SCRIPTA MEDICA (BRNO) – 79 (1): 19–34, *February 2006*

A VINCA ALKALOID EFFECT ON MICROTUBULUES OF HELA CELLS

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Abstract

The study evaluated the effect of selected vinca alkaloids on the microtubule network of HeLa cells and the recovery of its disruption. Model experiments were used for demonstration of the quantification of microtubule network changes induced by vinca alkaloids using computer-assisted image analysis.

Cells were treated with vincristine or vinblastine at various concentrations from 2 μg/l to 800 μg/l for 60 min, or with vincristine at a concentration of 4615 μg/l and 9230 μg/l for 60 min. Microtubules were detected by means of indirect immunofluorescence. The damage was examined in a fluorescence microscope. Also, cells were treated for 60 min with vincristine at concentrations of 20 μg/l or 800 μg/l and the recovery process was studied in time intervals of 6, 7, 8, 9, 10 hours, or 8 and 12 hours, respectively. Differences in the arrangement of microtubules were assessed by means of a software for quantification of the cytoskeleton changes in cells treated with vincristine at a concentration of 20 μg/l and in untreated control cells. The microtubule network recovery process in time intervals of 6, 7, 8, 9, and 10 hours was quantified after treatment with vincristine at a concentration of 20 μg/l for 60 min using the software for quantification of the cytoskeleton changes.

Key words

Cytoskeleton, Microtubule disruption, Cytoskeleton recovery, Vincristine, Vinblastine

Abbreviations used

MT, Microtubule; MAPKs, mitogen-activated protein kinases; PBS, phosphate-buffered saline

INTRODUCTION

In eukaryotic cells the cytoskeleton is formed by three major structural elements – microtubules, microfilaments, and intermediate filaments (*1*). The cytoskeleton plays a specific role in cell division, maintenance and changes of cell

shape, in intracellular contacts, interaction with membranes, extracellular matrix, and in cell motions.

Microtubules are filaments with the largest diameter of all cytoskeletal components. Microtubular network is important for the execution of many cell functions. They play an important role in cell division. The microtubular diameter measures about 25 nm. Microtubules are composed of 13 equally spaced protofilaments (2).. Tubulin is the basic protein of the microtubules and molecules of tubulin are arranged in dimers containing both its forms: α-tubulin and β-tubulin. Microtubules are continuously changeable structures (*3*) and polymerisation and depolymerisation of MTs is regulated by extracellular and intracellular factors (*4*). The presence of GTP at microtubule ends is necessary to maintain the stability of the polymer (*11*). The cytoskeleton can be damaged through the effect of many external factors or chemical agents (*5–10*). The opposite ends of free microtubules show different sensitivities to microtubule depolymerising agents such as low temperature, Ca++ or colchicine (*12*). The mitotic spindle is a self-organising structure that is constructed primarily from microtubules. Among the most important spindle microtubules are those that bind to kinetochores and form the fibres along which chromosomes move.

Vinca alkaloids – vincristine and vinblastine – are microtubular toxins of chemically similar nature (*13*) that disrupt microtubule function by binding to a site on β-tubulin and suppressing microtubule dynamics. Although they are closely related in physical and chemical properties, they have various effects on the human body.

Vinca alkaloids perturb the kinetochore-microtubule attachment. This activates a checkpoint pathway that ensures proper attachment of chromosomes to the mitotic spindle (*14–16*): When microtubules fail to attach to one or more kinetochores as a result of drug treatment, the checkpoint components continue to generate signals that inhibit the metaphase/anaphase transition that delays cell cycle progression and induces programmed cell death (*17).* At higher drug concentrations, vinca alkaloids induce the assembly of spiral filaments of tubulin, which, consequently, can interact laterally and form paracrystals (*18*). This action is similar to the action of colchicine, but is different from that of paclitaxel, which promotes the polymerisation of tubulin polymers to form abnormal stable microtubule structures. Treatment with high concentrations (100 nM) of vincristine cause disruption of microtubule organisation, which, in turn, prevent accumulation of p53 in the nucleus. By contrast, treatment with lower drug concentrations (3 nM) that are known to suppress microtubule dynamics but do not alter microtubule organisation enhance nuclear accumulation of p53 and its downstream transactivated elements above physiological levels (*19*).

Vincristine and vinblastine have been widely used to treat cancer (e.g. acute leukaemia, rhabdomyosarcoma, neuroblastoma, Hodgkin's disease), to synchronise cell cycle, or to look for defects in the mitotic checkpoint (*13*).

MATERIALS AND METHODS

Cell line

In our experiments we used the stable heteroploid line of HeLa cells obtained from the Department of Biology, Faculty of Medicine, Masaryk University in Brno. The cells were grown on uncoated coverslips in a Dulbecco's Minimal Essential Medium (DMEM) (PAA Laboratories GmbH, Linz, Austria), supplemented with 10 foetal bovine serum (PAA), 2 mM glutamine (PAA), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (PAA Laboratories, Austria) in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C. The cells grew as monolayers. They were subcultured (three times) per week.

Exposure to vincristine and vinblastine

In the first series of experiments, to investigate the action of vincristine and vinblastine onto the microtubule network, a solution containing 1 mg of Vincristini sulfas in 1 ml of medium (Vincristine-Teva, inj.), or an original solution containing 5 mg of Vinblastini sulfas in 5 ml of medium (Gedeon Richter), was mixed with 3 ml of growth medium in each of the Petri dishes so that the final concentration of vincristine, or vinblastine was 2, 10, 20, 30, 40, 80, 100, 200, 400, 800 μg/l, or 4615 μg/l and 9230 μg/l (vincristine only). Each concentration was in two dishes. The cells were exposed to the drugs for 60 min at 37 °C. After the treatment, the samples were washed three times for 4 minutes concurrently with control samples in phosphate-buffered saline (PBS, pH 6.9) and processed for immunofluorescence microscopy.

In the second series of experiments the cells were cultivated for 2, 5, 10, 20, 30, and 60 minutes at 37 °C in media with a final vincristine concentration of 20 μg/l, or for 5 minutes in a medium containing vincristine at a concentration of 800 μg/l. The samples were washed three times for 4 minutes concurrently with control samples in PBS, pH 6.9, and processed for immunofluorescence microscopy. The third series of experiments was performed with vincristine at a final concentration of 20 μg/l. The cells were exposed to the drug for 60 min. After the treatment, the drug-containing medium was poured off and monolayers were subjected to three washing procedures concurrently with control samples with phosphate-buffered saline (PBS) (pH 6.9). Two slips with the cells were then subjected immediately to fixation and detection of the microtubular network as well as two slips which were cultivated for 60 min in vincristine-free growth medium as a control. The other Petri dishes were refilled with fresh growth medium and incubated for another 6, 7, 8, 9, and 10 hours in order to evaluate the recovery processes. Recovery progressed at 37 °C in a humidified atmosphere of 95 % air and 5 % CO2. The other control monolayers treated with a growth medium only containing vincristine-free DMEM were cultured in the same conditions as the vincristine-treated cells recovered for 10 hours. After fixation, the microtubular components were visualised and viewed in a fluorescence microscope. A similar attempt was provided for a vincristine concentration of 400 μg/l. The cells were exposed

to the drug for 60 min, and after the washing procedure, they were recovered for 8 or 12 hours in the same condition as last mentioned. Microtubular components were visualised and examined in a fluorescence microscope.

To quantify the cytoskeleton changes, cells were incubated in a fourth series of experiments at 37 °C in a medium containing vincristine at a concentration of 20 μg/l for 60 minutes. They were cultivated on two coverslips in two Petri dishes. The control cells on two slips were cultivated parallelly. The medium was poured off and the cells were subjected to three washing procedures with PBS (pH 6.9) and fixation. The samples were then subjected to visualisation of the microtubules and to structure analysis using the software for quantification of the cytoskeleton changes.

To quantify microtubular changes during the recovery processes, we performed the same experiments as in the third series. The cells were exposed to vincristine at a concentration of 20 μg/l for 60 min and recovered for 6, 7, 8, 9, and 10 hours in fresh growth medium. Two slips with the cells were subjected to fixation and visualisation of the microtubular network immediately after the treatment as well as two control slips. Recovery progressed at 37 °C in a humidified atmosphere. The other control monolayers were treated in the same way as the vincristine-treated cells recovered for 10 hours. The samples were fixed and subjected to visualisation of the microtubular components and to structure analysis using the software for quantification of the cytoskeleton changes.

Visualisation of microtubular network

Cells were washed three times for 4 min in the phosphate-buffered saline (PBS, pH 6.9) and fixed by 3 % paraformaldehyde in PBS. Thereafter, the cells were permeabilised by 0.2 % Triton X-100 solution in PBS. The microtubules were detected by means of the tubulin monoclonal antibody TU-01 (Institute of Molecular Genetics, Prague, Czech Republic), diluted 1:300 by PBS, and a secondary antibody SwAM/FITC (conjugated swine anti-mouse globulin/fluorescein isothiocyanate; Institute for Sera and Vaccines, Prague, Czech Republic), diluted 1:100 by PBS. The cells were washed in the phosphate buffer three times for 5 min between the application of individual agents. The samples were then closed in the Vectashield mounting medium (Vector Laboratories, Inc., Burlingham, CA, USA) and examined in a fluorescence microscope (Leitz Laborlux S, Wetzlar, Germany).

Images acquisition

Microtubular networks were photographed on an Ilford PAN 40 black-and-white film with a camera or they were captured with a cooled CCD Santa Barbara camera. Primary magnification was 1000x. Some pictures photographed with the film camera were scanned and processed by the programme Adaptive Contrast Control (ACC), version 5.0, for better setting off of the cytoskeleton. Cytoskeletal networks intended for cell structure analysis were photographed with the CCD camera (primary magnification 400x) with a resolution of 510 x 340 pixels and a bitmap depth

of 16 bits per pixel. Twenty to twenty five pictures for each slip were captured (approximately 2 x 25 pictures for each experimental group) in that case.

Microtubule structure analysis

The images acquired with the CCD camera were processed by the "FFT magic" software. The ratio of the area of cell microtubules in the image, in fact observable projection of the three-dimensional microtubular network, to the total cell area in the image was taken as the parameter characterising the quantity of microtubules in the cytoplasm. To obtain information about the area of the microtubules, we used filtering in the frequency domain using a discrete Fourier transform. We used intensity histogram expansion, gamma correction, and intensity thresholding (*20*).

Statistical methods

Data acquired with the "FFT magic" software were statistically analysed and presented as box graphs with medians, quartiles for the distribution of values, and maximum and minimum values or as a graph of arithmetical means and confident intervals (95 %). We used the Mann-Whitney *U* or Wilcoxon rank sum test for the difference in medians and a Kolmogorov-Smirnov test for different distributions. We used multiple comparisons of medians in the analysis of the recovery process. Both the calculations and the graphs were processed by Statistica 6.0 software (StatSoft, Inc., USA).

RESULTS

Untreated control cells (*Fig. 1*) showed a microtubule network regularly distributed along the whole cell content. Cells exposed to drugs at concentrations of 2 μg/l for 60 min did not show considerable changes in the distribution of microtubules. Cells exposed to vincristine or vinblastine at concentrations of 10 (*Figs. 2, 3*), 20, 30, 40, 80, 100, 200, 400, and 800 μg/l for 60 min showed changes in the arrangement of the microtubular network. The network of cytoplasmatic microtubules at concentrations of 10, 20 μg/l was thinned down, and individual fibres had a wavelike shape. The network damage increased with increasing concentration of cytostatics. The microtubules were more thinned down and fragmentation of fibres occurred. At a higher concentration of 400 μg/l, sometimes blebs were formed (*Fig. 4*). Cells exposed to vincristine at concentrations of 4615 μg/l and 9230 μg/l formed paracrystals (*Fig. 7, 8*). No significant difference was detected in vincristine and vinblastine treated cells.

When cells were exposed to vincristine at a concentration of 20 μg/l for 2, 5, or 10 minutes, no noticeable changes occurred in the microtubule network. The 20-min treatment at a concentration of 20 μg/l caused disruption of microtubules. The network was thinned down, and individual fibres had a wavelike shape. The cells exposed to vincristine at a concentration of 800 μg/l for 5 minutes showed a severely defective microtubular network.

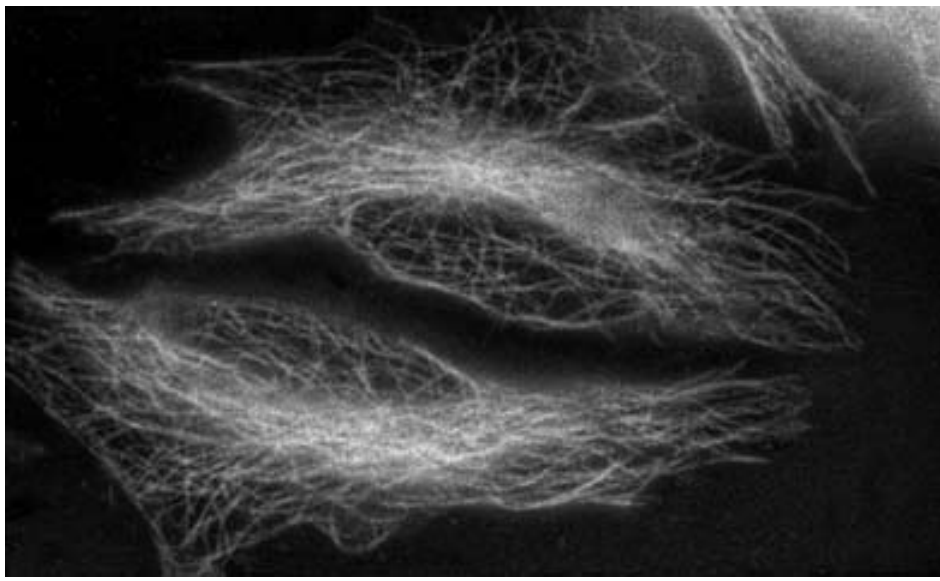

Fig. 1
Microtubules of untreated control cells of HeLa line. The network is regularly distributed along the whole cell content. Figures 1–8 were processed by the programme Adaptive Contrast Control (ACC), version 5.0.

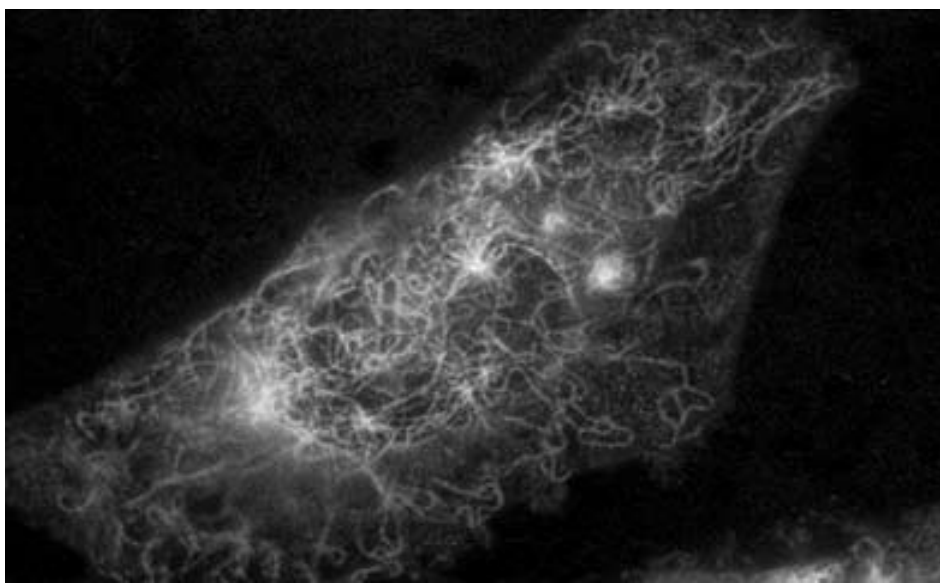

Fig. 2
Microtubules of HeLa line cells treated with vinblastine at a concentration of 10 μ/l for 60 min. The network of cytoplasmatic microtubules is thinned down, and individual fibres have a wavelike shape.

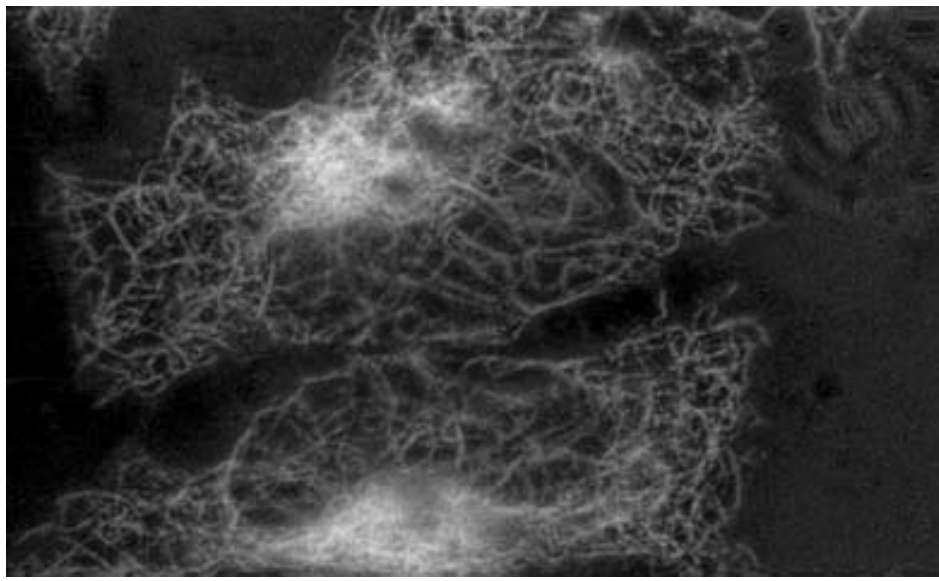

Fig. 3

Microtubules of HeLa line cells treated with vincristine at a concentration of 10 μ/l for 60 min. The network of cytoplasmatic microtubules is thinned down, and individual fibres have a wavelike shape.

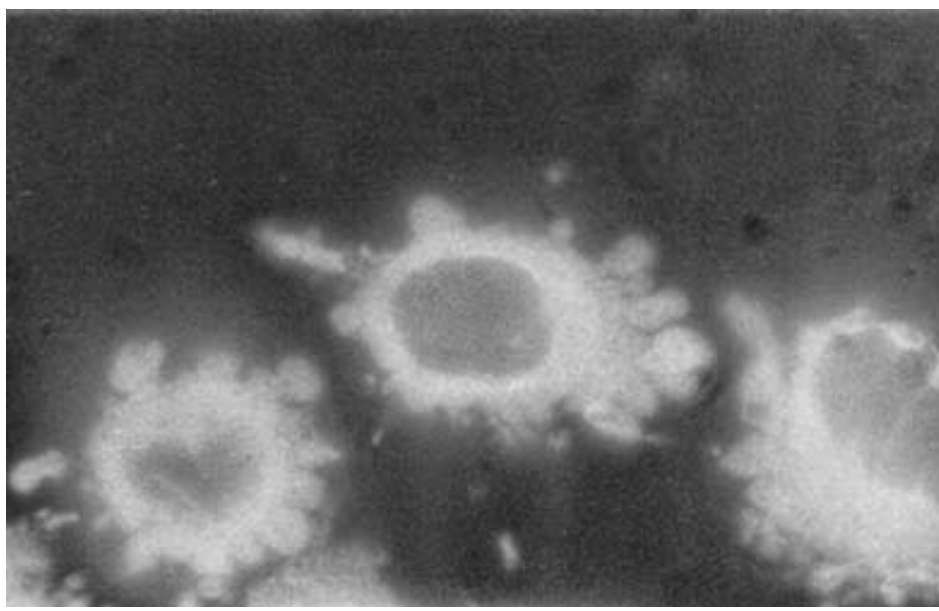

Fig. 4

Cells of HeLa line treated with vincristine at a concentration of 400 μg/l for 60 min. There are blebs formed on the periphery of the cells. The microtubules perished and free tubulin was detected along the whole cell content.

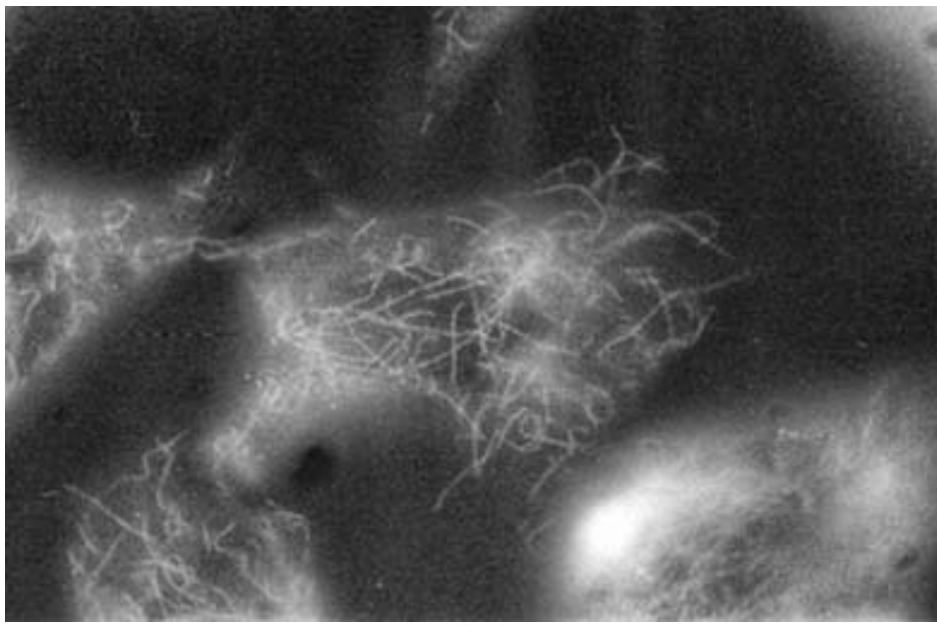

Fig. 5
Microtubules of HeLa line cells treated with vincristine at a concentration of 400 μ/l for 60 min. Microtubules recovered for 8 hours. The network is partially restored.

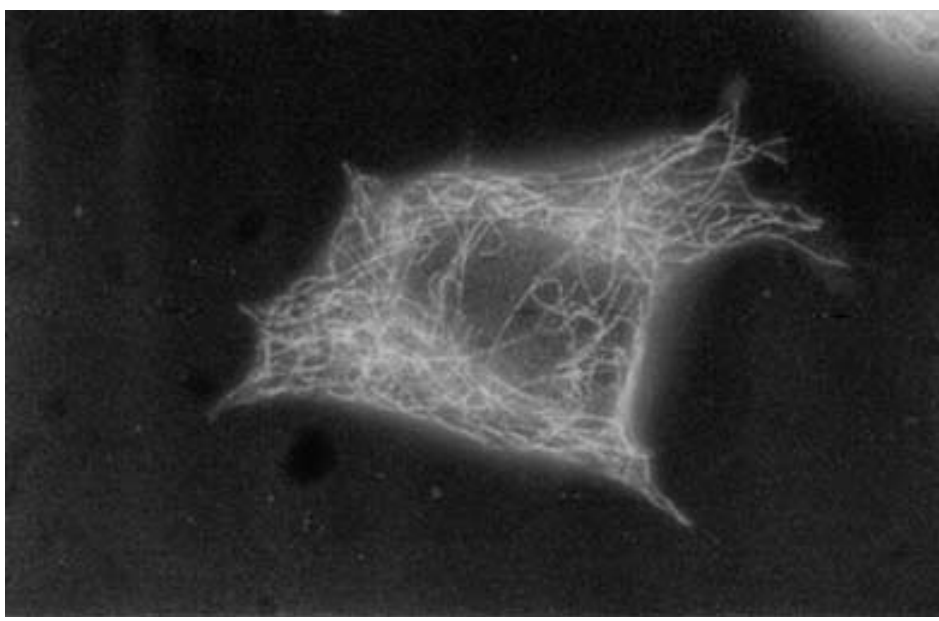

Fig. 6
Microtubules of HeLa line cells treated with vincristine at a concentration of 400 μ/l for 60 min. Microtubules recovered for 12 hours. The network is nearly restored.

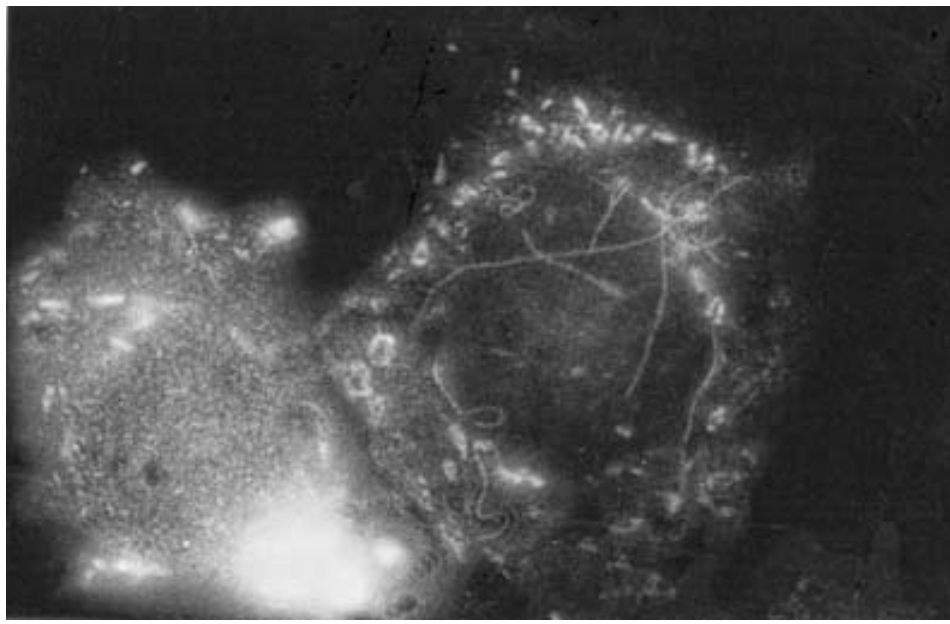

Fig. 7
HeLa cells treated with vincristine at a concentration of 4615 μg/l for 60 min. Paracrystals are formed.

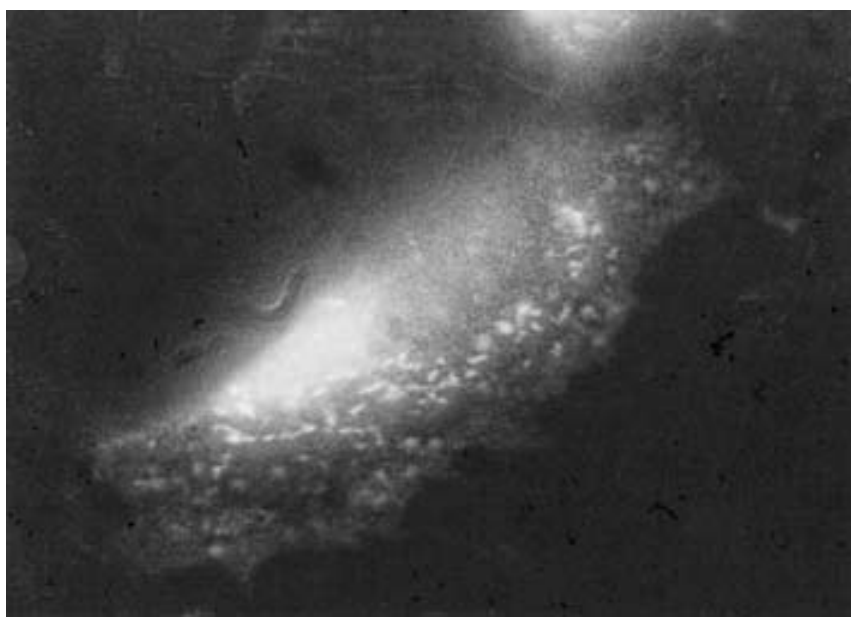

Fig. 8
HeLa cells treated with vincristine at a concentration of 9230 μg/l for 60 min. Paracrystals are formed.

All the cells with the recovering period of 6 hours in a drug-free growth medium following vincristine treatment showed damage of microtubules. The cells after a 7-hour recovery period had their microtubular network either fully restored or still damaged. After recovery for an 8-hour period, some cells showed a partly defective (thinned-down) network, but the majority of the cells showed restored microtubules. When the cells were allowed to recover for 9 hours, the microtubules were spread out comparably to those observed in untreated control cells. The control cells showed their microtubule network regularly distributed along the whole cell volume. When cells were recovered after treatment with vincristine at a concentration of 400 μg/l for 8 hours, the cytoskeleton was partially restored afterwards (*Fig. 5*). The microtubules were thinned down, and individual fibres had a wavelike shape. After a recovery period of 12 hours, the network was also damaged (*Fig. 6*), only several cells showed nearly restored microtubules.

In a fourth series of experiments the results of microtubule structure measurements were statistically analysed. The data obtained from a slip constituted a group. The groups obtained from the two slips treated with vincristine at a concentration of 20 μg/l were compared with one another. The observations are demonstrated by a box plot (*Fig. 9*). The values obtained from the two slips were homogeneous (consistency in medians and in distributions) and therefore were summed up for the final analysis. The groups obtained from both control slips were compared in the same manner (*Fig. 10*) and summed up for final statistical evaluation, because they were homogeneous. Further, we compared the control and vincristine-treated groups (*Fig. 11*). The hypothesis about consistency in medians was rejected ($P=0.00006$) and the hypothesis about consistency in distributions was rejected, too ($P=0.0003$). The decrease in the quantity of microtubules in the cytoplasm was significant in vincristine-treated cells.

To quantify cytoskeleton changes during the recovery processes, we summed up all data obtained from the binate slips with the cells recovered for a particular period (6, 7, 8, 9, 10 hours) and from slips with non-recovered cells (*Figs. 12, 13*). Multiple comparisons of medians were performed (*Tab. 1*). There were significant differences in non-recovered cells and all other groups (recovery periods of 6, 7, 8, 9, and 10 hours). The quantity of microtubules in the cytoplasm was significantly higher ($P<0.05$) in cells with a recovery period lasting from 6 to 10 hours than in the non-recovered cells. The results prove recovery processes and restoration of the microtubular cytoskeleton.

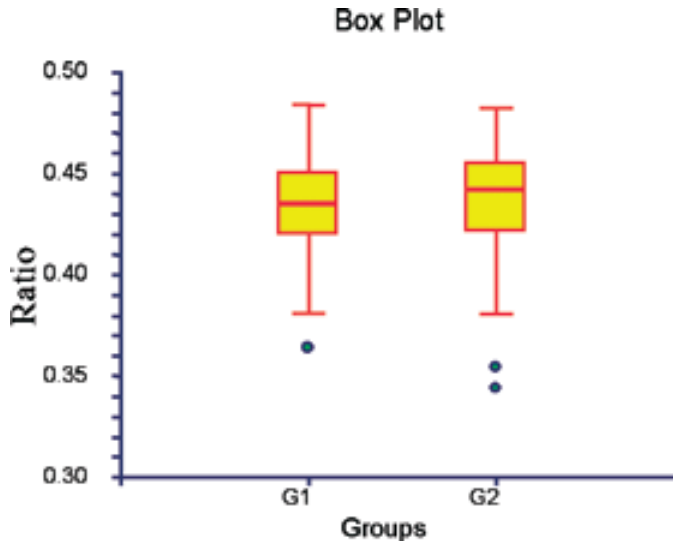

Fig. 9

Box plot. Data were obtained from two slips treated with vincristine at a concentration of 20 μg/l. G_1 – first slip treated with the drug. G_2 – second slip treated with the drug. Dots present outliers. In Figures 9–13 the ratio is the parameter characterising the quantity of microtubules in the cytoplasm and is obtained as the ratio of the area of cell microtubules in the image, in fact observable projection of the three-dimensional microtubular network, to the total cell area in the image.
In Figures 9–14 data were obtained by "FFT magic" software.

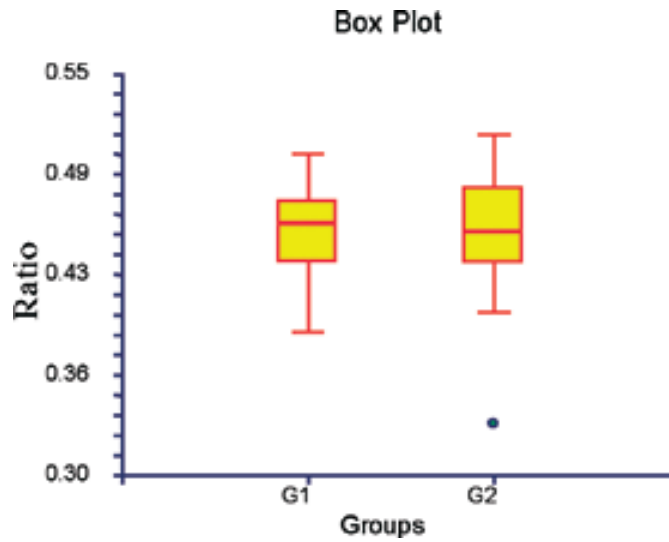

Fig. 10

Data were obtained from two untreated control slips G_1, – first control slip. G_2 – second control slip. The dot presents the outlier.

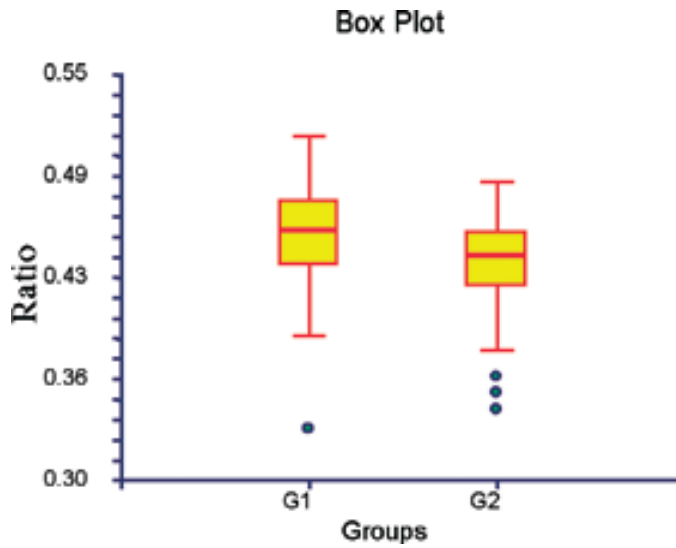

Fig. 11

Box plot. Data were obtained from untreated control slips (G_1) and from slips treated with vincristine at a concentration of 20 μg/l (G_2). Groups were obtained by the sum of data from two control slips (G1), or from two treated slips (G_2). Dots present outliers.

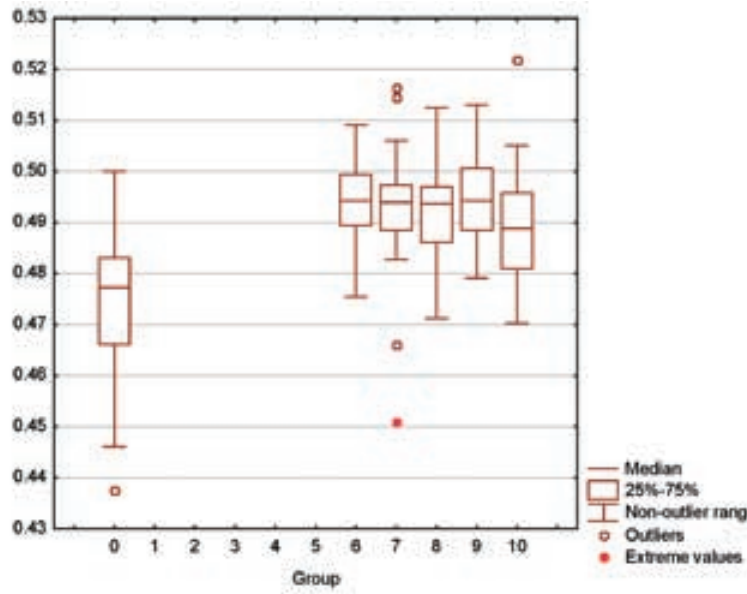

Fig. 12

Box plot of recovery processe. Recovery progressed 0, 6, 7, 8, 9, 10 hours. Data were acquired by „FFT magic“ softwere

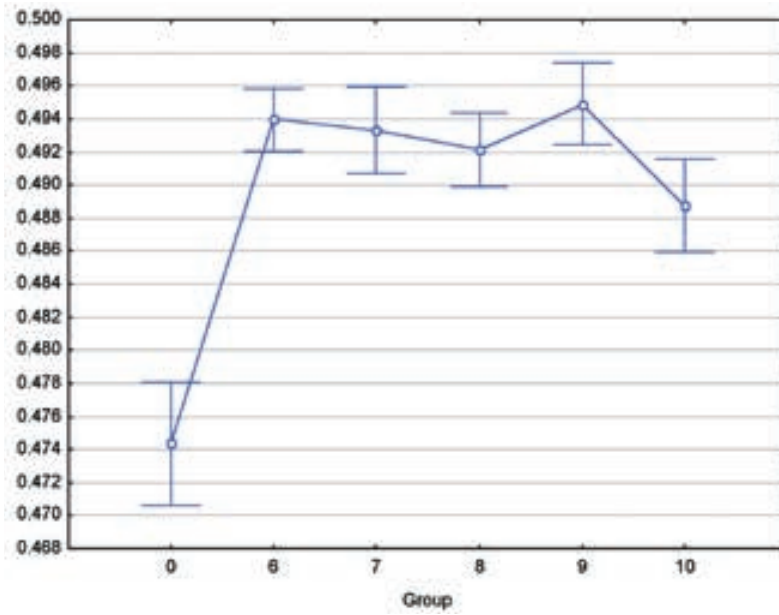

Fig. 13
Graph of arithmetical means and confident intervals (95 %).
Recovery progressed for 0, 6, 7, 8, 9, 10 hours.

Table 1
Table of multiple comparison of medians, p-values (levels of significance).
Recovery progressed for 0, 6, 7, 8, 9, and 10 hours. P-values less than 0.05 were printed bold.

Recovery (hours)	0	6	7	8	9	10
0		**0.000000**	**0.000000**	**0.000000**	**0.000000**	**0.000125**
6	**0.000000**		1.000000	1.000000	1.000000	0.052672
7	**0.000000**	1.000000		1.000000	1.000000	0.073603
8	**0.000000**	1.000000	1.000000		1.000000	0.657816
9	**0.000000**	1.000000	1.000000	1.000000		**0.033792**
10	**0.000125**	0.052672	0.073603	0.657816	**0.033792**	

DISCUSSION

The interaction of antitumour agents with compounds of the cytoskeleton is a theme studied in many papers (*5-8, 9, 7, 18, 21-24, 25*). *Lobert* (*22*) studied the interaction of vinca alkaloids with tubulin, and compared vinblastine and vincristine. She studied e.g. the affinity of the drug for tubulin heterodimers. Vincristine exhibited a higher overall affinity for porcine brain tubulin than vinblastine, but the affinity of the drug for tubulin heterodimers was identical for the two drugs.

Under our experimental conditions we did not mark any differences between the two drugs. Some authors also studied the recovering processes of the cytoskeleton after treatment of cell cultures with physical factors or agents interfering with cytoskeleton compounds. Vincristine caused a sequence of morphological changes in sensitive cells from three pleiotropic resistant MCF-7 human breast carcinoma cell lines mixed with vaginal adenocarcinoma cells. The cells were selected in serially increasing drug concentrations. These changes included precipitation of tubulin and disappearance of tubular structure. The changes occurred initially within 3 hours of incubation, but were expressed in all cells after 6 hours. If, after 3 hours of drug exposure, the cells were subcultured in drug-free media, the cytoskeletal structure reformed within 10 hours. The maximal recovery of the cytoskeletal structure occurred 22 hours after drug removal and was sustained up to 36 hours (*23*). Treatment with vincristine (*24*) eliminated the microtubule bundles, leaving only tubulin paracrystals. Within 24 hours after washing out the vincristine, the microtubule bundles repolymerised in cultured hippocampal neurons.

We have only one comparable method of evaluation of microtubular changes using computer-assisted image analysis. *Fink-Puches et al*. (*26*) evolved a method of quantification of cytoskeletal components using an IBAS image analysis system and demonstrated their approach on cytoplasmic microtubules of two murine melanoma cell lines. In addition, they investigated quantitative changes after application of the microtubule inhibitor nocodazole. They presented a decrease of the assembled tubulin after treatment with nocodazole and a less delicate structure of the remaining microtubules. This was indicated by a reduction of the parameters used. They also showed significant differences between the high and low metastatic cell lines. They used confocal laser scanning microscopy. We have no possibility of comparing the mathematical approach because the description of image analysis is not detailed in this paper, but we suppose to have probably a more appropriate approach on account of using histogram expansion, gamma correction (*20*). Our results indicate the method as efficacious to quantify loss of microtubules in cells treated with vincristine and efficacious enough to quantify microtubular recovery processes and restoration of the microtubular cytoskeleton.

CONCLUSIONS

The cells showed changes in the arrangement of microtubules even at the 10 μg/l concentration of cytostatics after 60-min exposition. Its damage increased with increasing concentration of cytostatics. No differences were recorded between the two cytostatics. At concentrations of 4615 μg/l (vincristine) and 9230 μg/l (vincristine) tubulin paracrystals appeared. Disruption of the microtubules was also time-dependent. It was more serious with prolonged cell treatment. The network of microtubules recovered in a time-dependent manner after vincristine at a concentration of 20 μg/l for 60 min, and after vincristine at 400 μg/l for 60 min.

The results obtained with the method of microtubule structure analysis confirmed our previous microscopic studies of cells treated with vincristine at a concentration of 20 μg/l; the results provided evidence of a loss of microtubules, and the method also confirmed our previous studies of the recovery process. Quantification of microtubular network changes by using computer-assisted image analysis is sensitive enough to demonstrate and specify changes caused by vincristine at a concentration of 20 μg/l and microtubular recovery processes.

A c k n o w l e d g e m e n t s

The research was supported by grants N 301/01/1102 and 301/03/H005 from the Grant Agency of the Czech Republic.

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SLEDOVÁNÍ POŠKOZENÍ CYTOSKELETU NÁDOROVÝCH BUNĚK

S o u h r n

V této studii byl hodnocen účinek vybraných Vinca alkaloidů na síť mikrotubulů HeLa buněk a průběh reparace mikrotubulů. Modelové experimenty byly použity pro nalezení možnosti kvantifikace cytoskeletálních změn indukovaných Vinca alkaloidy pomocí počítačové obrazové analýzy.

HeLa buňky byly ovlivněny vinkristinem nebo vinblastinem o koncentraci od 2 do 800 μg/l po dobu 60ti minut nebo vinkristinem o koncentraci 4615 μg/l a 9230 μg/l po dobu 60ti minut. Mikrotubuly byly detekovány metodou nepřímé imunofluorescence a poškození bylo stanoveno pomocí fluorescenčního mikroskopu. Buňky byly také ovlivněny po dobu 60ti minut vinkristinem o koncentraci 20 μg/l nebo 800 μg/l za účelem studia reparace cytoskeletu. Proces reparace byl hodnocen v časových intervalech 6, 7, 8, 9, 10 hodin, nebo 8 a 12 hodin (v tomto pořadí). Změny v uspořádání mikrotubulů byly u buněk ovlivněných vinkristinem o koncentraci 20 μg/l po dobu 60 min a u buněk neovlivněných hodnoceny pomocí softwaru pro kvantifikaci cytoskeletálních změn. Dále byl s použitím softwaru pro kvantifikaci změn cytoskeletu studován proces reparace mikrotubulů v časových intervalech 6, 7, 8, 9 a 10 hodin, po ovlivnění vinkristinem o koncentraci 20 μg/l po dobu 60 minut.

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