Photodamage of dental pulpa stem cells during 700 fs laser exposure

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Material and methods

Cells

Human stem cells from the dental pulp of adults (given by S. Gronthos, NIH, Bethesda, USA) were cultivated in α-modified Eagle's Medium (MEM) by Gibco BRL Life Technologies (Paisley, Scotland) while adding 20 % FCS, 2 mM L-glutamine, 100 µM L-ascorbate-2-phosphate, 100 u Penicillin and 100 µg/ml Streptomycin at 5 % CO_2 and 37 °C in 25 ml and 75 ml cell culture flasks (Greiner, Frickenhausen, Germany).[1] The cells were treated for experiments and cultivation with 0.25 % trypsin, 5 mM glucose, 0.05% EDTA in PBS for 5 minutes at 37 °C. After having been thus detached from the base of the culture flasks, the cells were incubated in cell chambers (MiniCeM, JenLab GmbH, Jena, Germany) for laser microscopy.

Comparative studies were conducted on Chinese hamster ovary (CHO) cells, which are available in many international laboratories as reference cells. The CHO cells were incubated in Dulbeccos HAM-F12 Medium (Gibco BRL) with 10 % FCS, L-glutamine and an antibiotic mix of Penicillin, Streptomycin and Amphotericin B at 5 % CO_2 and 37 °C. Trypsinisation corresponded to that of the stem cells.

Individual cells were marked by special diamond engravings in the exterior glass window. In case of the detection of cellular damage, these engravings were easily located by applying the phase-contrast technique.

Laser microscopy

An 80 MHz Ti:Sa Laser, Mai Tai (Spectra-Physics, Mountain View, USA) was applied for femtosecond-laser microscopy in the near infrared (NIR) spectrum. The laser power at microscope entrance and objective plane (power at the sample) were determined by the measuring instrument Fieldmaster (Coherent, Santa Clara, USA) and the measuring head LM2 and varied by grey filters when necessary. The measured values were specified as corrected in the presented protocol. This correction results from a limited measurement area and altered radiation conditions in the medium when compared to air.

Laser microscopy was realised via a modified LSM 410 (ZEISS, Jena, Germany) with a 40x/1.3 oil immersion objective. The microscope scan modus 512 x 512 with a laser scan time t = 16 s was applied for cell irradiation. The cells were scanned ten times at the same focus plane. These experiments were realised at a central wavelength of 800 nm. After irradiation, the cells were

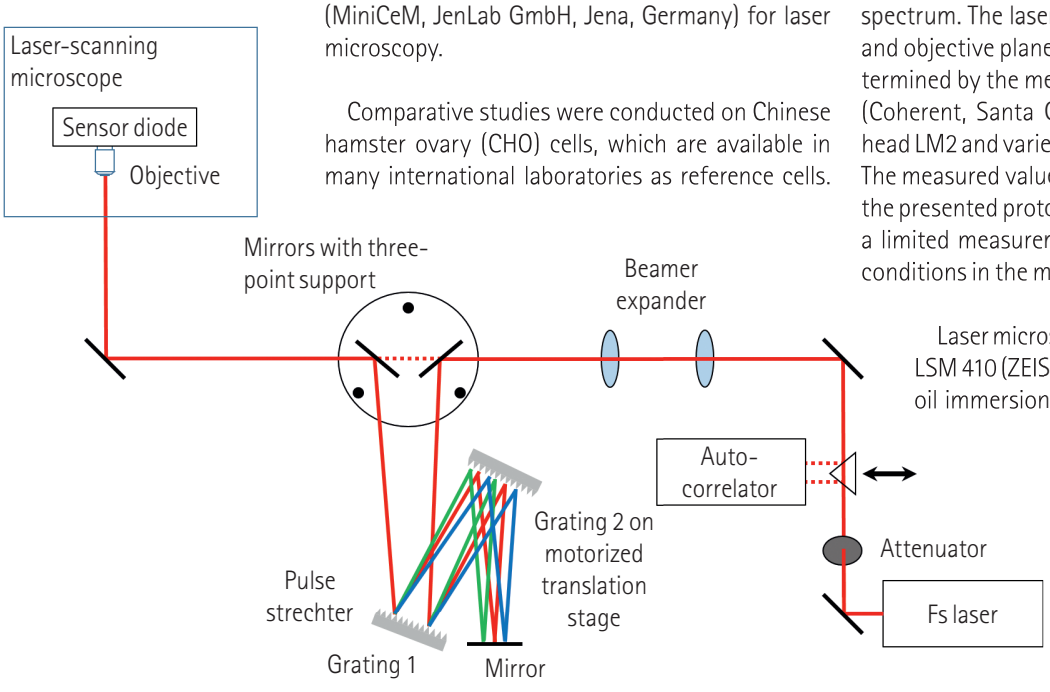


Fig. 1: Femtosecond laser system with pulse-stretching unit.

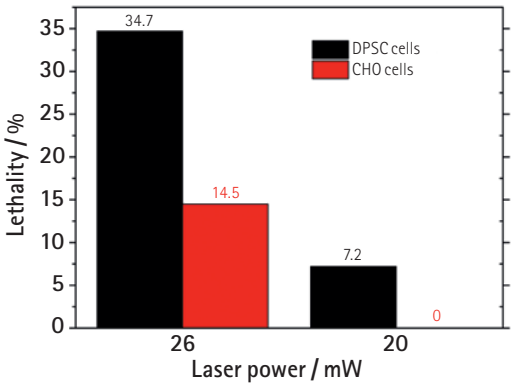


Fig. 2: Laser-induced damage rate (lethality).

transferred to an incubator in order to guarantee optimal conditions for further growth, cell division and repairing processes.

Pulse-stretching unit, generation and measurement of 700 fs pulses

In order to increase the pulse duration at the sample to 700 fs, a pulse-streching unit was implemented in the light path in front of the microscope. This unit consists of coated mirrors and two parallel arranged gold-sputtered gratings with a grating constant of 600 lines/mm. The second grating was mounted on a motorised stage with micrometre precision. The pulse width was varied in dependence on the grating distance. The laser beam received a spatial dispersion by the first grating, which was compensated at the second grating (Fig. 1).

The pulse duration was initially determined at the laser exit with the autocorrelator MINI (APE, Berlin, Germany) with 88 fs at a central emission wavelength of 750 nm, 80 fs at 800 nm and 91 fs at 850 nm, hypothesising a Gaussian function. In general, measurements at the focal plane of the objective proved difficult, as divergent beams exist. A flat, non-linear measurement diode was employed, thus facilitating the measurement at the focal plane of high-aperture objectives. The autocorrelation function (ACF), which can be fitted with either Gauss-, Lorentz or Secanthyperbolicus-based analysis programmes in order to calculate the pulse duration.

Life-/Dead-Test

In order to examine the vitality of dental pulp stem cells (DPSC), a test by Molecular Probes (Eugene, Oregon, USA) was applied. A mixture of 2 µM calcein AM and 4 µM ethidium-homodimer-D1 was added to the cell chambers and incubated for 20 min at 37 °C.

Live cells were stained by calcein (emission in the green spectrum), dead cells by ethidium-homodimer-D1 with an emission in the red spectrum (nucleus). Calcein AM is a non-fluorescent dye which easily permeates the cell membrane of live cells and is transformed by an enzymatic reaction to the strongly green fluorescent calcein which cannot pass the intact membrane. Ethidium-homodimer-D1 is a red fluorescent so-called dead-cell staining agent which can only permeate damaged cell membranes and is significantly intensified by binding to DNA. The Life-/Dead-Kit was incubated 5.5 h after irradiation. Fluorescence was achieved by two-photon excitation.

Verification of laser-induced ROS-formation

The formation of ROS was verified in situ via two-photon excitation of the membrane-permeable fluorophore dihydroflurescein (DHF) according to the method by Hockberger et al.[2] First, the cells were incubated with the marker (10 µM, Fluka, Germany) and irradiated after 15 min incubation time. Only one ROI (region of interest) was subjected to irradiation. Surrounding cells were used as control. After irradiation, a full-frame scan was realised with the low power of 4 mW in order to visualise the effect.

Results

The pulse-stretching unit was adjusted in a way that allowed for each laser wavelength a pulse duration of 700 fs ± 50 fs in the focus. Single DPSC cells were scanned 10 x and the effect compared to the non-irradiated surrounding cells was determined. In addition, comparative experiments were accomplished using CHO cells. Cells or cell clusters were selected which were widely spaced in order to inhibit any intercellular communication as far as possible. During scanning, the transmission signal was detected and displayed as a picture on the monitor. A total of 325 cells was subjected to morphological examinations as well as a life-/dead-test, 50 cells underwent ROS examination. The results were compared to earlier findings with a pulse width of 170 fs.

Morphological changes

So-called black spots resulted from specific irradiation power perameters in locations with significant granulation in the cells. The laser power was gradually increased by 2 mW in order to measure the threshold value for the appearance of the first laser-induced morphological changes (Tab. 1). Under these conditions and with optimum focus, the minimal power for the appearance of black spots was 20 mW for the DPSC cells and 22 mW for the CHO cells (Tab. 2). At a power of 26 mW, 30 % of the DPSC cells and only 15 % of the CHO cells presented these morphological changes. Cells with laser-induced black spots generally revealed morphological changes within the following five hours thus indicating a photodamage effect.

Occurance of black spots in DPSC cells

mW	Total of irradiated cells	Cells with spots
16	10	0
18	10	0
20	69	1
26	72	21

Table 1

Occurance of black spots in CHO cells

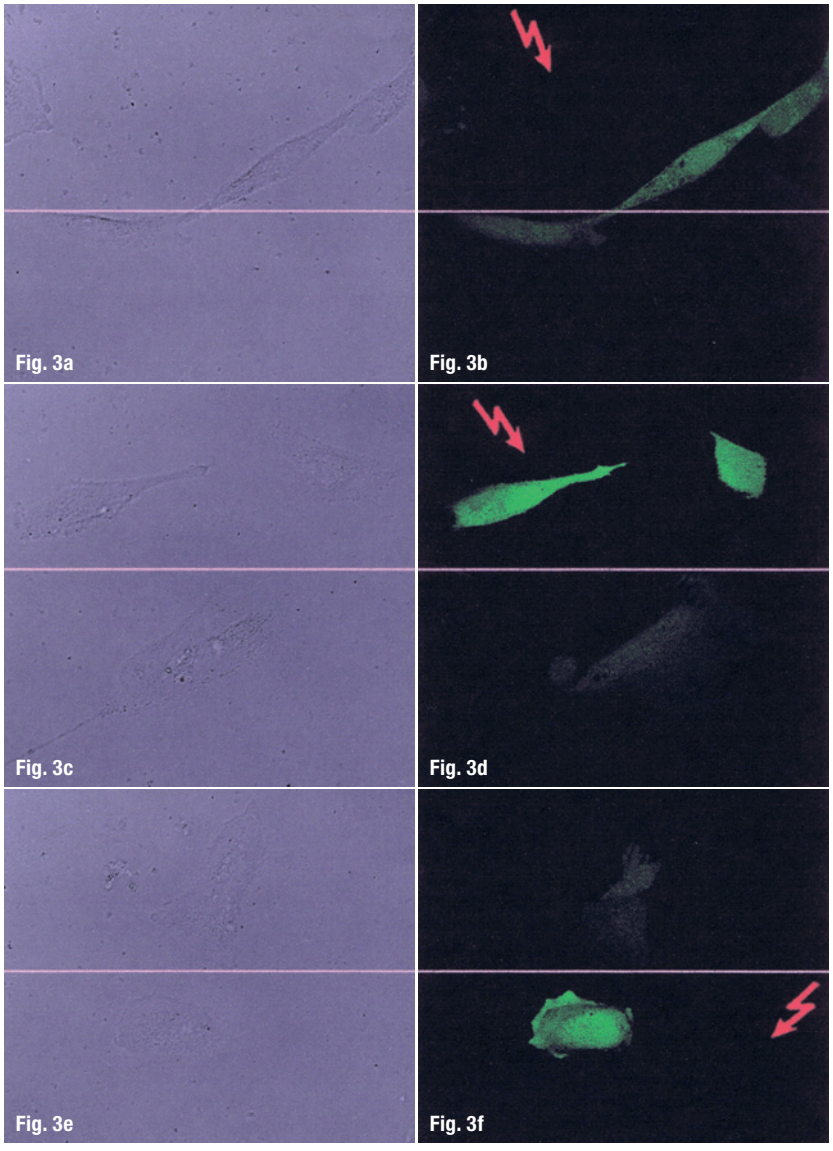
mW	Total of irradiated cells	Cells with spots
16	10	0
18	10	0
20	69	0
26	72	11

Table 2

Life-/Dead-Test

First, the DPSC cells were irradiated with different power parameters (4 mW, 12 mW, 16 mW, 20 mW and 32 mW). The irradiated cells were marked, incubated after 5.5 hours with the Life-/Dead-Kit and after 1 hour of incubation tested concerning their fluorescence behaviour. At an irradiation of 20 mW, 64 of 69 DPSC cells revealed a green cytoplasm fluoresence, while a red fluorescence was observed in 5 DPSC cells (Tab. 3). This corresponds to a damage of 7%. When the power was raised to 26 mW, already 35 % were subjected to a lethal effect. However all of the CHO cells tolerated a power of 20 mW, with 15 % of them dying at 36 mW (Tab. 4 and Fig. 2). The comparison with experiments of shorter pulse widths, at which already 73 % of the DPSC cells were damaged at a power of 20 mW, shows that the longer pulse width of 700 fs is better tolerated by the cells.

Figs. 3a–f: Verification of the laser-induced formation of ROS.



Verification of the laser-induced formation of ROS (reactive oxygen species)

The irradiation showed that no detectable DHF signals occurred at average power lower than 35 mW. As can be seen from Figure 3 (upper part of a and b) ROS signals were detected at a higher power of 37 mW in both upper irradiated cells. Weak fluoresence signals occurred in the lower non-irradiated cell too. However, this cell was linked to the irradiated cells via membrane contacts. If the power is only insignificantly increased, its effects become more pronounced. In Figure 3c the irradiated cells show a significantly higher intensity. This effect is also confirmed by the considerable fluorescence of the irradiated cell (Fig. 3f). Thus, destructive oxygen radicals are formed during irradiation of a pulse duration of 700 fs. In comparison to the 170 fs experiments, a significantly higher average power is necessary to achieve detectable ROS formation.

Conclusions

DPSC are less sensitive to irradiation with femtosecond NIR laser at a pulse width of 700 fs under the described irradiation conditions than to shorter pulse width of 170 fs. At average power parameters of 20 mW, up to 10 % of the cells were subjected to lethal effects within six hours after irradiation. At an average power of 26 mW, still two thirds of the cells survived. At the shorter pulse width all cells would be subjected to a lethal effect.

The observed lesser sensitivity at higher pulse widths and constant pulse energy corresponds to the results of earlier studies on Chinese hamster ovary

Lethality of DPSC cells

mW	Total of irradiated cells	vital	lethal	% lethal
16	10	10	0	0
18	10	10	0	0
20	69	64	5	7
26	72	47	25	35

Table 3

Lethality of CHO cells

mW	Total of irradiated cells	vital	lethal	% lethal
16	10	10	0	0
18	10	10	0	0
20	69	68	0	0
26	72	65	11	14,5

Table 4

cells (CHO) at an irradiation wavelength of 780 nm.[3] In these earlier studies, it was concluded that the damage is subject to a two-photon effect, for which a damage effect E can be expected according to the formula

$E \sim P^2/\tau$ with P: average laser power and τ: pulse width.

As in comparison an increase of the pulse width by factor F = 700 fs / 170 fs = 4 exists, it follows that a power increase by factor S = 1.7 to 2 should be necessary in order to achieve the same destructive effect.

This relation cannot be confirmed exactly basing on the presented data, but a factor S > 1.25 can be assumed, as already 7 % of the DPSC cells died at an average power of 16 mW and a pulse duration of 170 fs, but 20 mW at 700 fs were necessary to achieve the same effect.

DPSC cells react slightly more sensitive at a pulse width of 700 fs than CHO cells. At an exposure of 26 mW laser power only 15 % of the CHO cells were damaged in comparison to 35 % of the DPSC cells. The detected ROS formation indicates a photochemical damage process._

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Kurz & bündig

Die humanen Stammzellen aus der Zahnpulpa Erwachsener (erhalten von S. Gronthos, NIH, Bethesda, USA) wurden in α-modifiziertem Eagle´s Medium (MEM) bei 37 °C in 25 ml und 75 ml Zellkultur-Flaschen (Greiner, Frickenhausen, Deutschland) kultiviert. Die Zellen wurden für Versuche und Zucht mit 0,25 % Trypsin, 5 mM Glucose, 0,05 % EDTA in PBS für ca. 5 min bei 37 °C behandelt. Die so vom Boden des Kulturgefäßes abgelösten Zellen in Zellkammern (MiniCeM, JenLab GmbH, Jena, Deutschland) für die Lasermikroskopie inkubiert. Vergleichend fanden Untersuchungen an Chinesischen Hamsterovar-(CHO-)Zellen statt, die weltweit in vielen Laboren als Referenzzellen vorliegen. Einzelne Zellen konnten markiert werden, indem ein Spezialdiamant in das äußere Glasfenster Gravierungen erzeugte. Diese konnten bei Detektion von Zellschäden leicht mit dem Phasenkontrastverfahren lokalisiert werden.

Auf eine Bestrahlung mit einem Femtosekunden-NIR-Laser mit einer Pulsdauer von 700 fs reagieren die DPSC-Zellen nach vorliegenden Bestrahlungsbedingungen weniger empfindlich als bei einer kürzeren Pulsdauer im Bereich um 200 fs. Bei mittleren Leistungen von 20 mW erleiden bis zu 10 % der Zellen letale Wirkungen innerhalb von 6 Stunden nach der Bestrahlung. Bei einer mittleren Leistung von 26 mW überleben immer noch 2/3 der Zellen. Bei der kürzeren Pulsdauer wären bereits alle Zellen einer letalen Wirkung unterlegen. Die DPSC-Zellen zeigten sich bei der Pulsdauer von 700 fs etwas empfindlicher als CHO-Zellen. So wurden bei Einwirkung von 26 mW Laserleistung nur 15 % der CHO-Zellen im Vergleich zu 35 % der DPSC-Zellen geschädigt. Die detektierte ROS-Formation deutet auf einen photochemischen Schädigungsprozess hin.

Literature