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# RELEVANCE OF LASER IRRADIANCE THRESHOLD IN THE INDUCTION OF ALKALINE PHOPHATASE OF HUMAN OSTEOBLAST CULTURES

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**Abstract**

Induction of matrix synthesis by low level laser has been demonstrated extensively. However, the question of dose- or power-intensity-dependency is underinvestigated. To address this issue we chose human osteoblast cell cultures and measured their alkaline phosphatase (ALP) activity after laser irradiation.
The cell cultures were irradiated periodically by 690nm radiation via optical transmission fibers based laser needles, reaching into the culture dishes.
Osteoblasts showed no induction of ALP activity using a single laser needle stimulation with a laser irradiance of 51mW/cm², an increase of about 43% at 102mW/cm² irradiance (two needles per well) and a 9-fold increase at 204mW/cm² irradiance (four needles per well), leaving the temperature of the culture medium uneffected.
We conclude that the osteoblastic response in ALP activity to a laser stimulus shows a logarithmic relationship with a distinct threshold rather than a linear dose-dependency. Secondly, the laser irradiance rather than the dosis is relevant for the impact of the laser.

**Introduction:**

The use of non-ablative lasers in medicine to treat connective tissues has become increasingly popular. Applications include the treatment of skin as in psoriasis [1, 2]) and wound healing [3-5]. The aim of non-invasive lasertherapy is reduction of inflammation, thus lessening pain and the reconstitution of tissue by fibroblast proliferation and extracellular matrix builtup. In contrast to the topical use of LLLT where the exposure to light is obvious the effect on hard tissues like bone and cartilage is far less taken for granted. The purpose of this study is to investigate the effect of LLLT on cells derived from hard tissue, osteoblasts. Their osteoanabolic activity can be monitored easily in the form of the key enzyme in bone formation, alkaline phosphatase. A stimulatory effect of LLLT on osteoblast alkaline phospatase activity has serious clinical implications both in fracture healing [6, 7] and inflammatory bone disease. As pointed out, the penetration of the target tissue by laser emitted light is crucial for its therapeutic effect. The main questions for the LLLT practitioner are what laser irradiance to use for how long to treat. In this context we thus addressed this question analyzing the importance of laser irradiance during the stimulation of human osteoblast cell cultures in relation the irradiation dose.

**Materials and methods:**

*Cell culture*. Human primary osteoblasts were derived from femoral heads of patients undergoing hip replacement. Parallel experiments were carried out using the osteosarcoma cell line SaOS-2. All cells were kept under standard conditions (37°C, humidified incubators with 5% $CO_2$) in phenol-red-free Dulbecco´s Modified Medium supplemented with 10% calf serum. 30.000 cells were seeded per well in 48-well-dishes, became adherent over night and were serum deprived (0,5%) for another 24h before treatment.
*Laser treatment*. After a change of serum deprived medium the osteoblasts were exposed to laser light of 690nm wavelength via optical transmission fibers as used in therapy with the laser needle technology.
The 500µm wide laser needles are new medical instruments (Laserneedle GmbH Wehrden, Germany), which emit laser light of high area density and low beam divergence. They are used for non-invasive irradiation of articular spaces.

Different levels of irradiance were accomplished by using one (51mW/cm²), two (102mW/cm²) or four (204mW/cm²) needles reaching into the culture dishes using specially prepared dish covers. Distance to the cell layer and lens design of the needle enabled an even irradiation of the treated cell surfaces. The osteoblast were treated for the first 30min every four hours for 24h in total.
SaOS-2 cells were laser treated under the same conditions as the primary osteoblasts. However, the ALP activity after a periodic irradiation pattern (30min on, 3.5h off, 51mW/cm$^2$ irradiance) was compared to single irradiation (30min) after 24h. To assess a threshold value for the effectiveness of the irradiance on ALP activity we plotted ln(ALP [U/mg protein]) over 1/radiant exposure [mJ/cm$^2$]. The subsequent intersection of the linear phase of this concave Arrhenius plot with the abscissa was taken as a threshold value for the effectiveness of laser irradiance on ALP activity.
*ALP assay*. After each experiment the culture medium was removed, the cells were rinsed in phospate buffered saline, lysed in 250µl 25mM Tris-Phosphate (pH 7.8) buffer containing 1% Triton-X, and kept frozen until assessment of ALP activity. Cells extracts were diluted and mixed with an ALP-substrate (p-nitrophenylphosphate) leading to the formation of a yellow chromogen (p-nitrophenyl) measured photometrically (405nm). ALP activity was expressed according to the time of product formation and total protein content of each well derived from a Pierce assay in Units/mg protein.

*Temperature measurement*. In a separate series of experiments the temperature course in the culture medium was measured during laser treatment was measured using a highly accurate Ni-NiCr thermocoupler.
*Statistical analysis*. Data are expressed as mean values with standard error of means using the Mann-Whitney test to express significance using Prism 3.0 (Graphpad).

**Results:**
Human osteoblasts did not alter ($p>0.05$) their ALP activity due to an irradiance of 51mW/cm$^2$ (0.15±0.02 vs. 0.17±0.03 U/mg protein in controls). Doubling the irradiance to 102mW/cm$^2$ resulted in a significant ($p<0.005$) but fairly small increase of 43% (0.14±0.01 vs. 0.10±0.02 U/mg protein in controls). Using four LASERneedles® and thus using 204mW/cm$^2$ irradiance, however, the ALP activity of the cells increased ($p<0.005$) about eight-fold (1,47±0.03 vs. 0.18±0.04 U/mg protein in controls). Based on these findings shown in figure 1 we estimated an optical activation energy threshold at a radiant exposure of 178mJ/cm$^2$ according to the linear section of a concave Arrhenius plot. Protein contents remained constant during the experiment in controls and laser treated groups.
Fig 2 illustrates the effect of radiant exposure compared to irradiance on ALP-activity. A repetitive laser treatment (six times 30min within 24h) with 51mW/cm$^2$ irradiance adds up to a higher dose of energy exposed to the cells (550,8mJ) than a single treatment (30min) with an irradiance of 204mW/cm$^2$ (367,2mJ). SaOS-2 cells demonstrated higher constitutive levels of ALP activity than primary osteoblasts. The low irradiance in combination with a high total dose of irradiation even led to a 20% reduction of ALP activity, whereas the high irradiance with a low total dose of irradiation increased ALP activity by 40%.
Figure 3 shows the temperature course in cell cultures irradiation for 40min. One LASERneedle® (51mW/cm$^2$) left the target temperature of 37°C practically unaltered (+0.1°C), the maximum irradiance used led to a slight increase over time (+0.4°C).

**Discussion:**
The primary intend of this study was to prove the ability of the laser needle irradiation system to have an effect on bone cells. Primary human osteoblast cultures periodically irradiated with increasing irradiance (51, 102 and 204mW/cm$^2$) were analyzed for ALP activity. As shown in figure 1 the osteoblast cultures responded with an over eight-fold stimulation of ALP activity compared to non-irradiated controls. However, the maximum irradiance was necessary to achieve this. Half and quarter of the maximal irradiance left the ALP of the primary osteoblast unaltered. This pattern of reaction to laser needle irradiation strongly implies the existence of a threshold value of irradiance below which the osteoanabolic activity of the cells remains quiescent. The linear rise of irradiance in correlation to the logarithmic gain of ALP activity demonstrates a behavior that is described in the Weber-Fechner law. According to this law a stimulus cannot be perceived as distinct below a certain threshold. To manifest this threshold numerically we calculated an optical activation radiant exposure of 178mJ/cm$^2$ for this particular system.
In contrast to bone tissue in vivo, the in vitro osteoblasts were freely exposed to the laser needle irradiation, covered only by colorless growth medium. The question arose, whether the total radiant exposure (irradiance over time) could compensate the lack of irradiance for the osteoanabolic effect in the lower irradiance groups. To investigate this we chose a human osteosarcoma cell line (SaOS-2) and compared the effect of a periodic treatment (30 minutes every 4 hours with an irradiance of 51mW/cm² resulting in a total dose of 550,8J) to a singular treatment (30 minutes with an irradiance of 204mW/cm², resulting in a total dose of 367,2J). After 24 hours only the high irradiance increased the ALP in SaOS-2 cells, although the total dose of irradiation was much higher in the low irradiance group (figure 2). The rate of increase of ALP activity in this experiment was much lower due to the high constitutive levels of ALP in SaOS-2. This finding adds up to the fundamental role of irradiance in the effect of laser needle irradiation on bone tissue culture.
Seeing laser needle irradiation applied in vivo or in vitro, particularly using high irradiances with red color, the question of thermal effects constantly arises. To address this issue we used very precise thermal couple and measured the temperature during a laser treatment interval (figure 3). Even with the maximum irradiance, the temperature gain remained negligible.
Further studies will reveal whether a given irradiance threshold value for the induction of ALP activity will also account for other effects such as collagen formation. Future analysis of transcription factors and cytokines will shed more light on the effect of laser needle irradiation on bone cells.

**Conclusion:**
Our experiments clearly demonstrate that laser needle irradiation has an osteoanabolic effect on both primary and osteosarcomic cell line human osteoblasts assessed as a stimulation of alkaline phosphatase activity. The linear increase in irradiance resulting in a logarithmic rise in alkaline phosphatase activity implies a threshold value of irradiance according to the Weber-Fechner law. In accordance with that, the irradiance plays a superior role compared to the total irradiation dose.

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Figure 1

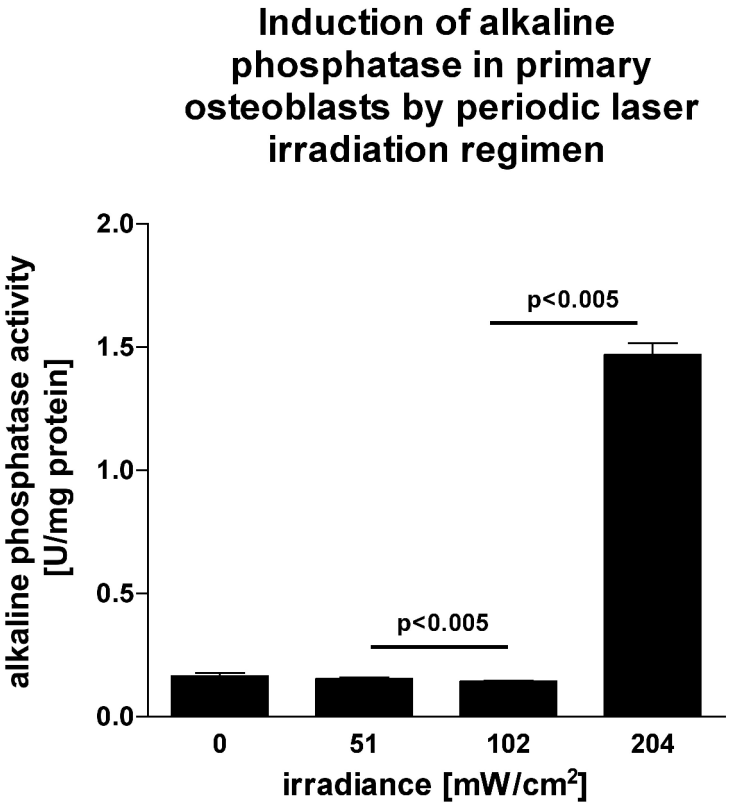

Induction of alkaline phosphatase in primary osteoblasts by periodic laser irradiation regimen
alkaline phosphatase activity [U/mg protein]
2.0
1.5
1.0
0.5
0.0
p<0.005
p<0.005
0
51
102
204
irradiance [mW/cm2]

Figure 2

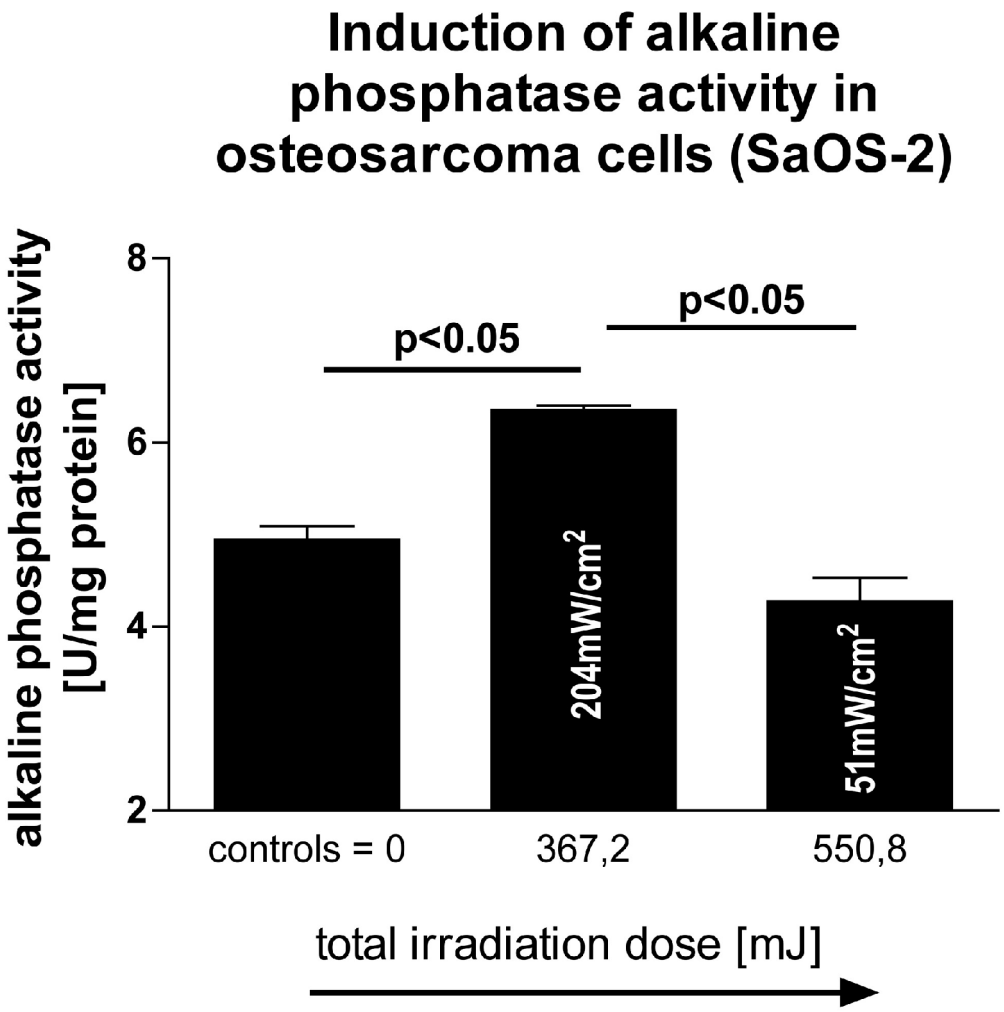
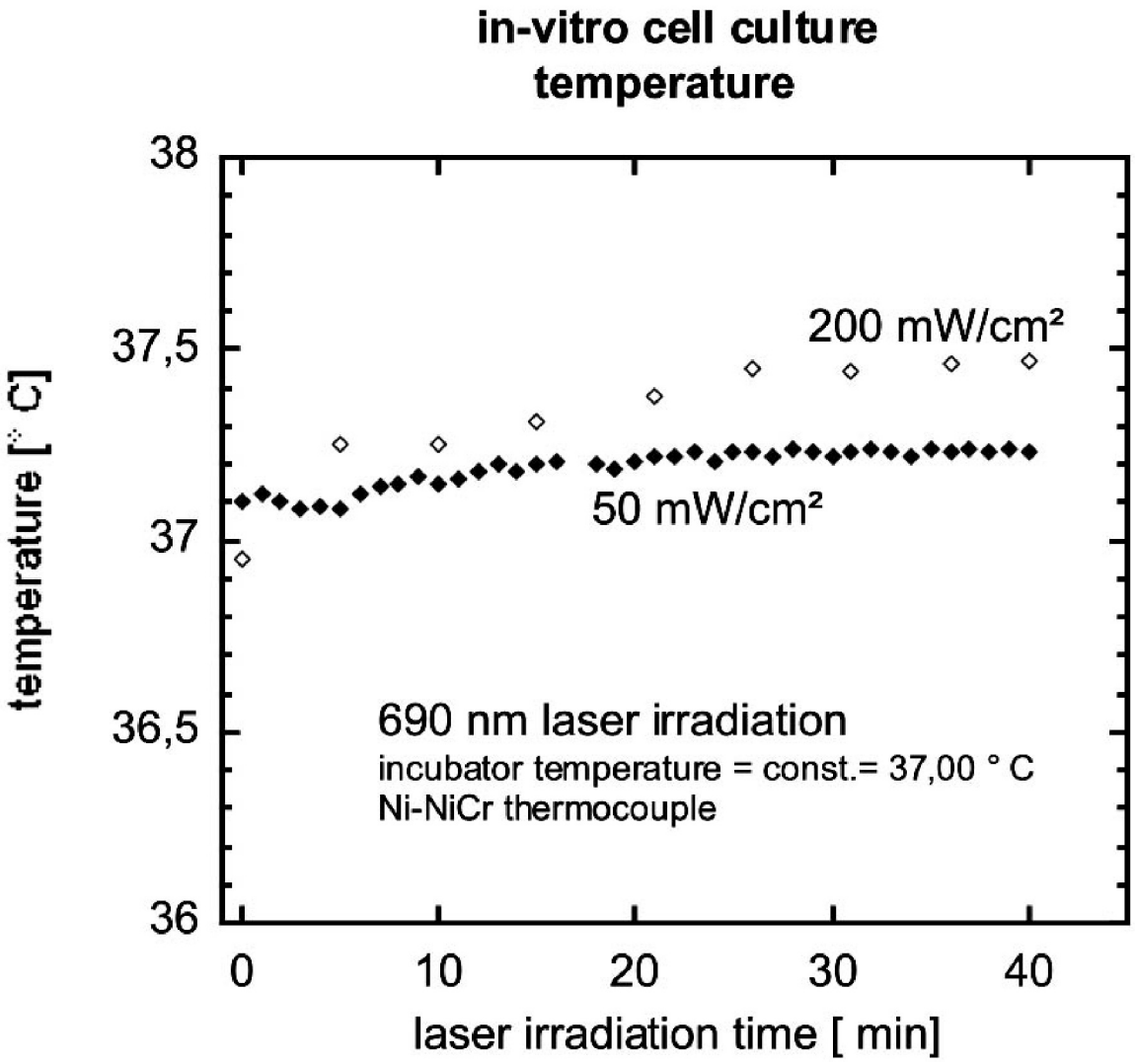

Induction of alkaline phosphatase activity in osteosarcoma cells (SaOS-2)
alkaline phosphatase activity [U/mg protein]
8
6
4
2
p<0.05
p<0.05
204mW/cm2
51mW/cm2
controls = 0
367,2
550,8
total irradiation dose [mJ]

Figure 3


in-vitro cell culture
temperature
38
37,5
37
36,5
36
temperature [° C]
200 mW/cm²
50 mW/cm²
690 nm laser irradiation
incubator temperature = const.= 37,00 ° C
Ni-NiCr thermocouple
0
10
20
30
40
laser irradiation time [ min]