Research article

The effect of Low-Level LASER therapy on osseointegration. Can LASER therapy improve bone/implant contact? A preliminary study on rats

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Abstract: Bone fractures can lead to disability and a prolonged rehabilitation period. In some patients, the risk of complications can increase if the healing process is not efficacious or if the metallic implants are loose. Photobiomodulation is a physiotherapeutic method of treatment that can stimulate the cell proliferation and reduce the symptomatology. We evaluated the effect of LASER therapy on the osseointegration of smooth titanium implants in 12 female rats that suffered fractures on both tibiae. The LASER therapy was performed second day after surgery on half of the rats on both tibia for 7 sessions, every 48 hours. Both tibia were removed in the control and experimental group at 2,4 and 6 weeks after finalizing the last photobiomodulation session. The methods of assessment were micro-CT scans, histology and mechanical tests. The micro-CT imaging show no significant differences between the 2 groups. LASER therapy could prove as an important tool in orthopedic patients and can potentially reduce the rehabilitation time.

Keywords: Titanium implants, Rehabilitation, LASER therapy, Photobiomodulation, Bone fractures.
1. Introduction

Fractures can occur at any age, however, comorbidities and individual risk factors could lead to prolonged disability and reduced quality of life. Fracture healing can be complicated by non-unions or pseudarthrosis which can further increase the rehabilitation time. In elderly patients, the risk of refracture can increase due to additional pathologies such as osteoporosis or neurological impairment. There is a need for researching methods that can accelerate bone healing in order to allow rehabilitation for regaining function and to return individuals to their previous life. Metallic implants are used in orthopedic surgery in apposition and stabilizing fractures or substituting different worn-out components (prostheses). The most commonly used alloys are stainless steels, commercially pure titanium, Ti-6Al-4V, cobalt-based alloys, zirconium, and tantalum and were selected because of their biocompatibility [1].

Biocompatibility is the property of a material to perform its function inside a living organism without biologically changing or damaging the tissues [2]. Biocompatibility does not imply a better tissue-implant interaction. Titanium implants either pure or alloyed with Al or V are often used in surgery because an amorphous protective TiO_2 layer on the surface offers high resistance to corrosion [3]. There are methods that can improve bone-implant interaction by changing the implant’s surface (grit blasting, acid etching, LASER ablation..etc) or by adding different coatings (hydroxyapatite, ceramic, peptides..etc)[4]. The implant osseointegration can also be increased by stimulating the bone directly through physiotherapy. Physiotherapy is a treatment used by Rehabilitation Medicine physicians that involves electrical currents, ultrasound, LASERs, magnetic fields, or shockwaves to enhance biological effects in the surrounding tissue for faster and more efficacious healing.

The LASERs used in modern medicine are either for ablation (surgery) or tissue stimulation (physiotherapy). In Rehabilitation, the LASERs are used for tissue stimulation, anti-inflammatory and analgetic effects in pathologies that involve the musculoskeletal system and nerves such as tendinopathy [5], epicondylitis [6], neuropathy [7,8]. LASER irradiation in tissue stimulation is called photobiomodulation. The effects of photobiomodulation on bone growth and implant stability have been studied in stomatology [9,10], and in vivo experiments on rodents with promising results [11]. A systematic review conducted by Randa Zein et al on human patients concludes that the increase in osseointegration of the implants can be attributed to enhanced cellular metabolism with DNA and RNA synthesis that increase protein production. Higher irradiation doses with low power and low power with high doses produced a positive response, however high doses with high power could result in inhibitory responses. [12]. The Low-level LASER was used for assisting implant osseointegration in animals with favorable results, but the effect on human patients still lacks evidence. [13] However, the studies conducted on human patients are all in stomatological medicine on spongious bone and lack clear protocols and patient following.

The photo-biological effect of LASER therapy is due to the absorption of irradiation by chromophores such as melanin or hemoglobin, and the mitochondria seem to be the most susceptible. The production of ATP, the synthesis of DNA, and the intracellular Ca^{2+} increase [14]. The expression of bone morphogenic protein- 4 (BMP-4) and activation of transcription factors such as Runt-related transcription factor 2 (RUNX-2) gen increases in bone healing of irradiated animals [15].

This study aimed to evaluate the effect of photobiomodulation using a Low-level LASER, available in rehabilitation facilities, on the osseointegration and stability of titanium implants in rats, using an increased fluency and energy emission. With this occasion we developed a protocol that will be used also for further research aiming to
understanding the phenomena and establishing the optimal parameters for human patients.

1. Materials and Methods

The study was conducted at the Experimental Facility of our local University of Medicine and Pharmacy and the protocol was approved by the University’s Ethics Committee on 06.10.2022. The animals used in the experiment were 4-month-old white Wistar female rats (Rattus Norvegicus), 4 months old with a median weight of 266.5 g. The rats have a similar physiology to humans, are resilient to anesthesia and surgery with rapid healing, and have a size that allows for bone implant placement. The animals chosen were of the same age, sex, and similar weight to reduce the risks of bias and were checked before the start of the experiments by a veterinary doctor who supervised the interventions. After surgery, the animals were kept in cages at a temperature of 22°C with water and food ad libitum and a cycle of light and darkness of 12h.

A number of 12 rats were used in the experiment, with no deaths during the surgery and follow-up phase. All the rats in the study underwent surgery on the same day. After surgery, the animals were introduced at random into either the control or the experimental group. One of the rats from the group that received LASER therapy and was sacrificed at 4 weeks was removed from the study after removing the tibia due to osteomyelitis on both sides. One tibia was also removed in the group that received LASER therapy at the 6-week sacrifice time because the implant was mobilized during sampling. All rodents were subjected to fracture and the implantation of a Titanium rod in both tibiae. The implants were 1 cm long and 1 mm wide and made from pure commercial Titanium with no surface addition fabricated by the local Technical University, Department of Materials Science. Before being placed in the tibiae, the implants were immersed in 99% alcohol, dried, and placed in a UV chamber for sterilization. The animals were divided into two lots, 6 in each lot: lot 1 received LASER therapy for 14 days, once every 48 hours for 7 sessions while lot 2 (control) received no intervention. Each lot was divided into 3 groups according to the time of sacrifice 2, 4 and 6 weeks after finishing the photobiomodulation sessions. Each lot was divided into 2 groups, with or without LASER therapy.

2.1 Surgical intervention

The rats were anesthetized with a cocktail of Ketamine 10% and Xylazine 2% intramuscularly in the thigh using a syringe with a hypodermic needle. The lower legs were shaved using an electric shaver. A median incision was performed on the patellar tegument, and an 18G needle was used to drill a hole in the tibial plateau for implant placement. The implant was press-fitted in the tibiae and the tegument was sutured. A second incision was performed on the tibia’s external side, and the muscles were separated (atraumatic) exposing the bone. A fracture was executed by the same examiner with surgical pliers in the metaphyseal area of the tibial bone, beneath the fibular head without affecting the fibula. All fractures were stable at the moment of tegument suturing.

2.2 LASER protocol

The LASER therapy was performed on half of the animals, on both tibiae. To reduce the risk of reflection from the residual fur, the tibial fur was removed using electric clippers followed by an application of shaving cream. The first LASER session was performed 24 hours after surgery for 7 sessions, 1 every 48 hours for 14 days. Before the intervention, the rats received cocktails of small doses of ketamine and Xylazine (0.05 ml/0.02 ml) to reduce the risk of injury and ensure the comfort of the animals. The LASER used was Diode with a continuous beam, 0.4 W power, 830 nm wavelength, a fluency of 80 J/cm² for each tibia, from 2 points (40 J/cm² per point), one on the internal and one on the external side of the
fracture, for 3 min and 20 sec/tibia on an area of 1 cm², Energy per tibia per session 80 J, total energy per tibia 560 J (1120 J per irradiated rat). No burns were reported during the intervention.

2.3 Tissue sampling and Methods of assessment

After finishing the LASER treatment we started euthanizing 4 rats every 2 weeks, 2 from the control and two from the experimental group. Both tibias of the rats were removed, cleaned, and preserved in formaldehyde 10%. One of the tibias was randomly selected from each experimental group at the time of euthanasia for Micro-Ct scanning. All the tibias were then sent to the Department of Materials Science for implant removal and pull-force test measurement. After implant removal, all the tibias were sent for histological analysis.

1.3.1 Micro-CT scan:

The micro-Ct analysis was performed using the SKYSCAN 1172 X-RAY Microtomograph made by Bruker (Belgium). One sample was selected at random from each group for evaluation. The scan slice thickness obtained was 13.6 μm. The scan results were interpreted using the CTAn software (1.13.0.0, Bruker, Belgium). The 3 D image of an implant situated in the tibia can be seen in Figure 1.

![Figure 1](image.png)

Figure 1. Micro-CT scan- 3D image of a titanium implant inserted in the tibial bone with adjacent newly formed bone tissue in a rat sacrificed at 4 weeks after photobiomodulation

The results were obtained by defining a round region of interest with a 2mm diameter (Figure 2). The region of interest was centered on the middle of the implant, in the metaphysis of the bones, and data 1mm above and 1mm below this point was included. The evaluation was performed by the same blinded investigator. Tissue Volume (TV), Bone Volume (BV), and percent bone volume (PBV) BV/TV were calculated for all of the scanned samples and were compared.
1.3.2 Mechanical tests - pulling out test
The implant–bone adhesion was assessed through the pulling-out test, carried out on a Zwick Roell Z005 machine, accuracy class 0.5. First, the bone was cut and removed, taking great care not to touch the implant, leaving 2 free mm in length on all implants. Subsequently, the bone-free part of the implant was vertically fixed in the upper pneumatic grips of the testing machine, while the bone was pulled downwards from the upper cut side, using a 3D adaptive system conceived to apply the pulling-out force on the axis of the implant and not to produce any cross tension that induces supplementary friction force. The loading rate was 1 mm/min.

2.3.3 Histological examination
The samples were fixated in a solution of formaldehyde 4 % for 48 hours and were decalcified using Biodec (Bio-Optica Italy) for 30 days. The decalcified samples were sectioned along the long axis using a microtome blade after the extraction of the metallic implant with the residual canal included in all samples. The resulting samples were then introduced in the automatic tissue processor (Leica TP 1020) for dehydration and paraffin wax was added afterwards. After the samples cooled down, they were sectioned again using a 3 μm histological microtome. The staining was performed using Hematoxyline Eosin and Trichrome Masson. The examination and characterization were achieved using a Leica DM 750 microscope with a Leica ICC 50 HD video camera. The bigger samples required multiple shots that were added together in a single image that would cover the whole canal using Photoshop PS 6 with the function FotoMerge.

1.3.3 Statistical analysis
Statistical analysis was carried out using the MedCalc® Statistical Software version 22.013. Data was presented as values. Comparison between groups was carried out using the Student-T test (independent samples) for the control and experimental groups at each time of sacrifice. The null hypothesis was that there was no true difference between these groups. The alternate hypothesis was that there was a difference between the experimental and control groups. A p-value <0.05 was considered statistically significant.

2. Results
3.1 Histological findings

In both groups that were sacrificed 2 weeks after LASER therapy a periosteal connective tissue lamella was spotted that can be seen at the end of the red arrow in Figure 3. The trabecular bone in thinner or thicker lamella with no mineralization differences can be spotted beneath the connective tissue that anchors the implant to the bone through the medular space in both groups. In the photobiomodulation group, the lamellas are denser (not thicker) towards the distal end of the implant. When the implant was in direct contact with the compact bone, the bone proliferation around the titanium implant was more significant forming a sheath of compact bone in the LASER groups.

Figure 3. 2 weeks- Histomorphometric imaging after removing the Titanium implant without LASER therapy (first row) and with LASER therapy (second row) in Hematoxyline Eosine And Trichrome Masson staining

The production of bone in proximity to the implant was superior in both groups at 4 weeks after photobiomodulation compared to 2 weeks. The bone sheath that was fully formed around the implant can be spotted through the blue arrow in Figure 4. The connective tissue can also be viewed in the second figure, but there is no difference compared to 2 weeks. The bone production at the distal side of the implant (black arrows) is superior at 4 weeks compared to 2 weeks. There is no significant difference in bone structure between groups.

Figure 4. 4 weeks- Histomorphometric imaging after removing the Titanium implant without LASER therapy (first row) and with LASER therapy (second row) in Hematoxyline Eosine And Trichrome Masson staining.
In the lot that was sacrificed 6 weeks after finalizing the therapy, the results were similar to the 4-week lot. The bone sheath was fully formed and superior to the 2-week lot, and there were no significant differences between the LASER group and the controls.

3.2 Mechanical test- Pulling-out test

The results of the pulling-out test measured in N were graphically represented in Figure 5. The diagram shows the mean values for the LASER treated animals vs. the untreated ones, together with the maximum and minimum measured values. One of the rats in the lot sacrificed after 3 weeks that received LASER therapy was eliminated from the study due to osteomyelitis in both tibia which was observed during tissue sampling. In lot 6, one of the rats from the irradiated group presented a lower value (0.3 N) due to implant mobilization during preparation for the pulling-out test, so the value was removed from the data. In all tissue sampling intervals, the groups that received LASER therapy showed higher implant stability levels. 2 weeks after LASER therapy the mean of the group 2w+LASER was 18.47 N while the mean in the 2w was 2.52 N with a significant difference (p 0.0018). 4 weeks after LASER therapy the mean of the group 4w+LASER was 10.35 N while the mean of the group 4w was 1.75 N with a significant difference in favor of LASER therapy (p 0.004). 6 weeks after LASER therapy the mean of the group 6w+L was 34.01 N, while the mean of group 6w was 14.91 N with significant difference (p 0.03).

![Figure 5. The pulling out force values (N) at 4, 6, 8 weeks after surgery (2, 4, 6 weeks after LASER therapy for half of the animals)](image)

2.3 Micro-Ct imaging

Figure 6 shows the percent of bone volume vs. tissue volume value of the samples evaluated through micro-CT scan, as observed in the area of the fracture. No significant differences can be spotted at any evaluation time, however, the percentage was higher in the LASER group after 2 weeks, while it was similar after 4 weeks and became lower than the control group after 6 weeks.
3. Discussion

One of the main difficulties with studying the effect of photobiomodulation on bone growth and osseointegration is the difference between protocols and parameters used by different researchers as there is no consensus at the present time if tissue stimulation is influenced by a higher exposure time, energy absorption, or fluency. Implant osseointegration can be evaluated through different methods: mechanical tests (removal torque test, pull force test), X-ray imaging (micro-Ct, cone beam computed tomography) [16], devices (Periotest) [17], histology, or immunohistochemical analysis [18]. We chose 3 methods of evaluation to observe the implant/bone interaction through mechanical testing, imaging, and tissue changes. The histological findings show differences 2 weeks after LASER therapy in favor of the irradiated group, however, the differences could not be spotted between groups after 4 and 6 weeks. The bone density was better around the implant in all groups sacrificed after 4 and 6 weeks compared to the 2-week lot, and there was no resorption effect due to LASER irradiation. The micro-Ct scan results show differences in favor of the LASER therapy group 2 weeks after irradiation similar to the histological findings. However, at 4 weeks, the osseointegration was similar between the groups and after 6 weeks, the non-irradiated group showed more promising results. In clinical practice, better implant stability during the first weeks could lead to fewer complications and a lower fatality rate. The most notable differences were found during the mechanical tests, for each time of sacrifice, and the values were significantly higher in the photobiomodulation groups. Similar results were found by Campanha et al. [19], however, they reported no differences after 6 weeks. The protocol was similar to the one we used, but our results showed better implant stability in the test group even after 6 weeks. Compared to our study, Campanha et al. used screw implants, that provided fixation by shape in contrast to our experiment that used press-fit implants. Our model is far more sensitive to bone healing processes. Kim JR et al. found no difference in the removal torque test in rabbits after 6 weeks but significantly improved stability after 12 weeks in the LASER irradiated group, however, the exposure time was less (60 sec) and the animals were larger [20]. Differences in fluency can change the outcome of implant integration. Studies conducted by Gomes and Goymen that compared 20 J/cm² to 10 and 5 J/cm² fluencies in osseointegration show that better results are achieved if a higher fluency is being used [21,22]. Compared to our study, both authors chose to evaluate the results at one single euthanasia time (4 weeks) and there was no observation over time. As the bone
is a structure that is subjected to continuous modifications after injury, it should be evaluated at different times. A higher fluency (197 J/cm²) has been used in the study conducted by Omasa et al. with good results, however, it is not commonly used in hospital rehabilitation protocols [23]. The fluency we chose (80 J/cm²) can be obtained on most devices used in Rehabilitation facilities. At the 4 week interval, the values for both LASER treated and untreated animals are lower compared to the other time intervals. More, for the LASER treated ones, the error is extremely small, so the confidence is very high. If corroborated to the Micro CT findings, one could conclude that the osseointegration process is not evolving linearly. The protocol we used can be replicated in a rehabilitation facility because most Low-level LASER therapy devices can accommodate the fluency and energy emission and the duration of the procedure does not make the patient uncomfortable. We consider that the methods of assessment used can help in evaluating thoroughly the bone/implant interaction and that this preliminary study offers a starting point for an experiment on a larger population.

One of the limitations of this preliminary study is the sample size that was chosen following the Ethics Committee’s directives, however, we chose to use both of the rats’ tibiae to maximize the data from which to gather results. The results from this preliminary study will be used to direct the conception of more ample research. Even though further research on a larger lot is mandatory before passing to human patients, this preliminary study is conceived to be a basis for selecting the proper parameters to improve osseointegration of titanium implants in human patients. As the higher fluency and energy absorption was higher than in most studies we reviewed, there were no tegument side effects reported and there was a trend for better implant stability.

The penetrability even though sufficient for rodents could be too low for a more profound structure like the vertebral body, however, the superficial bone structures such as the distal radius could benefit from low-level LASER therapy.

5. Conclusions

The osseointegration of titanium implants can increase by using photobiomodulation at a fluency of 80 J/cm² for 7 days. The effects were most notably seen through mechanical testing. The results were most noticeable 2 weeks after photobiomodulation. Even though histology and micro-CT did not show significant differences between the 2 groups the implants were harder to remove and the stability was better clinically in the photobiomodulation groups. The results of this preliminary study are promising and can be used as a basis for more extensive research.


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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of the University of Medicine and Pharmacy “Iuliu Hatieganu” Cluj-Napoca, Romania, number AVZ291/16.11.2022 on the 19th of December 2022
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References


