Low-Level Laser Therapy (LLLT) as Photo Dynamic Treatment (PDT) using ora-laser d-lux 810nm Promotes Proliferation and Differentiation of Human Oral Fibroblasts evaluated *in vitro*

Objectives:

The aim of the present study was to investigate the effect of low level laser therapy (LLLT) as photodynamic therapy (PDT) using ora-laser d-lux 810nm on proliferation and differentiation of a human fibroblast cell line.

Background data: It was previously found that low level laser therapy (LLLT) enhances anti-bacterial effect of conservative periodontal treatment in experimental models. The low level laser therapy (LLLT) as photodynamic therapy (PDT) using ora-laser d-lux 810nm *in vivo* would require a therapeutic window where bacteria could be killed without adjacent normal tissue damage. Some oral bacteria are susceptible to killing by laser light after their sensitization with toluidine blue O (TBO).

The autonomous migration of specialized cells is an essential characteristic in both physiological and pathological functions in the adult human organism. Leukocytes, fibroblasts, and stem cells, but also tumor cells, are thus the subject of intense investigation in a broad range of research fields. A wide spectrum of methods have therefore been established to analyze chemokinetic and chemotactic cell migration, ranging from easy-to-handle two-dimensional surface migration assays to highly specialized three-dimensional and intravital analysis methods.

Cell migration

Cell migration is an essential characteristic of both physiological and pathological processes within the human organism. As early as 1863, Rudolf Virchow described the observation of motile cells, which he had isolated from the lymph fluid and from cartilage tissue. Today, we know that only a small portion of highly specialized cells is able to actively and autonomously migrate within the organism. These cells are: stem cells, leukocytes, fibroblasts, and tumor cells (the most prominent and well-studied motile cells, the sperm cells, will not be the subject of this report). Stem cells recently came into focus for migration studies, as researchers recognized that stem cells do not only reside in the tissue, e.g. the bone marrow, but actively migrate to certain areas of tissue regeneration such as the liver.

In contrast, the migratory capacity of leukocytes to move through the body in order to keep the organism under immunological surveillance and to respond to pathogenic invaders is well described. The most active migrating cells among leukocytes are natural killer cells, T lymphocytes, macrophages and dendritic cells, and neutrophil granulocytes. Neutrophils, with a maximum speed of 15 to 20 A m/min, are the fastest cells of all. In comparison to these cells, fibroblasts are slow moving cells (0.2 to 1 A m/min). Their activity is necessary during tissue regeneration after injury. The morphology of these fibroblasts and their manner of migration

resembles the migration of tumor cells, and they share some structural elements with these cells, e.g. the focal adhesion contacts, which coordinate the regulated adhesion between the extracellular matrix (ECM) and the intracellular cytoskeleton via integrin receptors. In contrast to the aforementioned physiological migration, the pathological migration of tumor cells is a component of the tumor progression with resulting invasion in surrounding tissues and metastasis development in distant organs. Tumor cells are the slowest migrating cells in comparison to all the others mentioned herein (0.1 to 0.3 A m/min). In addition to these, there are other cells that show motility, but not autonomous migration, e.g. epithelial cells in the colon, which slowly, but continuously migrate from the bottom of the crypta to the top of the villus, where they are exfoliated from the underlying substratum.

In order to investigate cell migration in detail, researchers have invented a plethora of assays, each with certain advantages and disadvantages. Thus, before the investigation of migration can begin, the goals of the migration experiments should be carefully considered and the appropriate migration assay accordingly chosen.

Because the majority of assays allow only a start- to end-point determination but not the observation of the intermediate stages of cell migration, it is necessary to decide at the onset if the behavior of cells during migration is going to be an integral focus of the investigation. These start- to end-point assays are naturally much easier to handle and are more useful for screening purposes than assays which allow or even require a constant observation of cells during migration.

A second point of consideration is whether it might play a role if the cells show directed movement towards a substance (chemotaxis) or if the cells show undirected, random migration (chemokinesis) and whether this is a subject of the investigation. An experimental setting with a gradient of a chemoattractive substance displays a much higher grade of complexity, especially for slow moving cells like fibroblast cells.

Thirdly, and perhaps most importantly, it must be determined how reductionary the experimental setup can be to answer the scientific question. This is certainly a general issue that has to be addressed in every experimental endeavor and as such would seem to be trivial. Each laboratory experiment is only an approximation of *in vivo* conditions, but simplification is necessary to keep the number of variables limited in order to allow for a reliable interpretation of the results.

3D migration assays

While 2D assays and filter assays are largely used for screening purposes, 3D assays are generally more complex in their experimental setup and are thus largely unsuitable for the investigation of such hypotheses. The eligible applications of 3D assays are the analyses of the

migration process *per se*. In principle, the investigated cells are embedded within a matrix in 3D assay, whereas the composition of the matrix varies as widely as ECM components used for the coating of surfaces in 2D assays. The most commonly used 3D matrix is collagen type I-based, as it is the predominant ECM component in the body and it is the main constituent of the connective tissue (for instance in the human periodontium). So far, 27 different collagen types have been characterized, and the expression of some of these is preferential or even restricted to certain tissues, e.g. collagen type II is the predominant component in cartilage, and collagen type XXVI is specifically expressed in the testis and ovary. It is noteworthy that not all collagens are equally able to form fibrils; some are termed as fibril-associated collagen with interrupted (triple) helices (FACIT). Besides collagens, other matrix components are used as constituents for artificial matrices, or mixtures of ECM components are used to imitate the *in vivo* conditions of special tissues. Fibronectin is an ECM molecule, which is also frequently used to constitute 3D matrices.

In order to continuously visualize the migratory behavior of the cells, the method of choice for analysis in 3D assays is time-lapse videomicroscopy. Usually, a video camera is mounted on a regular microscope and coupled to a time-lapse video recorder or another recording system. This method combines the advantages of maximum temporal resolution and long observation times. Thus, both the exact migrational components of individual, fast-moving neutrophils and slow-moving fibroblast cells can be dissected and analyzed. In such continuous movies, the individual migration paths of cells can be analyzed for several parameters summarized as the migratory behavior, e.g. with regard to the velocity of migration, the frequency and duration of breaks between migratory active phases, or how tortuous a path is.

3-D Cell Migration High Content Screening System Technology (METAVI LABS TECHNOLOGY) / 3-D collagen matrix assays

In 3-D collagen matrix assays, the cells are embedded in a matrix of collagen, usually type I collagen because it is the predominant component of the extracellular matrix in the body. Cell migration is continuously visualized using time-lapse videomicroscopy. This is typically done using a videocamera mounted on a light microscope and coupled to a recording system such as a time-lapse videorecorder. This method allows both maximum temporal resolution and long observation times. 3-D collagen assays are the best approximation of *in vivo* cell migration conditions.

MetaVì Labs has developed a patent pending system for tracking thousands of individual cells in natural 3-D collagen, in morphology as well as position, with no fluorescent tagging or staining of the cells. Tracking cells in natural collagen is critical because the 3-D matrix simulates actual body tissues. Cellular responses to migration stimulants and inhibitors can be measured precisely because the reaction of each individual cell is tracked. MetaVì Labs' technology is custom designed from the bottom up for this purpose. All of the image processing algorithms and software are designed from scratch for this purpose. The microscope technology is also custom designed for high volume individual cell tracking. Rather than cobbling together bits of software, computer and microscope technology from other fields, the engineering team decomposed the problems in time-lapse microscopy, object identification and tracking, data-management, high-content-screening, and came up with the own designs for each element, to ensure that solution would do the job precisely, efficiently, and on a scale to support high throughput screening. In order for the results to be clinically relevant, the experimental results must be repeatable over time and place. Therefore, each step in the process is precisely controlled.

Materials and methods:

Cultured fibroblast cells were irradiated using laser irradiation (810 nm; 0,3 mW power output). On the second and third day after seeding the fibroblasts were exposed to laser irradiation. The laser-induced effects of ToluidinBlu (TBO) on normal human gingival fibroblasts have been studied *in vitro*. For the assessment of viability, the "MetaVì Labs 3-D collagen matrix **PASS screening** Assay" was used.

Major Technical Compounds

Image Processing

- Motion stabilization
- Focus drift
- Collagen contraction
- Collagen-blur
- 2-D image slices of 3-D cells
- Tracking with ill-defined cell membranes
- Dramatic cell morphology changes over time
- Very high computation resources required for high resolution imagery

Microscopy

- Z-axis tracking
- Repeatable micro-focus control
- Multi-well scanning with sub-micron position return
- Scanning 96 wells in 15 second loop time
- A small compact form factor that can be scaled up for high throughput drug screening with live cell

Data Management

- Tracking of experimental parameters
- The imaging processing stage produces 50 M-bytes of data per well
- High throughput screening generating terabytes per month of information
- Visualization and interpretation of the data

MetaVì Labs PASS screening assay / Time-lapse microscopy with automated analy*sis*

The PASS assay (Fig. 1) combines all the advantages of 3-D collagen matrix assays with the benefits of high-throughput, fully automated (hands-off) tracking technology. Cells do not need to be labelled with any dyes or tags that could interfere with cell motility or interact with substances being tested in the assay. Whole cell populations, thousands of cells, can be tracked simultaneously in real-time. MetaVì Labs' analysis software can analyse migration and morphology data by population, by sub-population, or by closer examination of individual cells. The PASS assay is the only validated, consistent cell migration assay available for high volume preclinical screening applications.

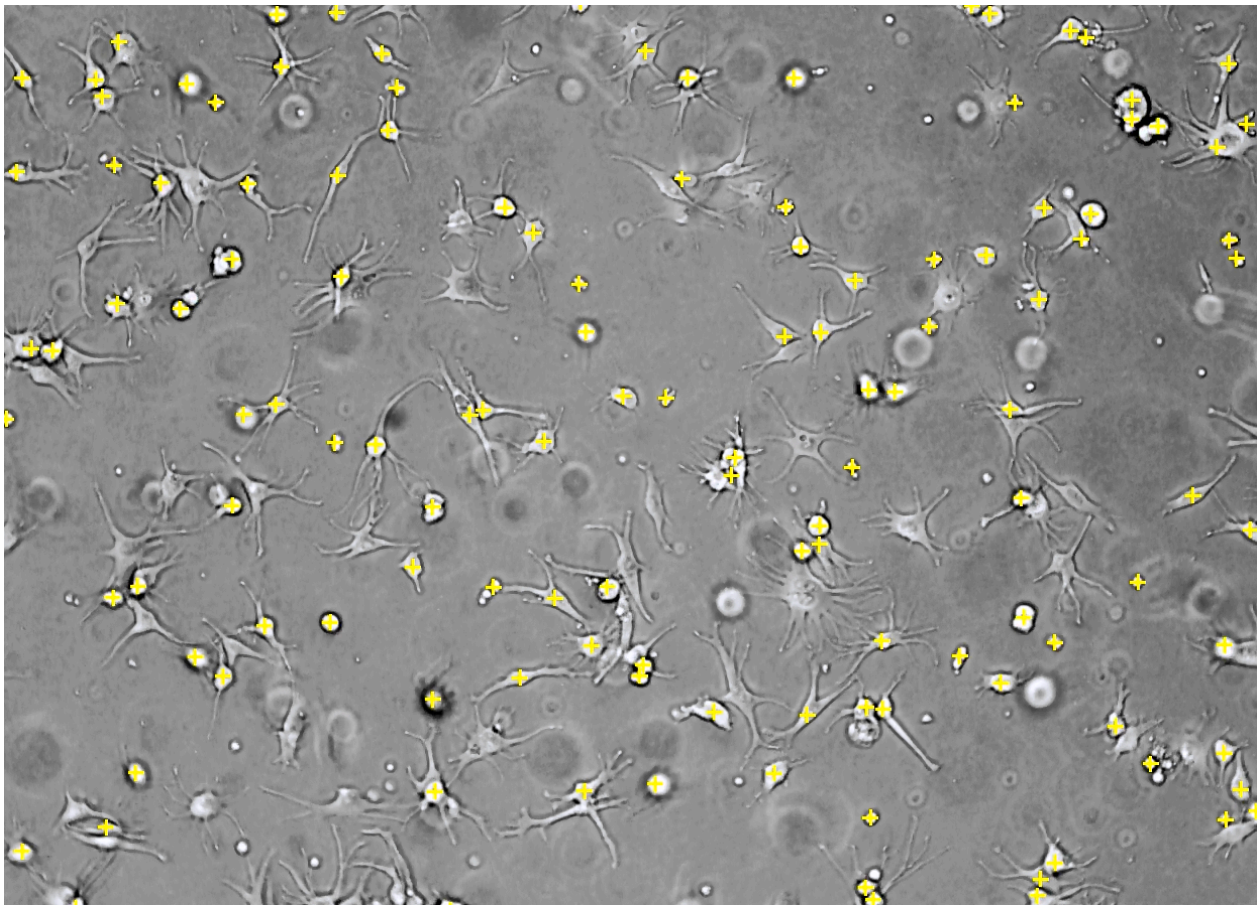


Fig. 1: MetaVì Labs PASS screening assay. With MetaVì Labs PASS, the overall anti-metastatic effect of substance can be evaluated on multiple cell lines with metrics that can be used for standardized comparison.

With MetaVì Labs PASS, the overall anti-cellular effect of substance can be evaluated on fibroblast cell lines with metrics that can be used for standardized comparison (Fig. 2).

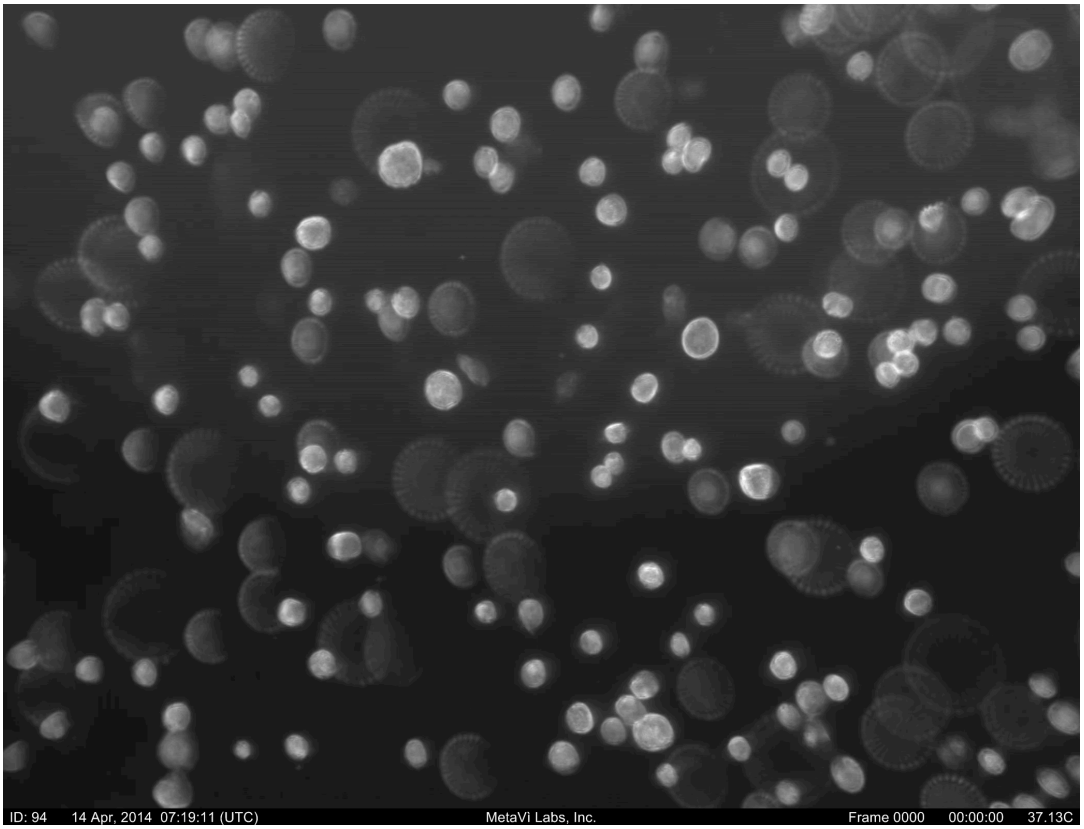


Fig. 2: MetaVì Labs PASS assessing gingival fibroblasts

Results:

Two sets of tests (MetaVì Labs 3-D collagen matrix Assay) were performed with human dermal fibroblasts derived from PromoCell (Heidelberg, Germany). The cells were harvested at the indicated date and passage number below. After centrifugation (3 minutes at 220g), the cells were resuspended in 1 ml PBS. Four aliquots were generated, each containing 60,000 cells. ToluidinBlau / toluidine blue O {TBO} (Dr. Köhler Chemie) or PBS were added to the same amount, resulting in toluidine blue O {TBO} concentrations of 50% and 5% (v:v). Two of each samples were treated with laser-light (810 nm) for 1 minute at 0.3 Watt (PDT, sensitization with toluidine blue O {TBO}). After that (resulting in a total incubation time of 5 minutes) all samples were spun down and resuspended in 50 µl PBS. 100 µl collagen solution was added to each sample, resulting in a collagen concentration of 1.67 mg/ml. Locomotor behaviour was analyzed for a 10 hours period.

Set 1 (50% toluidine blue O {TBO})

Set 2 (5% toluidine blue O {TBO}

At a concentration of 50% toluidine blue O {TBO}, the locomotor activity of the fibroblasts was significantly impaired (results not showed), whereas 5% toluidine blue O {TBO} did not affect the part of locomoting cells (Fig. 3-Fig. 5). PDT treatment had no influence on the locomotory behaviour.

All the following charts showed imaging the Experimental Set 2 (5% toluidine blue O {TBO}) using different series of the experimental setting.

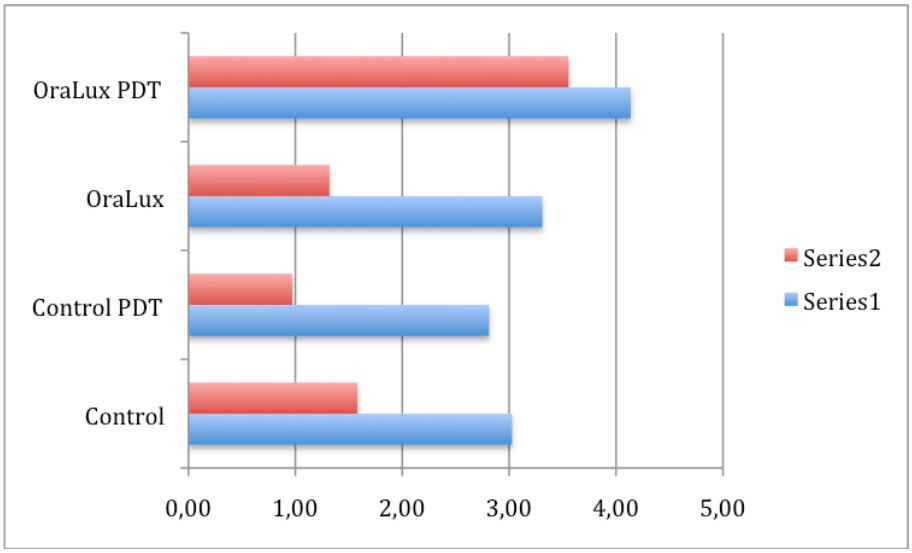


Fig. 3: Percent Migrated shows the quantity of cells that are migrating each moment over time. The source video is divided into 15 minute time intervals, and for each interval, all cells visible during that interval are categorized as either migrating (meaning they moved more than a threshold radius of 3 µm) or stationary

Concommitantly, the speed of locomotion was significantly reduced in those cells treated with 50% toluidine blue O {TBO}, but showed only a weak tendency to a reduced speed in those samples treated with 5% toluidine blue O {TBO} (Fig. 4). Again, PDT treatment did not influence the speed of locomotion in neither control samples nor OraLux-treated samples.

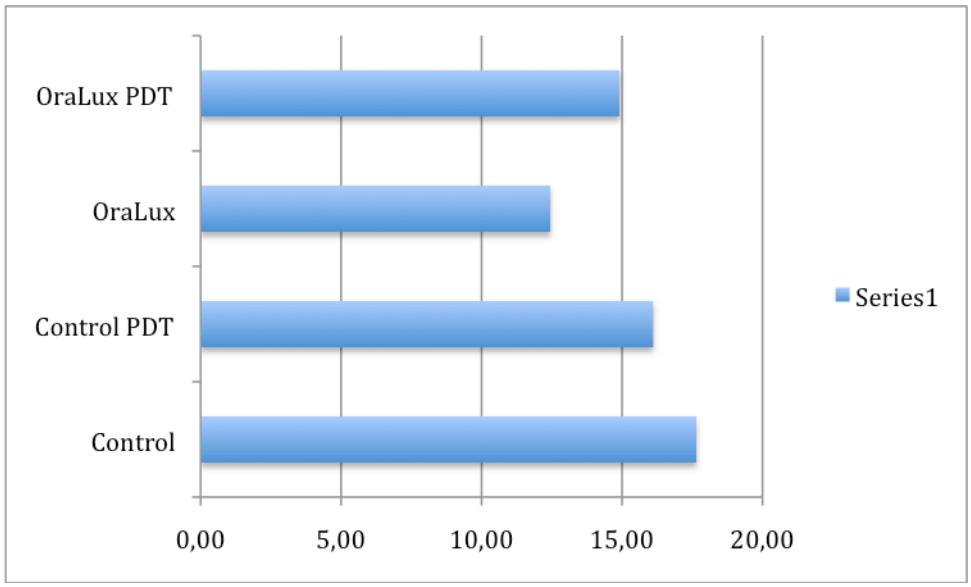


Fig. 4: Total Path length of all cells divided by the total time cells were visible

As a combined analysis of locomotor activity and speed, the average distance that each cell of the observed populations travelled, was significantly reduced at 50% toluidine blue O {TBO}, and showed only a slightly tendency to reduction at 5% toluidine blue O {TBO} without PDT treatment and no tendency to reduction at 5% toluidine blue O {TBO} with PDT treatment in comparison to the controls.

For further analysis, the migratory activity was categorized into minimum distances reached by the migrating cells (3, 4, 5, 10, 15 and 20 µm). Interestingly, although 50% toluidine blue O {TBO} led to a strong reduction of the migratory activity, there was still a small part of cells which showed strong migratory activity comparable to untreated cells (results not showed). 5% toluidine blue O {TBO} only led to a slight reduction in those cells of moderate activity (4 and 5 µm distance reached).

The number of active intervals is significantly reduced by 50% toluidine blue O {TBO}, and the number of rest intervals is concomitantly significantly reduced towards '1' This provides evidence that fewer cells are migrating in response to toluidine blue O {TBO}, because each migrating cell must have at least one active interval, but those cells that are not migrating in the entire observation period have one rest interval. Therefore, the less the migratory activity in a population is, the closer the number of active intervals goes to '0' and the number of rest intervals goes to '1' (Fig. 5).

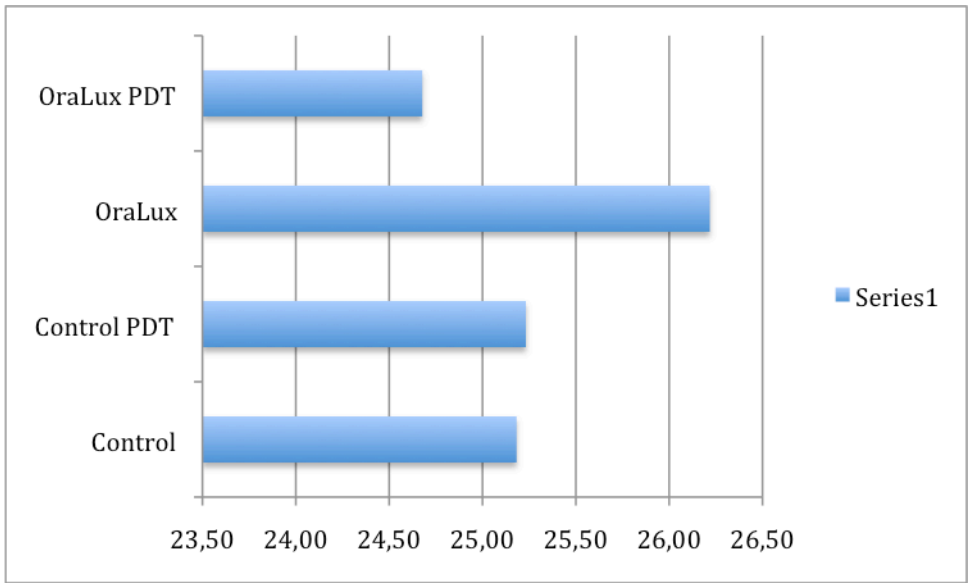


Fig. 5: 5% toluidine blue O {TBO} only led to a slight reduction in those cells of moderate activity (4 and 5 µm distance reached)

The average active interval length was reduced by 50% toluidine blue O {TBO}, which however only reached statistical significance in combination with PDT due to the high standard deviation.

At 5% toluidine blue O {TBO} the average active interval length tended to be longer. In none of the test, the average length of rest intervals was significantly altered (Fig. 6).

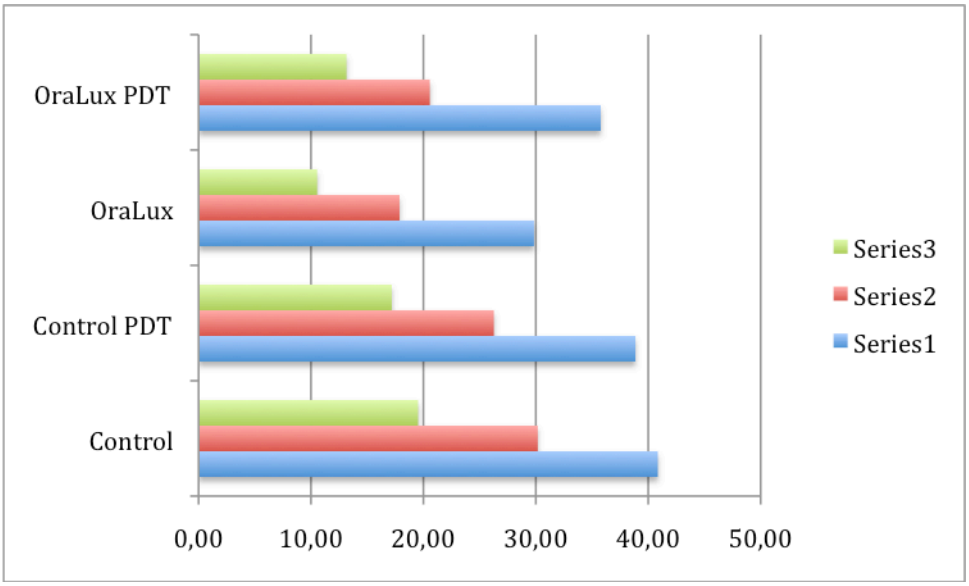


Fig. 6: At 5% toluidine blue O {TBO} the average active interval length tended to be longer. In none of the test, the average length of rest intervals was significantly altered.

Conclusions

High concentrations (50%) of toluidine blue O {TBO} affect the locomotory behaviour of human fibroblasts, whereas concentrations that are supposed to occur in physiological conditions (5% and below) have only weak influence on the locomotory behaviour. PDT treatment does not influence locomotory behaviour. **We conclude that LLLT as photodynamic therapy (PDT) using ora-laser d-lux 810nm promotes proliferation and maturation of human fibroblasts *in vitro*.**

Further outcomes

Rapid advancements in the field of regenerative medicine have led to many current efforts to exploit Photodynamic treatment (PDT) as therapeutic agents. However, current *ex vivo* cell manipulations common to most regenerative approaches create a variety of technical and regulatory hurdles to their clinical translation, and even simpler approaches that use exogenous factors to differentiate tissue-resident cells carry significant off-target side effects. We show that non-ionizing, low-power laser treatment (LLLT) can instead be used as a minimally invasive tool to activate an endogenous latent growth factor complex, transforming growth factor–β1

(TGF-β1), subsequently differentiates host cells to promote tissue regeneration. LLL- treatment induced reactive oxygen species (ROS) in a dose-dependent manner, which, in turn, activated latent TGF-β1 (LTGF-β1) via a specific methionine residue. Laser-activated TGF-β1 was capable of differentiating human dental cells *in vitro*. These findings indicate a pivotal role for TGF-β in mediating LPL-induced dental tissue regeneration. More broadly, this work outlines a mechanistic basis for harnessing resident dental cells with a light-activated endogenous cue for clinical regenerative applications.

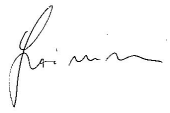
Disclosure notes

This collaboration report consists of the following elements:

1. Terms of agreement regarding intellectual property, data ownership, bi-directional first right of refusal, confidentiality, publishing and other issues regarding the RESEARCH PROJECT.
2. This Project has been individually contracted.

All experiments were conducted and analyzed in good scientific practice.

A detailed analysis of the individual cell behaviour (i.e. frequency and length of rest vs. active periods and further migrations parameters) are intellectual property of Molthera GmbH (videos, row data).



Prof. Dr. Wolf-D. Grimm
-scientific project leader-