LASER LIGHT EFFECTS ON THE CYTOSKELETON OF HELA CELLS

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A b s t r a c t

We present partial results of an ongoing study on the action of physical factors on cytoskeleton of tumour cells. HeLa cells were used. Microtubules and microfilaments were visualised by immunofluorescence using Leitz Labor LUXS microscope. The cells have been irradiated by a low-power therapeutic laser BEAUTYLINE BTL-10. Changes in microtubules and microfilaments after irradiation at wavelength of 830 nm, in modulated pulse mode and non-modulated continuous mode were observed. The laser used gave constant power of 45 mW and densities of radiation energy of 24, 144 and 180 $J.cm^2$. The area of 0.15 cm^{-2} has been irradiated. In continuous mode the pulse frequency was not modulated and in modulated pulse modes frequencies of 10, 146 and 5000 Hz were used. The exposure times ranged from 80 seconds to 10 min. Our results show damage and rarefaction of the cytoskeletal network in the whole volume of cells at long exposure. In contrast, short exposure produced changes only in the cell periphery. Our preliminary results show that the damage of microtubules is markedly time- or power density-dependent. We were unable to show any difference in changes caused by the pulse modulated and continuous laser light exposure.

K e y   w o r d s

Low-power therapeutic laser, HeLa cells, Microtubules, Microfilamentes, Immunofluorescence

INTRODUCTION

Laser is a source of coherent visible (ultraviolet or infrared) monochromatic light. The first laser used in medicine was an invasive (high-power) device for coagulation of retina (1961). Positive effects of low-power laser light were reported by *Solon a Gould* in 1971. Mester studied biostimulatory effects of laser since 1974. In Czech Republic, Rosina used the non-invasive laser only in 1984 *(1)*.

The power of low-frequency lasers does not exceed 500 mW. The wavelength of laser light exploited in non-invasive laser therapy ranges from 630 to 904 nm. In epidermal and mucosal applications, lasers emitting red light (639–700 nm) are the most advantageous, for the shorter wavelength the smaller penetration of light inside the tissue depth. Therefore, energy of light is absorbed in a thin layer, and these lasers are predominantly utilised in dermatology, dentistry, plastic and aesthetic surgery or gynaecology. The lasers emitting light in the range of 800–904 nm, which is able to penetrate into deeper tissue layers at the same

power, are used mainly in orthopaedics, physiotherapy and rehabilitation. *(1)*

There are three different effects of laser light on tissues: biostimulatory, anti-inflammatory and analgesic *(1)*. On the molecular level we distinguish two different effects: photochemical and photothermal *(2).* In our morphological studies on cytoskeleton we studied laser light effects on cellular level. There are many possible intracellular targets of laser light.

It was published already that the nucleus belongs among the most laser light-sensitive cytoplasmic structures, mainly due to its content of nuclear chromatine. It was shown by means of the Nd-YAG laser microbeam (l = 365 nm). The objective of laser microbeam irradiation of centriolar region (Nd-YAG laser, l = 265 nm) was to verify a possibility of influencing the mitotic process *(3).*

The effects of a low-power He-Ne laser (l = 632,8) on intermediary filaments and stress fibres were already studied by *Bolognani (4).*

The cytoskeleton is formed by three structural components: microtubules, microfilaments and intermediary filaments *(5, 6).* Each of these components forms an individual cytoplasmic network. It is presumed that these networks, formed exclusively by proteins, are mutually connected and interacting. The individual structural components of the cytoskeleton form also many specialised structures. Other proteins which are associated with cytoskeletal structures, are also important parts of the cytoskeleton. They mediate mutual contacts and function of cytoskeletal structures *(5).*

The principal property, in which a tumour cell differs from a normal somatic cell, is the uncontrolled proliferation and defective differentiation *(7).* In both these processes, namely the cytoskeleton plays a substantial role. The knowledge of possibility how to influence the cytoskeleton and its functions can also contribute to the explanation of regulation of tumorous transformation.

Our research is motivated by today's extensive use of low-power lasers in many branches of clinical medicine in which numerous therapeutic achievements are also referred *(8, 9, 10, 11, 12,13).* Unfortunately, there is only small number of reproducible experimental results, what could explain the healing effects of laser light considering the observations on molecular and cellular level.

MATERIAL AND METHODS

Biological object

In our studies on low-power laser light effects, the HeLa cells (a stable cell line derived from human epithelial carcinoma of uterine cervix) were used. Monolayer cultures of these cells were grown in Petri dishes on microscopic cover glasses, immersed in 1.5 ml of Eagle's minimum essential medium which contained foetal bovine serum, penicillin and streptomycin, at 37°C in presence of 5 % CO_2 for 24 hours.

Laser application

We used the low-power semiconductor laser BEAUTYLINE BTL-10 (Czech product, III.B class laser). It is a microprocessor-controlled instrument with programmable therapeutic modes and

also manual control of working parameters. The exposure time is calculated automatically. Settings of power, frequency of pulses, dose and irradiated area can be continuously changed. Specific user settings of working parameters can be stored in memory. The irradiation can be performed in various modes: continuous, pulsed, according to Claus and according to Nogier. In the Claus and Nogier modes, the laser beam is modulated in different frequency regimens. These regimens are exploited in laser acupuncture as well as electrotherapy and ultrasound therapy.

The laser probe used in our study emits convergent beam of infrared laser light (l = 830 nm). The energy density applied on cells was 24 or 180 $J.cm^{-2}$ (in continuous mode), and 24 or 144 J.cm-2 (in pulsed modes), radiation power always 45 mW. The area of 0.15 cm^2 has been irradiated. The continuous mode was not modulated; modulation frequency used in the pulsed mode was 5000 Hz, 10 Hz and 146 Hz in Claus and Nogier mode respectively. The exposure times used were 80 s (for continuous mode) and 100 s or 10 min. in pulsed exposure modes.

The cells were irradiated according to scheme in *Fig. 1*. The laser probe was immersed into the medium to a distance of 0.5 mm from the cell monolayer. The laser beam was targeted directly in the centre of the cover glass. A movable holder maintained constant positioning and targeting of the Petri dish with cells.

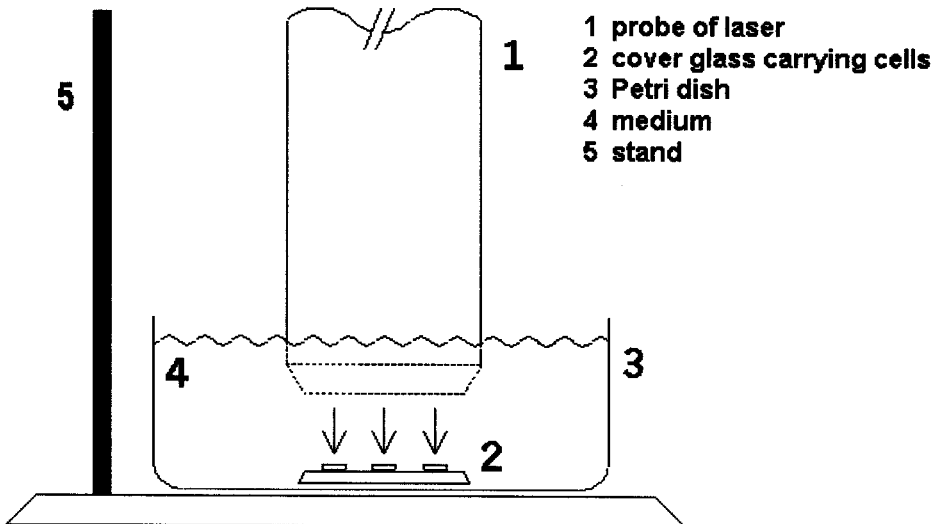


Fig. 1
A scheme of laser irradiation

Cytoskeletal structure detection

The detection of microtubules was performed by means of the indirect immunofluorescence method. We used primary tubulin antibody TU-01 (Institute of Molecular Genetics, AV CR, Prague) and secondary antibody SwAM-FITC (USOL, Prague). Microfilaments were detected by means of Phalloidin-FITC (Sigma).

Microphotographs were made with the fluorescence microscope Leitz Labor LUXS. The images were then digitised with scanner VISTA-S6E, and processed with Adaptive Contrast Control (ACC) software (produced by SOFO) to enhance the cytoskeletal structures and prepare the images for quantitative assessment.

RESULTS

Microtubules and microfilaments of non-irradiated HeLa cells

In the non-irradited cells, the cytoplasmic microtubules form a dense network equally distributed in the whole cell volume. The cells growing on the glass are broadly extended, and the peripheral parts of the cytoplasm are very thin in comparison with the central region around the nucleus so that this part of network seems denser (*Fig. 2*).

The microfilaments of control non-irradiated cells are parallel organised in thick bundles forming stress fibres. These fibres are stretched between different places of cell surface. In addition, they are also present as a network of thinner cytoplasmic structures (*Fig. 3*). Actin can be found also in the form of tiny grains, equally distributed in the cytoplasm.

Microtubules of irradiated cells

After 100 s action of continuous mode of laser light we found slight rarefaction of cytoplasmic microtubules in some parts of cell periphery. These microtubules often formed thicker bundles. The 10 min. laser exposure caused also partial fragmentation of microtubules which manifested itself mainly as well resolvable granules of tubulin positioned along the fragmented microtubule.

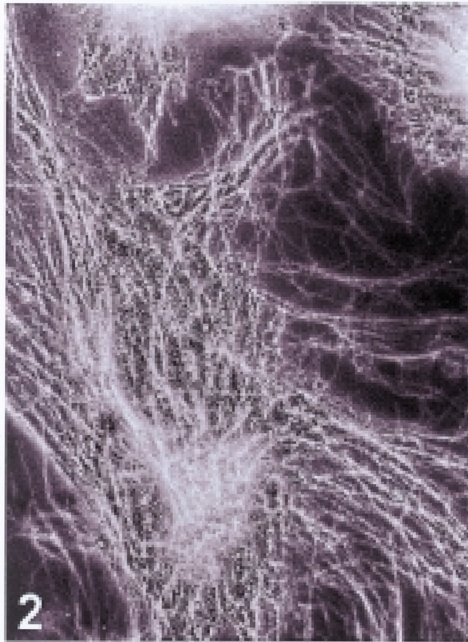
The pulsed and continuous modes of irradiation caused similar changes of microtubular network after both exposures 100 s and 10 min. After the 10-min. exposure, the loss of microtubules in some parts of cytoplasm and their beginning fragmentation were more distinct. (*Figs. 4, 5, 6, 7*).

Fig. 2
Microtubules in non-irradiated HeLa cells. Peripheral parts of the cytoplasm are very thin, cytoplasmic microtubules form a dense network evenly distributed in the whole cell volume

Fig. 3
Microfilaments in control non-irradiated cells. Thick bundles of stress fibres are stretched between different regions of the cell surface. There was a minimum of actin granules

Fig. 4
Microtubules of HeLa cell after 100 sec of laser irradiation in the continuous mode. There was a slight rarefaction of cytoplasmic microtubules in some peripheral cell regions (thin arrow). Some microtubules formed thick bundles (thick arrow)

Fig. 5
Microtubules of HeLa cell after 10 min of laser irradiation in the continuous mode. Partial fragmentation of microtubules into resolvable granules of tubulin positioned along the fragmented microtubule was seen (thin arrow)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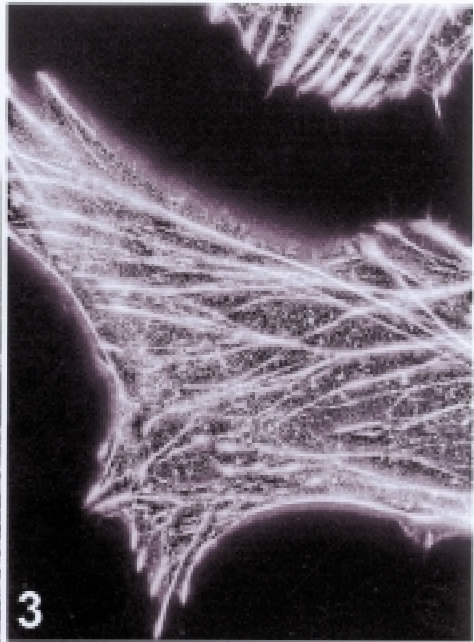
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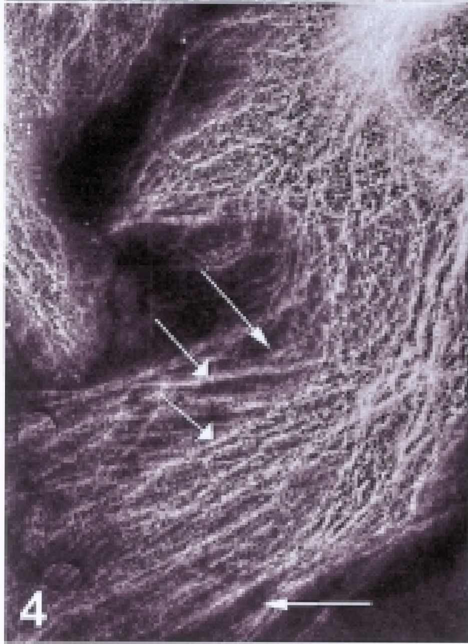
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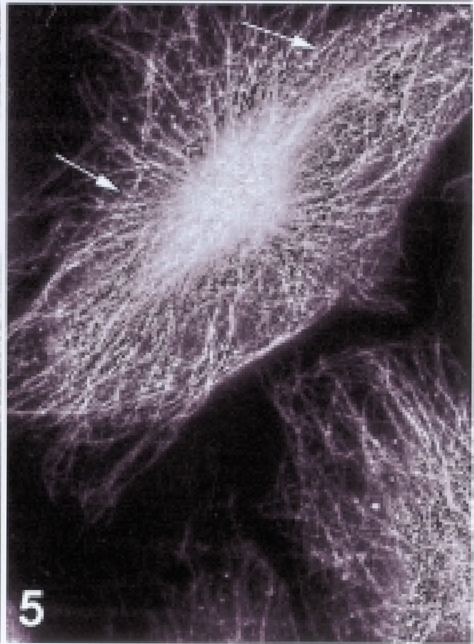
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The Claus mode exposure caused rarefaction of microtubules mainly in periphery (at 100 s exposure), and also fragmentation of long fibre segments (at 10 min. exposure).

After application of the Nogier mode we also observed the rarefaction of peripheral microtubules (at 100 s exposure, *Fig. 8*), and also their fragmentation along the whole fibre length (at 10min. exposure, *Fig. 9*). The microtubules remained radially oriented and were often organised in thick bundles.

When increasing the exposure time the rarefaction of microtubules occurred at first, followed by their fragmentation. Changes in cell shape were also often observed. In contrast to the continuous and pulsed mode, the cells became more spherical after application of Claus and Nogier modes.

Microfilaments of irradiated cells

After 100 s continuous application of laser light (*Fig. 10)* we found evident loss of stress fibres, mainly in central parts of the cell. After 10 min. exposure (*Fig. 11*) we could observe, besides disappearance of stress fibres in the cell centre, actin aggregates in form of thick, short and variously curved fibres which often made clusters. We could find also larger granulous actin aggregates.

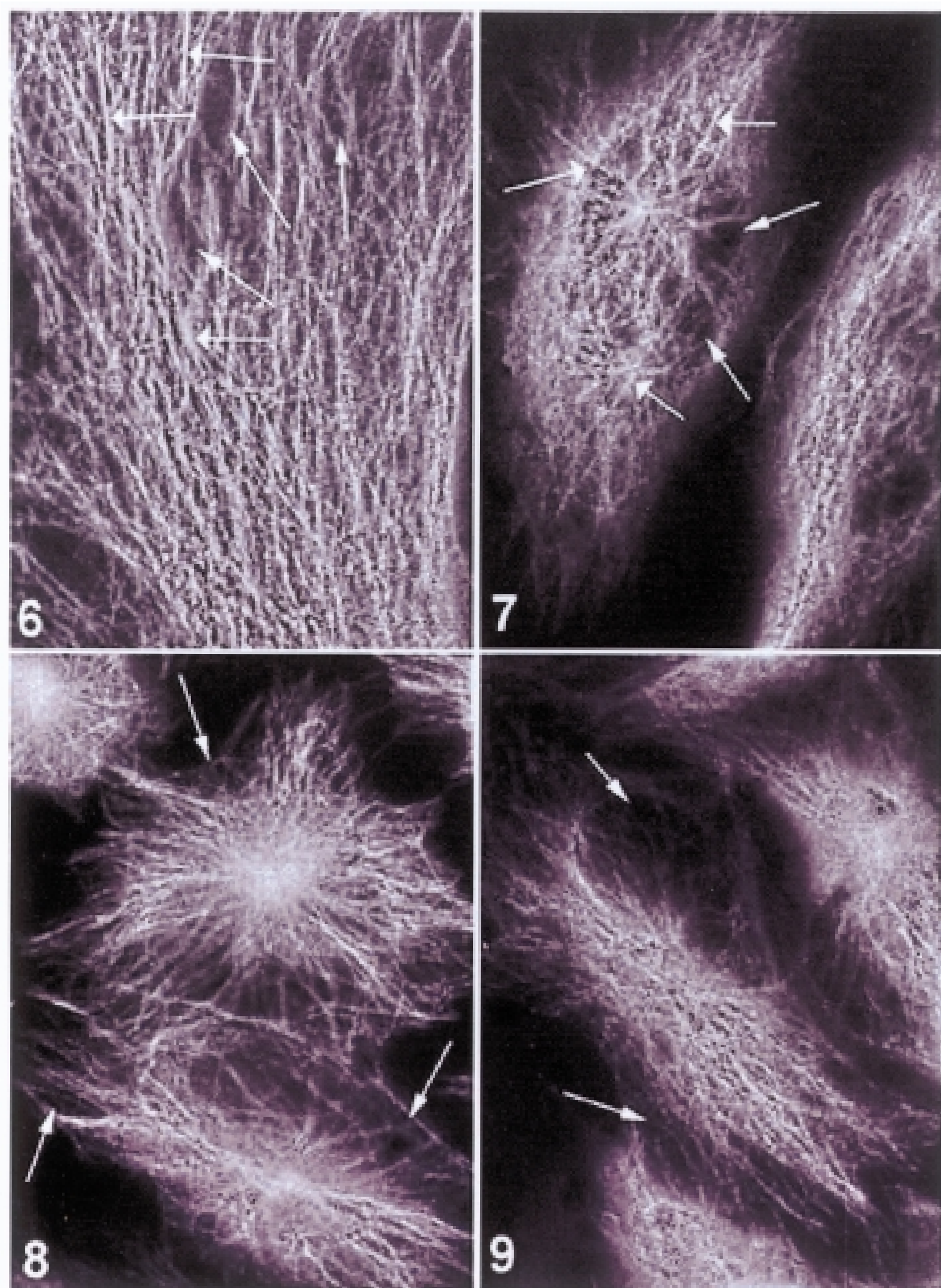
Pulsed mode of irradiation applied for 100 s (*Fig. 12*) caused general rarefaction of stress fibres, mainly in the cell centre. The 10min. exposure *(Fig. 13*) caused evident loss of stress fibres, mainly in central parts of the cell, and formation of atypical assemblies of stress fibres in cell periphery. Large granules of actin and their aggregates also occurred.

Fig. 6
Exposure to laser irradiation in the pulse mode for 100 s caused a dissipation of microtubules in some peripheral regions of the cell (thin arrow) and a thickening of some microtubular bundles (thick arrow)

Fig. 7
Ten-minute exposure to laser irradiation in the pulse mode caused the loss of microtubules in some parts of the cytoplasm (thin arrow) and the beginning of their fragmentation (thick arrow) was more distinct than after 100 s of laser treatment

Fig. 8
Peripheral rarefaction of microtubules (thin arrow) after 100 s of exposure to laser irradiation in the Nogier mode

Fig. 9
Rarefaction of microtubules (thin arrow), their partial fragmentation and thickening of microtubule bundles after 10 min of laser irradiation in the Nogier mode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After application of Claus irradiation mode for 100 s (*Fig. 14*) we could observe loss of stress fibres in the centre of the cell. Remaining stress fibres were shorter, and larger amount of tiny actin granules occurred in places without stress fibres. The 10 min. exposure (*Fig. 15*) caused disappearance of long stress fibres in cell centre, atypical clusters of stress fibres in periphery, formation of large actin granules dispersed in cytoplasm sometimes forming groups there.

After application of Nogier irradiation mode for 100 s (*Fig. 16)* we could observe rarefaction of stress fibres and formation of actin granules. The 10 min. exposure (*Fig 17*) caused disappearance of stress fibres in cell centre, atypical clusters of short stress fibres in periphery, and formation of large actin granules, often in groups.

Generally, when prolonging the exposure time the loss of stress fibres increased. These fibres were shortened and number of actin granules increased.

In both studied cytoskeletal structures, i.e. microtubules and microfilaments, the changes were found in the whole area of the sample, even in places, which were irradiated only by the scattered laser light.

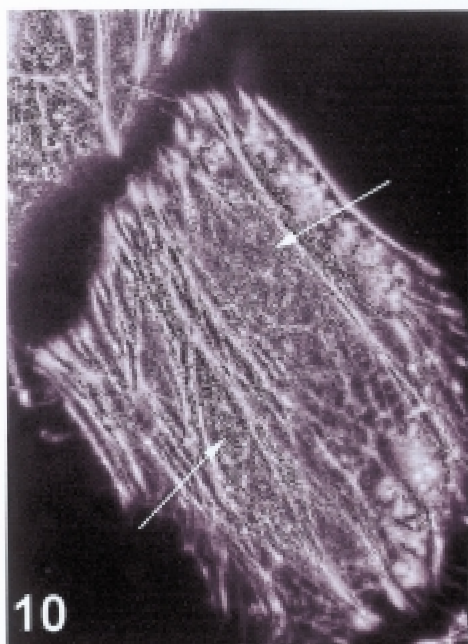
We presume that the changes of cytoskeletal structures are more influenced by the exposure time than the used operation mode, continuous or pulsed.

Fig. 10
The loss of stress fibres, mainly in central parts of the cell (thin arrow), after a 100 s continuous application of laser light.

Fig. 11
Disappearance of stress fibres in the cell centre (double arrow) after 10 min of exposure to continuous laser light. Actin aggregates produced thick, short and variously curved fibres (thick arrow) which often formed clusters. There were also large granular actin aggregates (thin arrow).

Fig. 12
Rarefaction of stress fibres, mainly in the cell centre (thin arrow), after a pulsed mode of irradiation applied for 100 s.

Fig. 13
Massive loss of stress fibres, mainly in central parts of the cell (thin arrow), and formation of atypical assemblies of stress fibres at the cell periphery after 10 min of exposure to pulsed mode irradiation. Large granules of actin and their aggregates also occurred (thick arrow).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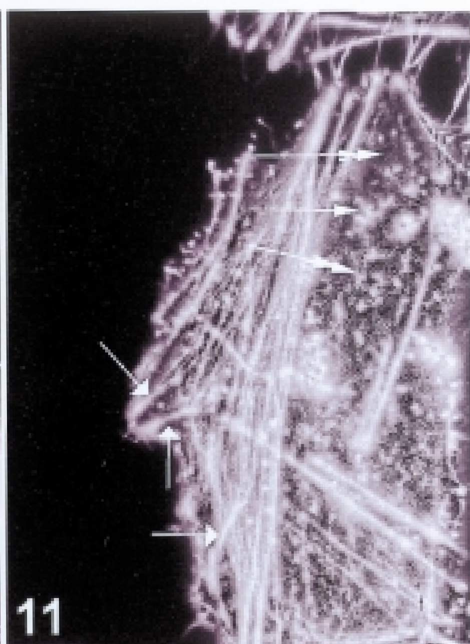
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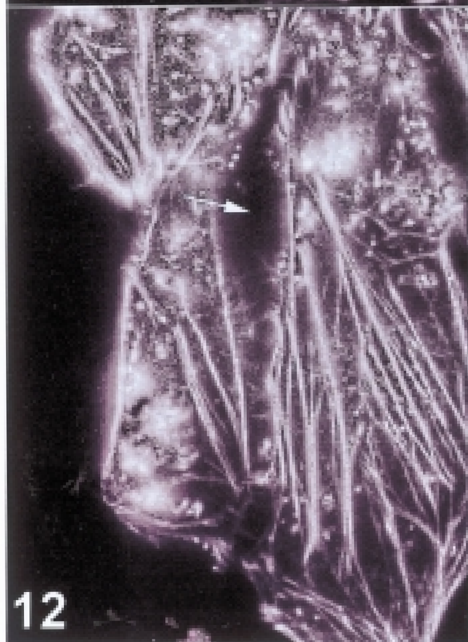
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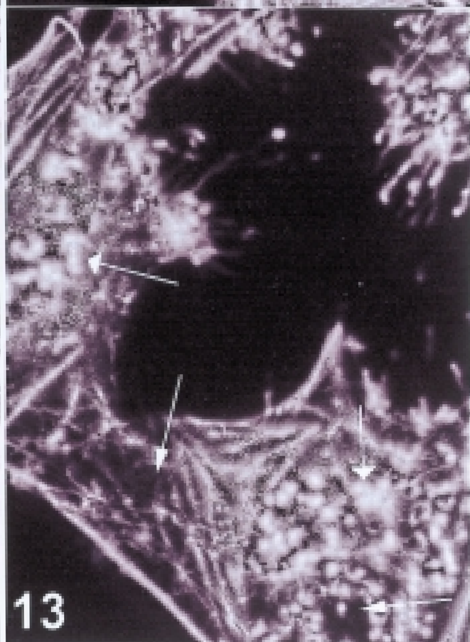
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DISCUSSION

The results presented here lead us to a conclusion that laser light can evoke certain changes of cytoskeleton under given experimental conditions. When using laser light with wavelength of 830 nm we can expect limited photothermal effect, i.e. changes in rotational and vibrational states of molecules (*1*). Resulting changes in dipole moments and electric charge distribution in protein molecules can destroy van der Waals bonds and evoke processes leading to observed fragmentation of cytoskeletal network.

The cytoskeletal structure changes caused by low-power laser are only seldom studied. There are many papers dealing with microirradiadion of selected cellular structures (*3*) but they cannot be used in this discussion because of considerably higher powers and very narrow laser beams used. Some resources are too vague in description of irradiation parameters or even contain claims about mechanisms of laser bioeffects which cannot be denoted as scientific (*14).*

The effects of low-power He-Ne laser (λ = 632,8 nm) on intermediary filaments and stress fibres were studied by *Bolognani et al. (4).* They described aggregation and bundle formation of intermediary filaments in cells after irradiation. The stress fibres seem be a good object for studies on subcellular reparation. Besides the cytoskeleton, Bolognani et al. studied also concentration and charge changes of ATP, ADP and AMP. Human emryonal cells has been irradiated for 5, 15 and 30 min. It was found that the ATP concentration does not change after 15 and 30 min. exposure. Under the same conditions, the ADP concentration decreased by 39 %, while the AMP concentration increased 6 times.

Fig. 14

Loss of stress fibres in the centre of the cell (double arrow) after application of the Claus irradiation mode for 100 s. Short stress fibres (thin arrow) and larger amounts of tiny actin granules (thick arrow) were observed in areas free from stress fibres.

Fig. 15

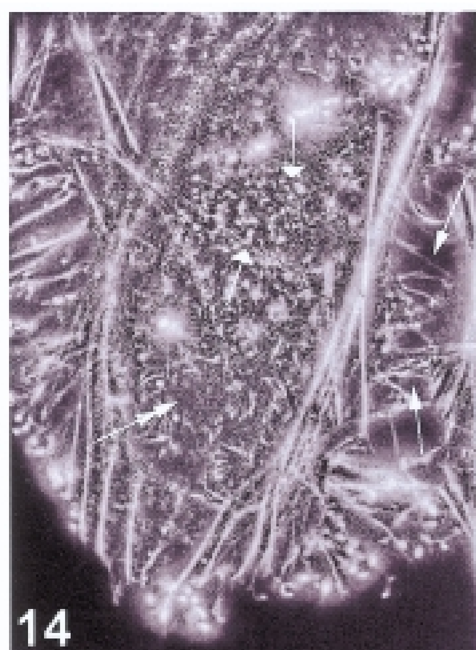
Disappearance of long stress fibres in the cell centre (double arrow) after 10 min of exposure to the Claus irradiation mode. There were atypical clusters of stress fibres at the cell periphery (thin arrow); large actin granules dispersed in the cytoplasm were sometimes grouped together (thick arrow).

Fig. 16

Rarefaction of stress fibres (thin arrow) and formation of actin granules (thick arrow) after application of the Nogier irradiation mode for 100 sec.

Fig. 17

Disappearance of stress fibres in the cell centre (double arrow) and atypical clusters of short stress fibres in the periphery (thin arrow) after a 10 minute application of the Nogier irradiation mode. Atypical clusters of short stress fibres appeared at the periphery and large actin granules (thick arrow), often grouped together, were formed.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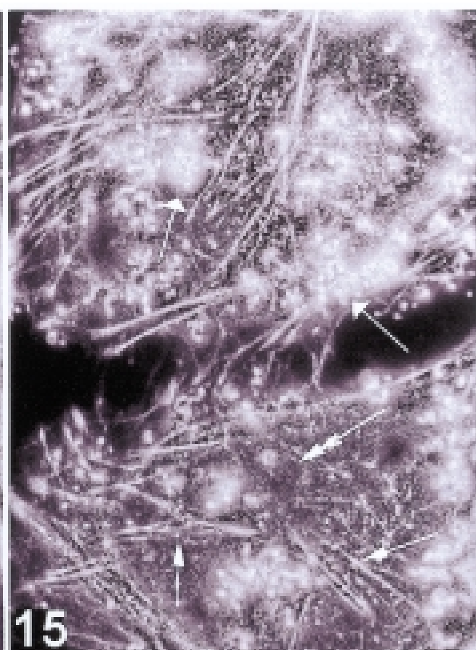
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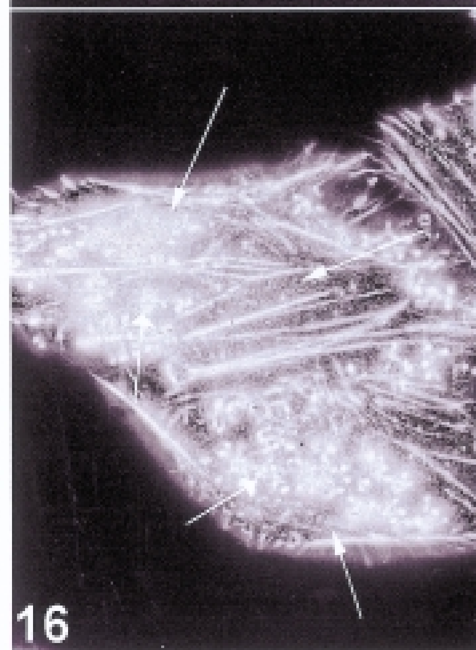
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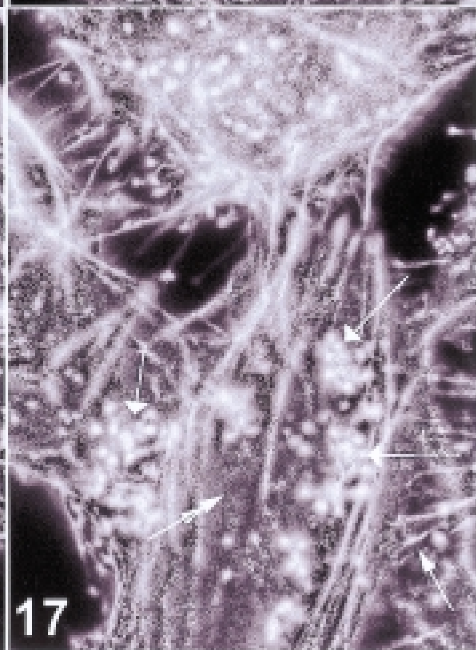
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We suppose that the loss of microtubules and microfilamts observed after laser exposure in our experiments is probably caused by depolymerisation of these structures for there are often found incomplete microtubules, shortened stress fibres and actin granules distributed individually or in groups in the cytoplasm. The cytoplasmic microtubules and microfilamets are very dynamic structures. They are depolymerised with possible consequent repolymerisation during the normal cell cycle or when the cells are exposed to numerous external factors, e.g. temperature (*15*), microsurgery (*16*), and ultrasound (*17, 18*). In our study the direct effect of laser light energy on these structures need not be the cause of microtubule and stress fibre decomposition. However, it can be caused by the effect on the associated proteins responsible for specific functional arrangement of the whole cytoskeletal network.

The damage found in the whole area of the cell monolayer can be caused by scattered light. The transmitting radiation scattered (back-scattered) on particles which size is comparable with the light wavelength. Therefore, this radiation can be absorbed and act on cytoskeleton even in places, which were not directly irradiated.

Our next experiments will deal with more detailed studies of structural and proliferation changes. It will be also necessary to compare laser light effects with those of normal light of the same wavelegth.

CONCLUSION

Changes in microtubules and microfilaments after laser irradiation at wavelength of 830 nm, in modulated pulse mode and non-modulated continuous mode were found in our experiments. We used constant power setting of 45 mW and densities of radiation energy of 24, 144 and 180 $J.cm^{-2}$. The area of 0.15 cm^2 has been exposed. The continuous mode was not modulated. In modulated pulse modes frequencies of 10, 146 and 5000 Hz were used. The exposure times ranged from 80 s to 10 min. Our results show damage and rarefaction of the cytoskeletal network in the whole volume of cells at long exposures. In contrast, short exposures (i.e. low power densities) evoked changes only in the cell periphery. Our preliminary results show that the damage of microtubules is markedly time- or power density-dependent. We were unable to show any difference in changes caused by the pulse modulated and continuous modes of laser exposure. Under given conditions we assume mainly the photothermal action mechanism.

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ÚČINKY NÍZKOVÝKONNÉHO LASERU NA CYTOSKELET HELA BUNĚK

Souhrn

Naše studie se zabývá účinky laserového záření na vybrané cytoskeletální struktury, mikrotubuly a mikrofilamenta u nádorových buněk. Jako model byly použity HeLa buňky. Cytoskeletální struktury byly vizualizovány imunofluorescenční technikou. Buňky byly ozařovány terapeutickým laserem BEAUTYLINE BTL-10 infračerveným zářením o vlnové délce 830 nm při režimu kontinuálním a pulsním. Plošná hustota energie aplikované na buňky činila 24, 144 a 180 $J.cm^{-2}$, ozařovaná plocha 0,15 cm^2, vyzařovaný výkon 45 mW při různé době a režimu působení. Při těchto experimentech kontinuální režim nebyl modulován, pro pulsní režim byla použita modulační frekvence 5000 Hz, režimu CLAUS byla modulační frekvence 10 Hz a v režimu NOGIER 146 Hz. Na změnu stavu mikrotubulárních a mikrofilamentárních má větší vliv doba působení než použitý režim. U HeLa buněk při kratších časových intervalech (80 či 100 s) docházelo k prořídnutí mikrotubulů i mikrofilament a jejich porušení především na periferii buňky. Při delší době aplikace laseru (10 min) bylo u HeLa buněk pozorováno masivnější porušení mikrotubulární a mikrofilamentární sítě nejen na periferii, ale i v celém objemu buňky. Při použití daného typu laseru a režimu ozařování můžeme předpokládat spíše nespecifický fototermický efekt ve smyslu ovlivňování rotačních a vibračních stavů molekul.

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