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Effects of ultraviolet exposure and near infrared laser tweezers on human spermatozoa

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Photostress has to be considered during optical micromanipulation of gametes. Ultraviolet light, including low-energy UVA (320–400 nm) radiation, as well as high-intensity near infrared (NIR) laser radiation may induce cell damage. A total number of 580 light-exposed sperm cells were studied in single-cell photostress experiments. Low-power (1.5 mW, 5.3 W/cm^2) UVA exposure with 365 nm radiation of a standard mercury microscopy lamp to human spermatozoa resulted within 109 ± 30 s in paralysis and within 310 ± 110 s in cell death. Cytotoxic effects during cell manipulation with laser microbeams were found to be partly based on non-linear excitation phenomena, in particular two-photon absorption by endogenous cell chromophores. Two-photon absorption will be more intense in the case of pulsed laser microradiation, but occur also during micromanipulation with highly focused continuous wave (cw) microbeams used as laser tweezers ('optical traps'). In particular, short-wavelength NIR traps <800 nm induce UVA-like biological effects (oxidative stress). For example, sperm trapping with 760 nm microbeams resulted in UVA-like autofluorescence modifications, paralysis within 35 ± 20 s and cell death within 65 ± 20 s. In contrast, laser microbeams at 800–1064 nm may act as relatively safe micromanipulation tools. In most optical traps multifrequency cw lasers are employed. Radiation of these lasers can magnify cytotoxic effects. Therefore, single-frequency laser operation should be preferred. In general, laser-assisted cell micromanipulation requires a new understanding of microbeam-cell interaction, including aspects of non-linear optics.

Key words: IVF/laser tweezers/photodamage/spermatozoa/UVA

Introduction

Laser-assisted gamete micromanipulation is used in basic gamete research as well as for in-vitro fertilization (IVF). Oocyte microsurgery, such as hole drilling in the zona pellucida and laser-assisted hatching, with pulsed ultraviolet (UV, 100–400 nm) and pulsed infrared (NIR, 700 nm–1 mm) laser microbeams has been reported (Blanchet *et al.*, 1992; Feichtinger *et al.*, 1992; Laufer *et al.*, 1993, Neev *et al.*, 1993; Tadir *et al.*, 1994; Enginsu *et al.* 1995; Germond *et al.*, 1995). On the other hand, continuous wave (cw) near infrared (NIR, 700–1200 nm) laser microbeams are employed as laser tweezers (optical traps) in sperm micromanipulation. Optical traps allow confinement of single motile cells in the focal volume of an objective of high numerical aperture and controlled three-dimensional movement of single cells by trap-generated optical forces (Ashkin and Dziedzic, 1987; Tadir *et al.*, 1989). In addition, optical determination of motility forces of spermatozoa and fluorescence imaging/spectroscopy on motile spermatozoa is possible (Tadir *et al.*, 1989; König *et al.*, 1995a,b; 1996a). Up to now, the effects of the high intensity trapping beams and of UV photostress on gamete metabolism have not been studied in detail.

We probed the effect of UVA and NIR photostress on human motile spermatozoa by optical force measurements, onset of paralysis, sensitive autofluorescence imaging, and viability analysing. Relative motility forces were determined by measurement of the minimum laser power (minimum trapping power) required to keep the cell in the trap. A further power decrease would allow the trapped cell to escape. Because of the linear relationship between laser power and generated optical forces, the minimum trapping power is an indirect measure of the relative cellular motility force (Tadir *et al.*, 1989, 1994; König *et al.*, 1996a). Onset of paralysis was detected by monitoring flagellar motion. Autofluorescence is in part based on the fluorescent intrinsic coenzymes β-nicotinamide adenine dinucleotide (NAD) and β-nicotinamide adenine dinucleotide phosphate (NADP). These pyridine coenzymes, mainly located in mitochondria, fluoresce only in the reduced state and act therefore as sensitive probes of cellular redox state and metabolic function (Chance and Thorell, 1959; Schneckenberger and König, 1992; König and Schneckenberger, 1994; König *et al.*, 1995b). Cell viability was detected by sensitive fluorescence detection using a viability kit. The kit contained the live-cell fluorophore SYBR14 and the dead-cell fluorophore propidium iodide (König *et al.*, 1995a).

Materials and methods

Optical trap and fluorescence detection

In order to perform photostress experiments, vital motile spermatozoa were confined in a single-beam gradient force optical trap. For that purpose, the NIR radiation (105 mW) of a tuneable cw Ti:Sapphire ring laser (Model 899-01, Coherent, Palo Alto, CA, USA) was

introduced into a modified laser scanning microscope (Axiovert 135M, Zeiss, Jena, Germany) The laser operated as multifrequency cw laser (several longitudinal modes) but could be transformed into a single-frequency cw laser by adjustment of an intracavity etalon (20 MHz linewidth) The parallel beam was expanded to fill the back aperture of a ×100 Zeiss Neofluar brightfield objective (numerical aperture NA = 1.3) Laser scanning images of trapped spermatozoa labelled with exogenous fluorophores such as the LIVE/DEAD FertiLight™ Viability Kit (Molecular Probes, Eugene, OR, USA) were obtained with 488 nm microbeams of an Ar^+-laser (power at the sample: 2.2 μW, scanning time: 1 s per image). For autofluorescence imaging, the UVA excited fluorescence was detected in the spectral range 430 nm–490 nm (bandpass 460 ± 30) with a slow-scan, cooled charge-coupled device camera (Model TE576/SET135; Princeton Instruments, Princeton, NJ, USA). A 50 W high-pressure mercury arc lamp equipped with a 365 nm bandpass filter was used as excitation source (1.5 mW, 5 3 W/cm^2) as well as low-power UVA exposure source.

The spot size (d) of the focused laser beam was assumed to be diffraction-limited and was approximated by d = λ/NA (λ = wavelength) The intensities were therefore 35 and 39 MW/cm^2 for 800 nm and 760 nm trapping beams at a power of 105 mW, respectively The set-up is described in more detail by König *et al* (1995b) For comparison, sperm cells were trapped with 1064 nm microbeams using a cw Nd:YAG laser as trapping source

Sperm preparation

Semen specimens were obtained from three donors with normal semen parameters according to the World Health Organization guidelines (WHO, 1994). Semen was layered on a discontinuous isotonic Percoll gradient (Pharmacia, Sweden), consisting of three layers, 1 ml each, of 95, 70 and 50%. After centrifugation for 15 min at 200 *g*, the bottom layer was removed, washed with HEPES-buffered fresh human tubal fluid (HTF; Irvine Scientific, Irvine, CA, USA) and centrifuged for 10 min at 100 *g* The pellet containing the spermatozoa utilized for experiments was then diluted in HEPES-buffered isotonic saline solution containing 1% human serum albumin. Spermatozoa were injected into microchambers consisting of two 0.16 mm thick coverslips separated by a 0 5 mm thick tape layer as spacer. Experiments were performed within 3 h following ejaculation. Single cell measurements on a total number of 580 sperm cells were carried out at a room temperature of 29°C.

Chemicals

For viability analysis, the LIVE/DEAD FertiLight™ Sperm Viability Kit (Molecular Probes) was used, which contains the live-cell fluorophore SYBRTM14 (final concentration: 100 nM) with an emission maximum at 515 nm and the dead-cell stain propidium iodide (final concentration. 12 μM) with an emission peak in the red spectral range. Cells were incubated 5 min prior to experiment.

Results

Vital sperm cells in the 800 nm optical trap exhibited a blue autofluorescence in the cell midpiece, the primary site of mitochondria. No significant change in autofluorescence pattern was found during 10 min of trapping for laser operation both with and without the etalon (measurements every 2.5 min, fluorescence acquisition time: 5 s, n = 20)

In contrast, a significant change in autofluorescence characteristics was found in the case of 760 nm traps (n = 40) The autofluorescence intensity increased within 1 min and the sperm head became the brightest fluorescent site. We detected a faster autofluorescence increase in the case of the multifrequency laser compared to the single frequency laser (laser with etalon).

Table I. Effect of long wavelength UV (UVA, 1 5 mW) and near infra red (NIR, 105 mW) photostress on 480 spermatozoa probed by mean cellular autofluorescence intensity (relative units) I_{AF} after 2 min light exposure (n = 20), mean minimum trapping power P_I after 2 min light exposure (n = 20), mean time (in s) t_M where loss of motility occurred (n = 20), and mean time (in s) t_{PI} where onset of intranuclear propidium iodide fluorescence (indicative for cell death) was observed (n = 20) Control cells remained in the dark; they were exposed shortly (1 s) to excitation light for I_{AF} (2 min), t_M/s and t_{PI}/s measurements and to 800 nm trapping radiation for P_I determination Errors indicate SD

λ/(nm)	I_{AF} (2 min)	P_I/mW (2 min)	t_M/s	t_{PI}/s
Control	100 ± 30	82 ± 38	>600	>600
365	200 ± 50	10 ± 18	109 ± 30	310 ± 110
760 (no etalon)	510 ± 100	0	35 ± 20	65 ± 20
760 (etalon)	180 ± 50	8 ± 8	132 ± 50	406 ± 160
800 (no etalon)	100 ± 30	62 ± 21	>600	>600
800 (etalon)	100 ± 30	66 ± 21	>600	>600

λ = wavelength.

In order to study the influence on UVA exposure, cells were confined in an 800 nm trap and permanently exposed to UVA light of the mercury lamp (365 nm, n = 20). Interestingly, a similar autofluorescence increase and fluorescence relocalization as in the case of 760 nm traps was found. The sperm head, in some cells in particular the acrosome region, became the brightest fluorescent site.

Autofluorescence changes induced by UVA exposure as well as by 760 nm traps were accompanied with reduced motility, paralysis and finally, loss of viability. No viability loss was detected in 800 nm traps or 1064 nm traps (105 mW). The results are shown in Table I.

Discussion

UV lasers as well as NIR lasers have been reported to be harmless tools in laser-assisted IVF. Our results demonstrate that possible cytotoxic effects with radiation at both spectral regions have to be considered

UV laser radiation may induce genetic as well as non-genetic damage to gametes Genetic damage is assumed to be a result of direct absorption of UV photons by DNA and RNA, or of indirect processes involving photo-oxidation reactions after excitation of different cellular absorbers, such as porphyrins or pyridine coenzymes (Peak *et al.*, 1987). Nucleic acids have major absorption bands in the spectral range 200–300 nm (maximum ~260 nm). Therefore, laser exposure to vital cells at this wavelength range should be avoided. For example, toxic effects during exposure of mouse embryos to 248 nm excimer laser radiation have been reported (Blanchet *et al.*, 1992).

Interestingly, long-wavelength UV, so called UVA (320–400 nm) radiation, may also induce irreversible cell damage. We were able to show that exposure to low-power 365 nm light resulted in cell killing. It is known that severe cell damage may occur via UVA-induced oxidative stress even though there

is no direct absorption by nucleic acids (Tyrell and Keyse, 1990; König *et al.*, 1995c, 1996b). We performed our UVA experiments with a 50 W high-pressure mercury lamp, which is normally used in fluorescence microscopy. Interestingly, the power of the 365 nm radiation of only 1.5 mW was sufficient to paralyse and finally kill the cells within a few minutes. Photodamage was accompanied by cellular autofluorescence modifications. These results confirm findings on NAD(P)H-attributed autofluorescence during photostress to culture cells (Schneckenberger and König, 1992; König *et al.*, 1996b). More work has to be done to explain the preferential autofluorescence site in the acrosome region of some cells.

Most microsurgery experiments have been performed with UV lasers. A potent alternative to UV microsurgery is IR microsurgery (Feichtinger *et al.*, 1992; Germond *et al.*, 1995). In the IR spectral range, the main cell absorber is water.

Optical traps at the short-wavelength part of the NIR spectral region may also induce cell death. The NIR spectral region is the so-called 'optical window' of cells and tissues due to the lack of efficient endogenous absorbers. Only low-intensity absorption bands of water, reduced haemoglobin, and certain cytochromes occur around 760 nm. However, these weak absorbers are not responsible for the observed cell damage in 760 nm traps: if a 100 mW NIR microbeam is focused to a diffraction-limited spot of ~0.5 μm diameter, light intensities of about $>10^7$ W/cm^2 occur. For comparison, the light intensity of sunlight at the earth's surface is ~0.1 W/cm^2 (100×10^6 less intensity). At this high laser intensity, an intracellular photon flux density of $>10^{26}$ photons/cm^2/s is present. This value is high enough to induce non-linear effects in the sperm cell, in particular, two-photon absorption (König *et al.*, 1995a). That means the cell that normally absorbs no NIR photons at low light intensities, is now able to absorb two NIR photons simultaneously (non-resonant two-photon absorption). This results in similar effects as for irradiation at half the incident laser wavelength. Therefore, 760 nm microbeams may create similar biological effects as 760/2 = 380 nm UVA radiation, and can induce oxidative stress. In contrast, 800 nm microbeams or microradiation at longer wavelengths, such as at 1064 nm, are less harmful. Trapping wavelengths >1300 nm are not suitable due to the onset of significant water absorption (water absorption coefficient for wavelengths >1300 nm: >0.1/cm in contrast to that at 700 nm: <0.003/cm) (Hale and Querry, 1973).

The photon flux density can reach higher values when multi-frequency cw lasers are employed in optical trapping. In this case, so-called longitudinal laser modes are superimposed which can result in laser pulse formation. In fact, we measured in our 'cw' Ti:Sapphire laser unstable light pulses with a repetition frequency of multiples of 180 MHz and a pulse duration of <500 ps. These laser pulses were most intense at a laser wavelength of 760 nm. For two-photon excitation, there is a squared dependence of two-photon absorption on power. Therefore, the higher the pulse power, the more efficient the two-photon absorption. The intense pulse formation at 760 nm is responsible for a most efficient cell killing rate with the multi-frequency cw laser.

In contrast, transformation of the multifrequency cw laser into a 'pure' cw laser (single frequency laser) reduced the damaging effect significantly. However, two-photon induced oxidative stress may still occur.

In conclusion, we recommend more basic studies on laser microbeam–gamete interaction and the use of long wavelength NIR, single-frequency cw radiation for optical micromanipulation of spermatozoa.

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