation and blood flow. Among the cells known to be stimulated by low doses of LLL are osteoblasts (STEIN et al., 2005) and endothelial cells (MOORE et al., 2005) that are protagonists in the process of bone regeneration. In addition, the anti-inflammatory characteristics of LLL could improve the postoperative period and contribute to tissue healing.

The ability to increase the activity of osteoblasts and osteoclasts (NICOLA et al., 2003) and to stimulate the production of matrix comes from the modulation of extracellular expression that contributes to the formation of new bone and reduction of the time necessary for bone healing. (MERLI et al., 2012). The reduction of healing time in the region submitted to laser therapy may also be derived from the biostimulation of undifferentiated mesenchymal cells, which are more rapidly induced to differentiate into osteoblasts and osteocytes.

According to the literature, LLL has beneficial properties when used in infrared spectrum. Difference in density and wavelength may influence the dispersion of irradiation in tissues. Various combinations of density, power and wavelength have been employed in bone repair studies. The protocol used in this study was a single application during the surgical procedure with wavelength specifications of 780 nm, energy density of 210J/cm^2, using 6J per point in times of 60 seconds each application.

However, even in the literature reports that these cells are stimulated by LLL, the present study found no statistically significant difference when comparing grafted

regions with autogenous bone submitted to the application of LLL with grafted regions not subjected to irradiation (LLL x ABL). This difference was not observed in the two 30 and 60 day histological evaluation periods. Observing this result, it is noted that the laser did not demonstrate the ability to accelerate and/or increase in volume the formation of new autogenous graft bone. The bone formation in the 30 and 60 day ABL group apparently originated from the osteoinduction, osteoconduction and osteogenesis properties of the autogenous graft, since the 30 and 60 day ABL groups did not present a statistically significant difference in relation to the AB of 30 and 60 days.

In spite of this, it can't be affirmed that the LLL did not present biostimulatory abilities in this work, since in 30 days, the group in which the laser was applied in a region filled only by clot (LLL group) presented a statistically significant difference when compared to the Group C of the same period. In this case, it is suggested that the LLL, without the graft association, that is, in a single application, is capable of stimulating bone production at the initial healing time of the CSD created in rat´s calvaria.

However, no statistically significant difference was observed between the LLL and C groups in the 60-day healing period. Considering that the 60-day C group had a mean NBF greater than the 30-day C group and that the 60-day LLL group did not present a statistically significant higher NBF in relation to the 30-day LLL group, it may be suggested that the laser Has the ability to accelerate the formation of new bone, that is, shorten the healing time. The unique application of the laser at the surgical moment may also be the reason for the positive result to have occurred only in 30 days.

In the literature it has been reported that the properties of modulating the inflammatory response and promoting cellular biostimulation cause a decrease in the healing time of some soft tissues (VALE et al., 2015; AGGARWAL et al., 2014; KARU et al., 2015; GARAVELLO et al., 2004). Studies have also found that the use of LLL can also reduce healing time in bone defects, such as the alveolus of extraction. When the LLL is applied to grafted alveoli, bone formation can be observed around 60 days similar to that found in 120 days in non-irradiated grafted areas. Findings

such as these corroborate the results found in the present study when comparing the LLL and C groups at periods of 30 and 60 days (MONEA et al., 2015).

However, this finding does not corroborate the results found in this study when comparing the ABL group with the AB group in both the 30 days period and the 60 days period, which no statistically significant difference was observed. The absence of statistical difference between the groups reaffirms the gold standard quality of the autogenous graft, which may prevent the benefit of the laser from being detected in the statistical analysis.

In addition, this discrepant result of the literature may be due to the LLL application protocol. In the present study, LLL was applied in a single session during the surgical procedure. In studies in which bone formation was faster, the application of LLL was continued postoperatively. In the study in extraction alveolus of Monea et al (2015), in which the healing time of the grafted and irradiated region was halved, laser application was daily for 21 consecutive days.

In a study conducted inrat´s femur, the LLL application protocol in grafted regions consisted of 7 sessions with 48 hours intervals. The results showed that the laser was capable of forming a greater amount of new bone than in the non-irradiated region in the same period of time. (PINHEIRO et al., 2003) In addition to the number of laser therapy sessions, the studies that show positive results from the laser have different intensity, wavelength and power specifications from the present study. (RASOULI GHAHROUDI, 2014; PINHEIRO et al., 2003)

Thus, it is suggested that the positive result found between the 30-day LLL and C groups was not observed between the AB and ABL groups probably due to masking over the laser effect that the osteogenic properties of the graft cause. The lack of beneficial laser results between AB and 60-day ABL groups can also be explained by the lack of continuity of the laser therapy sessions.

The results obtained in this work indicate that the application of the laser in a single session during procedure does not stimulate the formation of new bone in grafted regions. The benefit found in relation to the not grafted region was restricted to 30 days of healing, and it was not observed in 60 days.

4 CONCLUSION

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The LLL did not present capacity in the increase of NBF when applied with wavelength of 780 nm, energy density of 210J/cm^2, 6J per point in times of 60 seconds each application, in single session, in critical size defects created in calvaria of rats grafted with particulate autogenous bone. Also, no statistically significant difference was observed when comparing the same technique at times of 30 and 60 days of healing. The LLL group showed greater bone formation compared with C group only in 30-days analysis, suggesting capacity of accelerate the bone formation.

REFERENCES

REFERENCES

Aggarwal H, Singh MP, Nahar P, Mathur H, Gv S. Efficacy of low-level laser therapy in treatment of recurrent aphthous ulcers - a sham controlled, split mouth follow up study. *J Clin Diagn Res*. 2014;8(2):218-21.

Bosch C, Melsen B, Vargervik K. Importance of critical-size bone defect in testing bone-regenerating materials. *J Craniofac Surg* 1998 Jul;9(4):310-6.

Carvalho FB, Aciole GTS, Aciole JMS, Silveira L, Santos JN, Pinheiro ALB. Assessment of bone healing on tibial fractures treated with wire osteosynthesis ssociated or not with infrared laser light and biphasic ceramic bone graft (HATCP) and guided bone regeneration (GBR): Raman spectroscopic study. *Proceedings of SPIE* 2011;(7887):1-6.

Cunha MJ, Esper LA, Sbrana MC, de Oliveira PG, do Valle AL, de Almeida AL. Effect of low-level laser on bone defects treated with bovine or autogenous bone grafts: in vivo study in rat calvaria. *Biomed Res Int*. 2014;(2014):104230.

de Souza Merli LA, de Medeiros VP, Toma L, Reginato RD, Katchburian E, Nader HB, Faloppa F. The low-level laser therapy effect on the remodeling of bone extracellular matrix. *Photochem Photobiol*. 2012;88(5):1293-301.

Fardin AC, Jardim ECG, Pereira FC, Guskuma MH, Aranega AM, Garcia Júnior IR. Bone graft in dentistry: review of literature *Innov Implant J, Biomater Esthet* 2010;5(3):48-52.

Fekrazad R, Sadeghi Ghuchani M, Eslaminejad MB. The effects of combined low level laser therapy and mesenchymal stem cells on bone regeneration in rabbit calvarial defects. *J Photochem Photobiol B* 2015;151(11):180-5.

Garavello I, Baranauskas V, da Cruz-Höfling MA. The effects of low level laser irradiation angiogenesis in injured rat tibia. *Histol Histopathol.* 2004;19(1):43-8.

Garcia VG, Sahyon AS, Longo M. Effect of LLLT on autogenous bone grafts in the repair of critical size defects in the calvaria of immunosuppressed rats. *J Craniomaxillofac Surg* 2014;42(7):1196-202.

Gomes PS, Fernandes MH. Rodent models in bone-related research: the relevance of calvarial defects in the assessment of bone regeneration strategies. *Lab Anim* 2011;45(1):14-24.

Honma T, Itagaki T, Nakamura M, et al. Bone formation in rat calvaria ceases within a limited period regardless of completion of defect repair. *Oral Dis* 2008;14(5):457-64.

Jordan HV. Rodent model systems in periodontal disease research. *J Dent Res* 197;50(2):236-42.

Karu TI. Yearly review: effect of visible radiation on cultured cells. *Photochem Photobiol* 1990;52(6):1089-98.

Kitchen SS, Partridge CJ. A review of low-level laser therapy. *Physiotherapy* 1991;77(3): 161–168.

Manzano-Moreno FJ, Medina-Huertas R, Ramos-Torrecillas J, García-Martínez O, Ruiz C. The effect of low-level diode laser therapy on early differentiation of osteoblast via BMP-2/TGF-β1 and its receptors. *J Craniomaxillofac Surg* 2015;43(9):1926-32.

Monea A, Beresescu G, Boeriu S, Tibor M, Popsor S, Antonescu DM. Bone healing after low-level laser application in extraction sockets grafted with allograft material and covered with a resorbable collagen dressing: a pilot histological evaluation. *BMC Oral Health.* 2015;29(15):134.

Moore P, Ridgway TD, Higbee RG, Howard EW, Lucroy MD. Effect of wavelength on low-intensity laser irradiation stimulated cell Proliferation in vitro. *Lasers Surg Med* 2005;36(1):8–12.

Nicola RA, Jorgetti V, Rigau J, Pacheco MT, dos Reis LM, Zângaro RA. Effect of low-power GaAlAs laser (660 nm) on bone structure and cell activity: an experimental animal study. *Lasers Med Sci.* 2003;18(2):89-94.

Pinheiro AL, Limeira Júnior Fde A, Gerbi ME, Ramalho LM, Marzola C, Ponzi EA. Effect of low level laser therapy on the repair of bone defects grafted with inorganic bovine bone. *Braz Dent J.* 2003;14(3):177-81.

Rasouli Ghahroudi AA, Rokn AR, Kalhori KA, Khorsand A, Pournabi A, Pinheiro AL, Fekrazad R. Effect of low-level laser therapy irradiation and Bio-Oss graft material on the osteogenesis process in rabbit calvarium defects: a double blind experimental study. *Lasers Med Sci* 2014;29(3):925-32.

Schmitz J, Hollinger J. The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clin Orthop Relat Res* 1986;(205):299-308.

Stein A, Benayahu D, Maltz L, Oron U. Low-level laser irradiation promotes proliferation and differentiation of human osteoblasts in vitro. *Photomed Laser Surg.* 2005;23(2):161-6.

Vajgel A, Mardas N, Farias BC, Petrie A, Cimões R, Donos N. A systematic review on the critical size defect model. *Clin Oral Implants Res* 2014;25(8):879-93.

Vale FA, Moreira MS, de Almeida FC, Ramalho KM. Low-level laser therapy in the treatment of recurrent aphthous ulcers: A systematic review. *Scientific World Journal*. 20152;(2015):150412.