Clin Oral Impl Res 2000: 11: 540–545
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CLINICAL ORAL
IMPLANTS RESEARCH
ISSN 0905-7161

Biostimulation of bone marrow cells with a diode soft laser

Dörtbudak O, Haas R, Mailath-Pokorny G. Biostimulation of bone marrow cells with a diode soft laser.
Clin Oral Impl Res 2000: 11: 540–545. © Munksgaard 2000.

In recent years, the use of low-intensity red light in regeneration of soft tissue has been increasingly pursued. As far as hard tissue is concerned, the biostimulating effect of laser has already been demonstrated successfully in more rapid healing of tibial bone fractures in mice at a dosage of 2.4 J. However, the effect of light of a low dose laser directly on osteoblasts has not been investigated yet. The aim of this study was to determine the effect of continuous wave diode laser irradiation on osteoblasts derived mesenchymal cells. Three groups of 10 cultures each were irradiated 3 times (days 3, 5, 7) with a pulsed diode soft laser with a wavelength of 690 nm for 60 s. Another 3 groups of 10 cultures each were used as control groups. A newly developed method employing the fluorescent antibiotic tetracycline was used to compare bone growth on these culture substrates after a period of 8, 12 and 16 days, respectively. It was found that all lased cultures demonstrated significantly more fluorescent bone deposits than the non-lased cultures. The difference was significant, as tested by the Tukey Test ($P<0.0001$) in the cultures examined after 16 days. Hence it is concluded that irradiation with a pulsed diode soft laser has a biostimulating effect on osteoblasts *in vitro*, which might be used in osseointegration of dental implants.

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Key words: biostimulation – bone marrow cells – diode soft laser

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Accepted for publication 28 October 1999

In recent years, laser light, particularly soft laser, has been increasingly used for the treatment of injuries of soft and hard tissue. Scientific findings indicate good healing of soft tissue (Mester et al. 1971), a faster regeneration of severed nerves (Rochkind et al. 1986) and an increasing formation of new capillaries through the release of growth factors (Kovacs et al. 1974). The stimulation of DNA and RNA synthesis formation in the cell nucleus (Karu et al. 1982) and the transformation of fibroblasts to myofibroblasts (Pourreau-Schneider et al. 1990) are also well documented. Osteoblast proliferation, in particular, is of great clinical interest in the regeneration of lost bone. Favourable results were also achieved in examinations of hard tissue, as bone fractures in mice showed a faster formation of bone tissue with a tighter mesh of trabeculae after 3 weeks of daily irradiation with a Helium-Neon laser (HeNe laser) (Trelles & Mayoyo 1987). Lasing also resulted in an increase in the hard tissue in new bone formation around hydroxyapatite implants in the lower jaw of rabbits (Asanami et al. 1993). Ozawa et al. (1995) achieved a significant increase in the total area of bone nodules with a Gallium-Aluminium-Arsenide laser (GaAlAs) in a dose-dependent manner (10.8–108 J/cm^2/day).

The exact mechanism of action of cell biostimulation by laser or light is still unclear, but is the subject of several studies.

For example, a possible stimulating effect by increasing RNA synthesis (Ribari 1981) and a possible activation of components of the respiratory chain (Karu 1987) or extracellular components (Surinchak et al. 1983) are being discussed as causes of biostimulation. Such an extracellular component might be caused by singlet oxygen, as free radical, following irradiation with laser light (Lubart et al. 1990; Karu et al. 1983).

The formation of adenosine triphosphate (ATP) which is influenced by singlet free oxygen plays an important role in this respect (Kudoh et al. 1989;

Passarella et al. 1984). However, this stimulation was only achieved in a certain dosage range. Mester et al. (1968) demonstrated that cells in culture and tissue can be stimulated by means of a certain dosage. A too low dosage has no effect at all and a too high dosage is less effective, while a much too high dosage can lead to inhibitory effects.

It remains uncertain whether stimulation of bone growth is a general effect on mesenchymal cells or whether an isolated stimulation of osteoblasts by “Low Level Laser Therapy” (LLLT) is possible. So far, a stimulation has been achieved by means of a low power laser with a wavelength of 820 nm and a very high energy density (10.8–108 J/cm^2/day) (Ozawa 1995). No data are available yet on whether the application of other lasers with other wavelengths and lower energy doses has an effect on bone matrix production.

Therefore, the objective of this experimental study was to evaluate the effect of soft laser irradiation on osteoblasts *in vitro*.

Materials and methods

The method used to cultivate the rat bone marrow cells that were inoculated into each well, has been described in detail elsewhere (Davies et al. 1991; Davies 1990; Lowenberg et al. 1991). Briefly, marrow cells were obtained from the femora of young adult male Wistar rats. Both femora of each rat were removed and washed with α-minimal essential medium (α-MEM) containing antibiotics (1.0 mg/ml penicillin G, 0.5 mg/ml gentamicin and 3.0 μg/ml fungizone). The epiphyses were removed and the marrow was washed out using α-MEM supplemented with 15% fetal bovine serum, 50 μg/ml freshly prepared ascorbic acid, 10 mM Na β-glycerophosphate, 10^{-8} dexamethasone (DEX) and antibiotics at 1/10th of the concentration described above (test substrata). This cell suspension, with a total medium volume of 30 ml containing cells from two femora, was aliquoted onto the test substrata. The cultures were maintained in a humidified atmosphere of 5% CO_2 and the medium was changed after the first 24 h to remove non-adherent cells. When confluent monolayers were reached, the cells were passaged with a trypsin solution. Aliquots (5×10^4) of the cell suspensions were counted on a Coulter counter multisizer (Coulter Electronics Hialeah, FL) and plated on the bottom of test wells. The medium was then renewed three times a week.

A microscopic examination was carried out daily. The formation of *in vitro* bone in this cell culture protocol has been well characterized (Davis et al. 1991). A total of 30 cultures were irradiated, and 30 non-lased cultures were used as control group. The cultures were lased with a continuous wave (cw) diode laser with an output power of 21 mW and a wavelength of 690 nm on days 3, 5 and 7. The laser was fixed 1 cm above the respective cultures by means of a tripod, and the cultures were irradiated for 60 s. The overall energy density per irradiation thus was 1.6 J/cm^2. Tetracycline labeling with tetracycline HCL (conc.) powder was used to replace the antibiotic mixture in the medium daily, beginning on day 3 (Todescan et al. 1996). Ten cultures of the lased and 10 cultures of the non-lased group were treated with 90% alcohol on days 8, 12 and 16 in order to stop cell growth.

The intensity of the fluorescence particles of the deposited tetracycline was then determined by means of fluorescence intensity measurements of the individual dishes. For this purpose, a central circle of the cultures, 1 cm in diameter, was evaluated using the NHI image software program (Open Lab, Improvision, Great Britain). The fluorescence intensity values were compared with relative values (0–256) (no fluorescence intensity to greatest flu-

Table 1. Effect of laser light (irradiated group) on osteoblasts after irradiation on days 3, 5 and 7, respectively. The values correspond to the assessed relative values (0–256) of the intensity of fluorescence of the measuring field (diameter=1 cm) after 8, 12 and 16 days

Sample	Day 8		Day 12		Day 16	
	Control group	Irradiated group	Control group	Irradiated group	Control group	Irradiated group
1	16.8251	16.8534	25.9415	26.9908	40.1907	45.5672
2	16.8309	16.8454	29.8028	30.3321	40.2702	44.6709
3	16.8856	16.8830	26.0319	26.4534	43.4587	47.3234
4	16.8716	16.9905	34.1585	35.2710	46.4897	52.9840
5	16.8484	16.9343	30.5359	30.6090	39.3431	45.3090
6	16.8808	16.9971	27.8894	28.3438	44.3929	51.8750
7	18.5352	19.0634	41.4149	42.6578	47.7013	53.8722
8	21.0701	21.8733	36.1631	37.2178	42.7522	51.5546
9	21.8754	22.4105	40.2324	41.5644	45.6751	52.6777
10	21.9846	22.0622	39.6578	40.5320	49.0541	55.9800

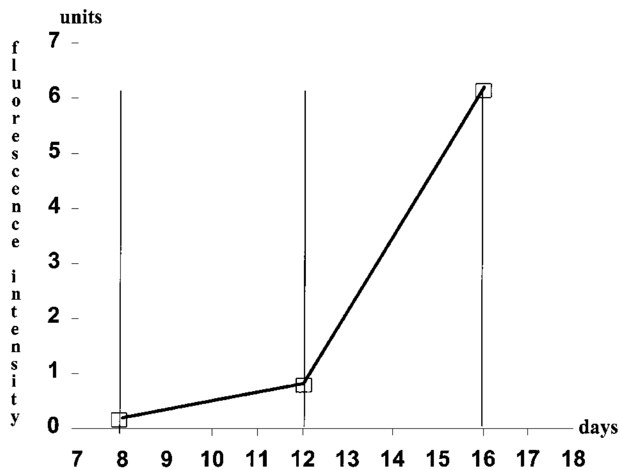

Fig. 1. Mean values (SD) of the intensity of fluorescence in the irradiated group and the control group after 8, 12 and 16 days.

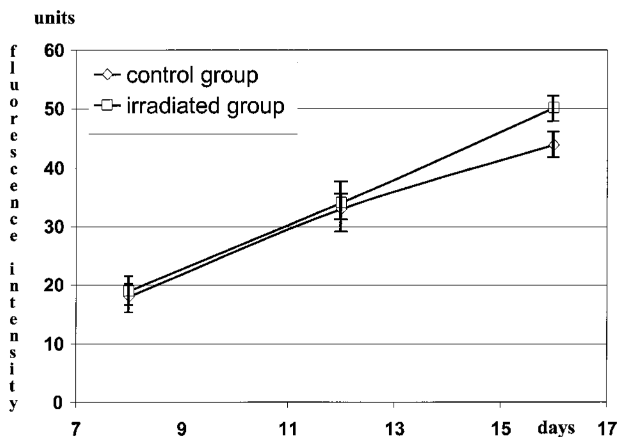

Fig. 2. Differences in mean values of the intensity of fluorescence between the irradiated group and the control group after 8, 12 and 16 days.

orescence intensity) from the measuring field (diameter=1 cm) after 8, 12 and 16 days.

Statistical data evaluation

Mean values and standard deviations were calculated. An analysis of variance was carried out in order to assess the group effect, a possible effect of time, and an interaction between the 2 groups and the 3 time points. Possible significant differences were assessed by means of the Tukey Test. *P*-values smaller than 0.05 were considered statistically significant.

Results

The cells showed a good growth behavior without any deficiency symptoms during the entire observation period. No changes were discernible macroscopically. The microscopic follow-up examinations revealed a higher growth of the irradiated cell populations after 12 days, compared with the respective control group.

The mean light intensity of the irradiated group was 34.3 units and that of the negative control group 31.9 units. The difference between these 2 groups was significant ($P=0.0347$). Furthermore, the time at which the cultures were examined (days 8, 12, 16) showed to have a significant ($P=0.0001$) effect on the light intensity values, irrespective of which group the samples belonged to (Table 1). The mean light intensity was 18.6 after 8 days, 33.6 after 12 days and 47.1 after 16 days (Fig. 1).

However, when both the group effect and the effect of time on the measuring values were considered, the *P*-value attained only borderline significance ($P=0.06$). Both a linear ($P=0.0319$) and an exponential ($P<0.0001$) significant increase was observed between the irradiated group and the control group. These trends were more strongly pronounced in the laser group than in the control group, which resulted in an increase in the difference between the 2 groups which was 0.2 on day 8, 0.8 on day 12 and as much as 6.2 on day 16 (Fig. 2).

Discussion

This study reports on the effects of low power laser irradiation on osteoblasts derived mesenchymal cells *in vitro*. It was found that laser light has a significant effect on bone matrix production.

In this study we used a new method, the tetracycline labeling, to label *in vitro* bone formation. The adsorption of the fluorescing agent tetracycline to the CaP minerals in bone and teeth is well documented in the literature and has been used in numerous *in vivo* studies to demonstrate new bone formation. Recently, this principle has been applied to demonstrate and quantify *in vitro* bone formation (Todescan et al. 1996). Based on this protocol, we determined the amount of mineral associated tetracycline by means of fluorescence intensity measurements of the individual dishes.

The inhibitory effect of tetracyclines on osteoblast proliferation and/or function is a well-known phenomenon which may affect the results of this study. Since this inhibitory effect would affect both the experimental and the control group, it is not taken into account in the further interpretation of the data. Moreover, an inhibitory effect was not obvious, based on daily routine assessment of the bone cultures.

By irradiating the cultures 3 times – on days 3, 5 and 7 – a significant increase in bone matrix pro-

duction was achieved in the lased group, compared with the control group.

Generally, lasing showed to result in a 10–15% higher deposition of detected tetracycline fluorescence in the irradiated group than in the control group. This biostimulating effect has also been demonstrated in other clinical studies examining other cell populations. However, the cell populations examined in these studies were exposed to much higher radiation doses. Rochkind et al. (1989) irradiated burn injuries in rats at 10 J/cm^2 over a period of 20 days and Trelles et al. (1987) lased bone fractures in mice at 2.4 J/cm^2 at each point over the same period of time, whereas the cell cultures examined in this study were irradiated three times at 1.6 J/cm^2. The extremely variable energy doses may be due to the fact that the irradiation field can be determined much more precisely in an experimental study than in a clinical study.

However, the energy doses used in this study – lasing was performed at an energy density of 1.6 J/cm^2 – are apparently within the range that induces a significant stimulation of bone matrix production *in vitro*. In contrast, other experimental studies have shown that lower doses have no effect and that too high doses have an inhibitory toxic effect on cell cultures. Van Breughel et al. (1992) tested the effect of laser light output (HeNe laser; wavelength 633 nm) on human fibroblast cultures. The greatest significant effects were obtained at a laser output below 2.91 mW, while 5.98 mW had no effect at all. The stimulating effect showed to be greatest in irradiation periods between 30 s and 2 min. Soundry et al. (1988) also analyzed the effect of a HeNe laser on human fibroblast cultures with an energy density of 1.2 J/cm^2. On the fourth day after irradiation, the irradiated group showed an accelerated growth compared to the control group. It remains to be seen if energy doses other than that used in this study (1.6 J/cm^2) have a different effect on bone matrix production.

Another finding of this study was that there was a small increase in fluorescence intensity between day 8 and day 12 and a marked increase between day 12 and day 16. Experiments with exponentially growing populations lased by means of various visible lasers have demonstrated a stimulating or indifferent or even inhibitory effect on the proliferation of cell cultures. Irradiation of human fibroblasts with a HeNe laser significantly increased the rate of cell proliferation during the exponential phase of growth and also enhanced the attachment of the cells (Boulton & Marshall 1986). Kovacs et al. (1982) noticed no change in mouse lymphoma cells, while Poon & Yew (1980) found inhibited mitotic activity of lens epithelium cells after irradiation with a HeNe laser. It is conceivable that the linear increase corresponds to a latent phase during which the osteoblasts adapt themselves to the respective environment. In this case, the increase in cells in bone matrix during the exponential phase would correspond to the period in which the osteoblasts of a generation unit reduplicate. If we assume that the slow replication phase of osteoblasts is 48 to 72 h, the significant increase occurs during the exponential phase in particular and is obviously supported by the energy of the laser light. The fact that the effect of irradiation is more pronounced in slow growing cultures has already been proved experimentally by Boulton & Marshall (1986) who irradiated cell cultures with different proliferation rates.

In summary, we conclude from these first results that irradiation with a diode soft laser results in a measurable biostimulating effect *in vitro*. Further studies on the application of this method in dentistry will show whether lasing might also help to achieve a better anchorage of dental implants in bone.

Résumé

L'utilisation de la lumière rouge à faible intensité dans la régénération des tissus mous est de plus en plus fréquente. En ce qui concerne les tissus durs, la stimulation biologique dûe au laser a montré son avantage dans la guérison de fracture osseuse du tibia chez les souris à une dose de 2.4 J. Cependant, l'effet de la lumière d'un laser à faible dose sur les ostéoblastes n'a pas encore été étudié. Le but de cette étude a été de déterminer les effets d'une radiation au laser à onde continue sur des ostéoblastes provenant de cellules mésenchymateuses. Trois groupes de dix cultures ont été irradiés trois fois (jours 3, 5, 7) avec un laser à longueur d'onde de 690 nm pendant 60 secondes. Trois autres groupes de dix cultures ont été utilisés en tant que contrôle. Une nouvelle méthode utilisant la tétracycline fluorescente a été utilisée pour comparer la croissance osseuse de ces substrats en culture après des périodes de 8, 12, 16 jours. Toutes les cultures qui avaient été irradiées avaient significativement plus de fluorescence au niveau de l'os que les autres: test de Tukey ($P<0.0001$) dans les cultures analysées après seize jours. L'irradiation avec un laser doux a donc un effet stimulant biologique sur les ostéoblastes *in vitro* ce qui pourrait être utilisé dans l'ostéointégration des implants dentaires.

Zusammenfassung

In den letzten Jahren setzte man mehr und mehr niederenergetisches Rotlicht bei der Regeneration von Weichgeweben ein. Das Hartgewebe betreffend konnte der biostimulative Effekt des Lasers (Dosis von 2.4 J) bereits erfolgreich mit einer beschleunigten Heilung von Tibiaknochenfrakturen bei Mäusen gezeigt werden. Der direkte Einfluss von niederenergetischem Laserlicht auf Osteoblasten wurde jedoch bis heute noch nicht untersucht. Ziel dieser Studie war es, den Einfluss einer Bestrahlung mit einem konstant gleichwelligen Diodenlaser auf von Mesenchymzellen abstammende Osteoblasten festzuhalten. Drei Gruppen mit je 10 Kulturen wurden dreimal (Tag 3, 5 und 7) mit einem gepulsten Diodensoftlaser mit Wellenlänge von 690 nm während 60 Sekunden bestrahlt. Drei andere Gruppen mit je 10 Kulturen dienten als Kontrollgruppen. Eine neu ent-

wickelte Methode, die mit fluoreszierendem Tetracyclin arbeitet, diente nach einer Zeitspanne von 8, 12 oder 16 Tagen zur Beurteilung des Knochenwachstums auf diesen Kulturen. Man fand heraus, dass alle mit Laser behandelten Kulturen signifikant mehr fluoreszierende Knochenablagerungen zeigten, als die nicht mit Laserlicht behandelten Kulturen. Der Unterschied war signifikant, wie der Tukey-Test ($P<0.0001$) bei den nach 16 Tagen untersuchten Kulturen zeigte. Man schloss daraus, dass *in vitro* die Bestrahlung mit einem gepulsten Diodensoftlaser einen biostimulativen Einfluss auf Osteoblasten hat, was uner Umständen bei der Osseointegration von Zahnimplantaten von Nutzen sein kann.

Resumen

En años recientes ha sido incrementada la propuesta del uso de luz rojo de baja intensidad en la regeneración de tejido blando. En lo concerniente al tejido duro, el efecto bioestimulante del láser ya ha sido demostrado con éxito en la más rápida cicatrización de fracturas del hueso tibial en ratones a una dosis de 2.4 J. De todos modos, el efecto de luz de láser de baja dosis directamente en los osteoblastos no ha sido investigado todavía. La intención de este estudio fue determinar el efecto de una irradiación continua de onda de láser de diodo en células mesenquimales derivadas de osteoblastos. Se irradiaron tres grupos de 10 cultivos tres veces (días 3, 5 y 7) con un láser blando de diodo pulsatil con una longitud de onda de 690 nm durante 60 segundos. Otros tres grupos de cultivo fueron usados como grupos de control. Un método de receinte desarrollo empleando antibiótico de tetraciclina fluorescente se usó para comparar el crecimiento óseo en estos substratos de cultivo tras un periodo de 8, 12 y 16 días respectivamente. Se encontró que todos los cultivos tratodos con láser demostraron un mayor depósito de hueso fluorescente significativo que los cultivos no tratados con láser. La diferencia fue significativa, probada con el test de Tukey ($P<0.0001$) en los cultivos examinados tras 16 días. Por ello se concluye que la irradiación con un láser blando de diodo pulsatil tiene un efecto bioestimulante en los osteoblastos *in vitro*, que podría ser usado en osteointegración de implantes dentales.

要旨

最近軟組織の再生における低エネルギー赤外線の使用がさかんに研究されている。硬組織に関するレーザーの生体刺激効果については、マウスにおいて２．４Ｊの線量で脛骨骨折の治癒が促進することが示されている。しかし低線量レーザーの骨芽細胞に対する直接的効果はまだ研究されていない。本研究は、連続波ダイオード・レーザー照射が骨芽細胞由来の間葉細胞に及ぼす効果を調べることを目的に行った。各々１０個の培養からなる３群を、波長６９０ＮＭ、６０秒間、パルス波ダイオード・ソフトレーザーで３回（３日目、５日目、７日目）照射した。さらに各１０個の培養からなる３群を対照群として用いた。蛍光抗生物質テトラサイクリンを用いる新規に開発された方法によって、各々８日、１２日。１６日後、これらの培養基質上の骨の成長を比較した。全てのレーザー照射を受けた培養は、受けていない培養に比べ蛍光骨の堆積量が有意に多かった。１６日後の培養において、Tukey Test による試験で有意差が示された（Ｐ＜０．０００１）。従って結論として、パルス波ダイオード・ソフトレーザーの照射は、in vitro において生体刺激効果を有しており、歯科インプラントの骨性統合に用いる可能性がある。

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