Effectiveness of gallium and aluminum Arsenide laser in bone repair

Efetividade do laser de Arseneto de gálio e alumínio no reparo ósseo

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RESUMO
Introdução - O conhecimento de métodos que estimulem a consolidação óssea vem adquirindo considerável importância atualmente. Dentre eles, estudos experimentais com laserterapia mostraram-se promissores. Objetivo - avaliar o efeito de diferentes doses de Laser de baixa potência (LBP) sobre o processo de consolidação óssea em Rattus norvegicus. Métodos - Utilizaram-se 45 ratos da espécie rattus norvegicus, divididos em quatro grupos, um controle e três terapêuticos (4J, 6J, 10J) com aplicação de LBP em lesões nas tíbias esquerdas. Após o sacrifício, analisaram-se as dosagens bioquímicas de cálcio, fósforo, fosfatase alcalina e a densidade mineral óssea média. Quanto aos níveis séricos de fósforo, observou-se maior concentração nos grupos terapêuticos. Resultados - Verificaram-se resultados distintos entre os grupos de animais irradiados, sendo encontrada maior concentração de fosfatase alcalina no de 6J, e conforme a medição da densidade mineral óssea, o de 4J apresentou maior valor, e o de 10J, o menor. Conclusão

- o laser favorece a remodelação óssea, porém conforme a análise radiográfica, os melhores resultados foram obtidos com dosagem de 4J, apesar da análise bioquímica, evidenciar altos níveis de fosfatase alcalina sérica no grupo de 6J.

Palavras-chave: Laser, Consolidação Óssea, Arseneto de Gálio e alumínio, Marcadores Bioquímicos.

ABSTRACT
Introduction - The knowledge of methods that stimulate bone healing has acquired considerable importance today. Among them, experimental studies with laser therapy have shown promise. Aim - to evaluate the effect of different doses of low power laser (LBP) on the bone healing process in Rattus norvegicus. Methods - 45 rats of the species rattus norvegicus were used, divided into four groups, one control and three therapeutic (4J, 6J, 10J) with application of LBP in lesions on the left tibia. After sacrifice, biochemical measurements of calcium, phosphorus, alkaline phosphatase and average bone mineral density were analyzed. Regarding serum phosphorus levels, a greater concentration was observed in the therapeutic groups. Results - There were different results between the groups of irradiated animals, with a higher concentration of alkaline phosphatase in the 6J, and according to the measurement of bone mineral density, the 4J had the highest value, and the 10J, the lowest. Conclusion - the laser favors bone remodeling, however, according to the radiographic analysis, the best results were obtained with a 4J dosage, despite the biochemical analysis, showing high levels of serum alkaline phosphatase in the 6J group.

Keywords: laser, bone consolidation, gallium and aluminum arsenide, biochemical markers.

1 INTRODUCTION

Among traumatic injuries, fractures are the most common type in humans. Under ideal conditions, complete repair of this type of injury occurs after 6 to 8 weeks and may vary according to the existence of external factors such as alcoholism, diabetes, osteoporosis, nutritional level and internal factors such as the type of fracture, existence of infection 1, 2, 3.

The acceleration of the fracture healing process results in great benefits for the patient and for society, such as reduced time to return to function, reduced medical costs and increased quality of life, decreased pain and increased mobility, in addition to preventing possible disabilities4. Numerous strategies have been tested in order to reduce the time period for healing and bone remodeling of the fracture, including the use of physical resources with low-level laser therapy5.

Low-level laser therapy is a resource widely used for several therapeutic purposes such as controlling inflammation, decreasing pain and accelerating the healing process.

Its actions are related to the absorption of the energy emitted by the laser beams through cell chromophores, such as the enzyme cytochrome c oxidase, present in the respiratory chain, inducing an increase in the production of ATP, regulation of the production of nitric oxide, intracellular calcium concentrations and modulation of several transcriptional pathways of the cell whose responses are dependent on the laser dose applied 1-3, 5.

Historically, several studies conducted since the 1980s have demonstrated varying effects of the low power laser action on the bone repair process. Such studies have evaluated such effects through biochemical, histochemical, histological analyzes or through images 6-13. The use of low-power laser therapy accelerates bone matrix synthesis due to increased vascularization and decreased inflammatory response, inducing a significant increase in osteocytes in the irradiated region and an increase in fibroblast growth factors both in vitro and in vivo14. However, there is still no consensus on which laser doses or which circumstances are ideal to maximize the photobiomulator effect of low power laser therapy on bone tissue5. Given the above, this study aims to verify the effect of different doses of low-power laser (LBP) on the bone healing process in rats.

2 METHODS

The project was, prior to the beginning of the experiments, submitted to the Research Ethics Committee of the Centro Universitário UNINOVAFAPI. All procedures related to the use of animals as an experimental model were performed according to the standards recommended in the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animals Resources, National Academy of Science, Washington, DC, 1996), by the Principles Ethics established by the National Council for Animal Experimentation Control - CONCEA (2009) and by the national legislation for animal vivisection in force (Federal Law 6,638 of May 8, 1979).

Forty-five male Wistar rats (Rattus norvegicus), with an average age of 3 months and body mass of 250 to 300 g, from the colony of the Vivarium of the UNINOVAFAPI University Center, were used. The animals were kept in collective cages (5 animals / box) with maintenance diet (Labina® - Purina) and water ad libitum, in an air-conditioned room with an ambient temperature of 25º C and a photoperiod of 12 hours of light and 12 hours of darkness.

The animals were divided into four groups arranged as follows: A (10 animals) - fracture and application of 4J laser; B (10 animals) - fracture and 6J laser; C (10 animals) - fracture and 10J laser; D, Control (15 animals) - fracture of the left tibia and application of disconnected laser (placebo).

After a two-week acclimatization period, the animals were anesthetized intraperitoneally with a solution composed of ketamine at a dose of 40mg / kg and xylazine at a dose of 5mg / kg of body weight, and fractures were produced using a 4mm dental drill. For this purpose, a longitudinal incision was made with a No. 24 scalpel blade and No. 4 handle and posterior continuity solution in the middle third of the left tibia (junction of the middle third with the distal third), performed using a 0,4mm. After a fracture was performed, still with the animal anesthetized. After induction of lesion, Pencivet® (0.1 mL / 100g ppu) was administered intramuscularly, and the animal was placed in an appropriate box, isolated from the others, until its complete recovery from anesthesia, when it was then returned to his cage in the vivarium.

One day after the fractures, the protocol of laser irradiation was started. The application of punctual laser light was performed at a single point, in the middle third of the incision, where drilling was performed with the drill. The animals were submitted to treatment once a day, five times a week, for four weeks.

For the laser irradiation, the Laserpulse model (Ibramed) was used, which uses aluminum and gallium arsenide (GaAlAs) with a wavelength of 830 nm as a laser.

At the end of the experimental period, to perform the sacrifice, animals in all groups were anesthetized with excessive doses of sodium thiopental (100 mg/kg, intraperitoneally). Immediately after induction of anesthesia, a wide laparotomy was performed followed by pneumothorax. Then, the fractured tibia was removed for histological analysis, as well as blood collection from the caudal vena cava for biochemical measurements.

The measurements of serum calcium, phosphorus and alkaline phosphatase were performed using Labtest® and Dolles® reagents, by colorimetric method following the manufacturer's instructions.

Radiographic analysis of the left tibias was performed using a Dabi Atlante® device, model Spectro 70X, with anteroposterior view and exposure time to X-ray beams of 3 seconds. The images were scanned with a specific scanner for radiographic images and the average density of the injured area was calculated using the software Digora© for Windows, delimiting a square with a size of 10x10 pixels for analysis.

The data were presented as mean ± standard error of the mean (SEM) and analyzed by paired t-test to compare differences within groups, ANOVA followed by Tukey's post-test for comparison between groups. The level of significance was set at $p<0.05$. Statistical analysis were performed by the GraphPad Prism 6.0.

3 RESULTS

Gallium and aluminum arsenide laser treatment for four weeks did not produce changes in serum calcium levels in the groups irradiated in relation to the control group (Table 1).

Table 1 - Serum calcium levels in rats with bone damage in the left tibia and submitted to four weeks of treatment with gallium and aluminum arsenide laser at doses of 4, 6 and 10J.

Group	Mean ± SEM
Control (n=15)	6,73 ± 1,17
4 joules (n=10)	9,37 ± 1,11
6 Joules (n=9)	7,68 ± 0,42
10 Joules (n=10)	7,41 ± 0,66

*p<0,05 in relation to the control group.

Contrary, serum phosphorus levels were significantly higher in irradiated groups than in non-irradiated groups ($p <0.05$), but not among irradiated groups (Table 2).

Table 2 - Serum phosphorus levels in rats with bone lesions in the left tibia and submitted to four weeks of treatment with gallium and aluminum arsenide laser at doses of 4, 6 and 10J.

Group	Mean ± SEM
Control (n=15)	5,468 ± 0,368
4 joules (n=10)	8,709 ± 0,407[a]
6 Joules (n=9)	7,772 ± 0,390[a]
10 Joules (n=10)	7,533 ± 0,684[a]

[a]p<0,05 in relation to the control group.

Regarding the effects of laser irradiation on serum levels of alkaline phosphatase, it was observed that the group irradiated with 6J showed significantly higher levels ($p <0.0001$) compared with the control group and with the other irradiated groups (Table 3). The analysis of bone mineral density of the injured areas showed that although all laser irradiated groups had significantly higher density results than the control group, the 4J group had better results than the others and the 6J irradiated group obtained significantly higher results to 10J.

Table 3 - Serum levels of alkaline phosphatase in rats with bone damage in the left tibia and submitted to four weeks of treatment with gallium and aluminum arsenide laser at doses of 4, 6 and 10J.

Group	Mean ± SEM
Control (n=15)	42,84 ± 3,339
4 joules (n=10)	4,407± 0,864[b]
6 Joules (n=9)	206,6 ± 35,17[a]
10 Joules (n=10)	2,837± 0,526b

[a]p<0,0001 in relation to the control group.
[b]p<0,0001 in relation to the group irradiated with 6J laser.

The radiographic analysis showed, by means of the average mineral density of the injured area, greater mineral density in the bone callus in the groups irradiated with LBP light. Among the groups irradiated with LBP, the one showing the highest density was 4J, where 70% of the animals had the highest density. The group irradiated with 6J showed higher density than irradiated with 10J, the difference observed being statistically significant (Table 4).

Table 4 - Mean density in area of bone failure in the left tibia of rats submitted to four weeks of treatment with gallium and aluminum arsenide laser at doses of 4, 6 and 10J.

Group	Mean ± SEM
Control (n=15)	42,88 ± 0,81
4 joules (n=10)	66,07 ± 2,24a
6 Joules (n=9)	56,33 ± 1,43b
10 Joules (n=10)	48,72 ± 1,23b

[a]p<0,0001 in relation to the control group.
[b]p<0,001 in relation to the control group.

4 DISCUSSION

To measure the results of laser irradiation in bone metabolism, biochemical markers were used, since these are substances that depict bone formation or absorption. Among the bone absorption markers the main ones are total alkaline phosphatase, bone alkaline phosphatase, osteocalcin and type I collagen peptide15.

In the present study, the serum calcium levels evaluated in the groups irradiated with gallium and aluminum arsenide laser were not different from those found in the control animals, while the serum phosphorus levels were higher in the irradiated groups when compared with the control group. Bone formation occurs when osteoblasts deposit the osteoid, which is later mineralized along the calcification front. In order to achieve satisfactory mineralization, an adequate concentration of calcium and phosphate ions is required, the presence of a calcifiable matrix, a nucleating agent and a control by promoting and inhibiting regulators. The deposition is associated with vesicles surrounded by a membrane that derives from the plasma membrane of osteoblasts16,17.

A study demonstrated that the laser action does not depend on connections with CNS since the laser irradiation was effective in the process of accelerating bone failures in rats with spinal cord injury and that such response is associated with activation of the genetic transcription of the RUNX2 gene that is the differentiation of osteoblasts18. Recent research suggests that the ability of the laser to induce bone repair is associated with the development of new vessels and the modulation of the inflammatory process through genetic modulation associated with COX2 and VEGF even during the initial phase of the repair process19. Stimulation with low-power laser induces modulation of genes associated with collagen expression20.

According to Ross21, the matrix vesicles located some distance from the cells where the mineralization should occur break and cause an increase in the local concentration of mineral capable of initiating the process.

Young and Heath22 state that the bone exists in two main forms, the interlaced bone and the lamellar bone, the first being an immature form with collagen fibers arranged in a disorganized way in the osteoid. The second, on the other hand, consists of parallel irregular bands of collagen arranged in sheets. Interlaced bone is the first to be formed during fracture repair, as it is formed more quickly; then it is remodeled and transformed into lamellar bone which is physically stronger and more elastic. Soon after the fracture occurs, a blood clot begins to form at the site, which will subsequently be replaced by highly vascularized collagen tissue (granulation tissue) that will progressively become more fibrous. Then, mesenchymal cells are differentiated into chondroblasts that will replace the granulation tissue with hyaline cartilage, thus forming the provisional callus, which is later strengthened by the deposition of calcium salts in the cartilage matrix. At the same time as this happens, osteoprogenitor cells in the endosteum and periosteum are activated and deposit a network of intertwined bone in and around the temporary callus. The temporary callus then gives way to bone callus. When the interlaced bone completely joins the fracture site, the bone union is made. The bone callus is then remodeled to form mature lamellar bone, thanks to the influence of functional forces.

One of the reasons that could justify the absence of changes in serum calcium levels in this study would be the fact that calcium has a physiological regulation mechanism that narrows the limits of its plasma level, not allowing variations beyond 2.4 mol/L , where any tendency to alter the plasma concentration of this ion is corrected within minutes to hours, largely by the action of parathyroid hormone, so that the serum

levels of calcium and phosphorus must be evaluated together since the concentrations of one directly influence the on the other23.

Although the increase in phosphorus levels in animals in the irradiated groups suggests that the bone healing process was underway more sharply than in control animals, the change in an isolated biochemical marker should be interpreted with caution, since this The marker does not constitute a gold standard for analyzing the bone formation process. One of the most used biochemical markers of bone remodeling today is alkaline phosphatase, whose activity depends on the activity of osteoblasts, usually measured in the form of total alkaline phosphatase, which corresponds to the sum of its various isoforms. The bone formation phase where alkaline phosphatase is most produced is that of collagen matrix formation, that is, before mineralization24-27.

The results obtained here demonstrating greater alkaline phosphatase activity in the group irradiated with 6J laser suggest greater effectiveness of the gallium and aluminum arsenide laser in bone repair at this dose.

In the study by Barushka, Yaakoby and Uron28, the effect of laser irradiation on bone repair in rats that had their tibiae pierced with a dental drill and subsequently subjected to the incidence of light from a 3J helium-neon laser was evaluated on the sixth day greater peaks of alkaline phosphatase, increasing calcium accumulation and greater filling of the intramedullary canal with interlaced bone, progressing from there to membranous ossification in the cortical zone. In addition, they observed an increase in calcium up to the eleventh day and that the smallest peak of bone mass occurred on the twelfth day, thus demonstrating that despite the decrease in serum alkaline phosphatase levels, bone repair progressed in its phases, further emphasizing the role of alkaline phosphatase in the deposition of collagen matrix, not least because the work also showed a rapid accumulation of newly formed bone and efficient osteoclastic activity in the bone remodeling of the cortical zone during the formation of mature compact bone, and even though the laser may have interfered not only in bone cells, osteoblasts and osteoclasts but also in the formation of new blood vessels, thus helping tissue repair more widely.

There are studies that show a reduction in the consolidation time and a better structural arrangement in the bone callus caused by the laser, such as the work carried out by Cerqueira et al. 29 showing significantly positive results with the dose of 4J in sheep submitted to osteogenic distraction, and by Liu et al. 30 using the 10J dose in rats. While in others, such results were not seen. Laughter 31, in a study with a GaAlAs laser with a wavelength of 830 nm at a dose of 4J in 30 rats suspended by the tail during the entire

experimentation period (15 days), did not observe results that showed effectiveness in laser therapy in the animals studied.

Léo et al 32 carried out a study to evaluate the effect of laser therapy (AsGa) of 10J and 15J applied on alternate days for 45 days in fractures surgically induced in the tibia of rats. After the experimental period, the animals were euthanized, the tibias excised and greater mechanical strength was observed in the tibias irradiated with 15 J compared to the non-irradiated control group, but there was no detectable radiographic difference between the groups regarding bone callus formation. Tran et al33, evaluated the effect of simultaneous laser irradiation at two different wavelengths (780 nm and 940 nm) on tibial fractures in dogs. The laser applications were daily and lasted 30 minutes each. After daily treatment for 21 days and radiographic control performed on the day of fracture induction, after 14 and 21 days, the authors observed better bone callus and bone marrow formation in the group of dogs that received laser irradiation.

The interactions and effects of laser irradiation at the intracellular level are of fundamental importance for the bone healing process. Thus, in a study by Oliveira et al. 34 evaluating the effect of biomodulation using a GaAIA laser with a wavelength of 830nm and 3J power in osteoblastic cells, an intense cluster of mitochondria in the perinuclear region was observed, which suggests a possible stimulus to cell division, in addition to causing changes in the shape of the mitochondria from filamentous to granular.

Stein et al. 35, through in vitro culture, showed the importance of LBE in the proliferation and differentiation of osteoblastic cells, demonstrating an increase of 31 to 58% in the cell count in the plates where the helium-neon laser with wavelength of 632 nm, probably related to the effect of laser on mitogenic activity via cell cycle induction. In a study by Otremski et al. 36 with male chicks irradiated with Helium-Neonium (He-Ne) laser at doses of 0.7, 1.5 and 3 J/cm2, no significant difference was observed in the radiological analysis in relation to the control group, but there was a small delay in the bone repair in this group, identified by histological analysis. Therefore, the literature supports the hypothesis that with different doses of laser, different results are obtained in the time of bone healing, as proposed in this research.

In the present study, radiographic analysis using Digora software to measure bone mineral density, showed that bone mineral density in the area injured by the drill was higher in animals irradiated with laser, thus showing better consolidation in groups irradiated with laser. Among the irradiated groups, the one that showed the best results

was 4J, as it presented the highest values of average mineral density in the area of the bone callus; and that of 6J was superior to that of 10J, as it presented values of average mineral density in an injured area greater than the 10J group.

When evaluating the effect of low power laser therapy on the consolidation of total fracture of rat femur, Sella et al37 observed that laser stimulation (AsGaAl, λ = 808 nm, energy density of 0.2 W/cm2, fluency of 37 J/cm2 per site, nominal dose of 2 J) resulted in an increase in the formation of bone matrix, formation of new bone matrix observed even through the expression of matrix proteins such as periostein and osteonectin 13 and 18 days after induction of the femur fracture in comparison to the untreated group.

Renno et al. 38 demonstrated that the use of the 830 wavelength laser at the 10 J dose stimulates osteoblastic proliferation, but not the osteosarcoma cell proliferation, while the 780 nm laser at the 1, 5 and 10 J doses causes a decrease in proliferation osteoblastic cells and an increase in the number of osteosarcoma cells, and that the laser at the wavelength of 670 nm at doses 1, 5 and 10 J did not cause changes in osteoblastic proliferation, although at dose 5J it causes an increase in the proliferation of cells in the osteosarcoma.

A study by Liu et al. 30 in transverse tibial osteotomies in rabbits showed, through histological and radiological analysis, a slight stimulation in the periosteum and endosteum and an increase in the size of the bone callus in the group irradiated with laser light at a dose of 40J. While the research by Cerqueira et al. 29 with laser irradiation of GaAlAs whose wavelength was 830nm, a greater distribution of mineralized bone trabeculae was observed in the mandibles of sheep irradiated with laser than in non-irradiated animals. It is noteworthy that in the aforementioned work, a bone distractor was used, where the laser had greater performance in the containment period than in the activation period.

Gurler and Gursoy 39, evaluated the effect of laser therapy on bone consolidation of rabbit jaws after osteotomy using a distractor. Therefore, after the surgical procedure and 5 days of latency period, the distractors were activated 1 mm per day for 5 days with laser application (6J, AsGaAl) in a punctual mode, radiating at 6 different points totaling 36 J/cm2 per day. The animals were euthanized after 15 or 30 days and it was observed that after 15 and 30 days the osteoblastic activity and vascularization was significantly higher in the irradiated group. Oliveira et al40 compared the effect of laser therapy (830nm, 100mW, 120J / cm²) and ultrasound therapy (US pulsed with 1.5 MHz burst,

200us, 1KHz, 30 mW / cm2) on bone defects in rat tibias with treatment in days alternate. After 14 days of experiment, the authors observed that both the laser and ultrasound produced a larger area of newly formed bone compared to the control group. Rabbits were submitted to a 3mm radius bone defect and subsequently treated for 3 weeks with laser therapy (830 nm, 4J / cm2). After the end of the study protocol, the authors concluded that laser therapy did not increase bone formation and did not decrease repair time41. Another study evaluated the effect of the combination of laser therapy (AsGaAl, 780 nm, 7.5 J/cm2) and platelet concentrate in bone defects induced in rats and found no increase in bone healing in the association group compared to the group treated exclusively with lasertherapy42.

5 CONCLUSION

In the present study, according to the analysis of the results obtained by radiographic evaluation, it is suggested that laser irradiation in the different doses used favors the bone remodeling process, with a better response being found in the 4J group. Therefore, taking into account that the serum levels of total alkaline phosphatase are influenced by other processes, in addition to bone metabolism, and that it is present mainly in the first phase of bone consolidation, the results suggest that the dose of 4J was better effect on bone repair than the others.

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