*Wojciech Dąbroś[1], Andrzej Klein[2], Barbara Tatar[2], Przemysław Holko[2], Anna M. Kordowiak[2]*

# Does Streptozotocin [STZ] Exert an Influence on Rat Hepatoma H35-19 Cell Line?

[1]Department of Clinical and Experimental Pathomorphology, Faculty of Medicine, Jagiellonian University, Kraków,
[2]Department of General Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology,
Jagiellonian University, Kraków

**The authors investigated the effect of streptozotocin (STZ) in low – micromoles (up to 500 μM) – or higher – millimoles (1-10 mM) – concentrations in culture media of the H35-19 cell line. Up to 500 μM, STZ did not show any cytotoxic or cytostatic action in the investigated cells; on the contrary, it triggered an "improved growth" of these cells, as an antibiotic effect of the drug was observed. The concentration of 1-10 mmoles of STZ in the medium inhibited proliferation and viability of the studied cells. This action depended (proportionally) on drug concentration and time (up to 72 h) of experiment. Statistical analysis of the results obtained by four methods: staining with MTT, neutral red (NR) or crystal violet (CV) and Biirker chamber counting (BC), demonstrated no significant difference in STZ impact between 48 h and 72 h of incubation, according to the Benferoni post-hock test. The results obtained by MTT showed an extremely high statistical significance ($p<0.001$) of the effect of concentration on the results, with a non-significant interaction ($p=0.2236$) and general time effect ($p=0.3600$). An extremely significant ($p<0.001$) interaction of the effect of time and concentration was observed in the results obtained by neutral red method, whereas a significant effect of general time and concentration was also observed, but according to [17] it is difficult to explain. The results obtained by crystal violet staining showed a highly statistical significance ($p<0.001$) in time and concentration effect on the data, without a significant interaction between the above-mentioned factors ($p>0.05$). Cell counting in a Biirker chamber demonstrated a highly significant time and concentration effect on the results, but the interaction was mildly significant ($0.01<p<0.05$); the time and concentration effect are said to be difficult to interpret. The main STZ effect exerted on proliferation and growth inhibition was noted after 48 h and 10 mM of STZ.**

**Morphological studies after 48 h and 5 mM STZ were performed, since these experimental conditions allowed for observing changes induced by the drug, when not all cells were destroyed by streptozotocin. At the studied streptozotocin concentration, the death of these cells occurred mainly by necrosis.**

## Introduction

Streptozotocin [2-deoxy-2-(3methyl-3-nitrosourea (1-d glucopyranose)] was isolated from cultures of *Streptomyces achromogenes* in 1960 [8, 25] as a broad spectrum antibiotic. In 1967, chemical synthesis of this drug was accomplished, but complete structural determination was reported in 1979. In normal state, streptozotocin is a fifty-fifty mixture of α and β anomers. Among a hundred analogues of streptozotocin [STZ] is chlorozotocin, a drug in which the methyl group was replaced by 2-chloroethyl, thus arriving at a clinically useful anti-tumor agent [1, 24, 25]. STZ has a broad-spectrum antibiotic activity and antineoplastic properties; however, it is also carcinogenic and, as reported by Bolzan and Bianchi [3], a single administration induces tumors in rat kidney, liver and pancreas. The 1-methyl-1-nitrosourea moiety of streptozotocin may be responsible for oncogenesis in the kidney, liver, peritoneum or pancreas of rat [2, 3].

Accumulation of the (3'-methyl-$^{14}$C) streptozotocin may suggest that a metabolite derived from the methyl-bearing ureido side chain of the drug may be specifically involved in the induction of tissue damage and the conse-

quent development of diabetes (as was shown by an autoradiographic study with rat kidney and pancreas [11]. Further experiments with streptozotocin revealed that it produced irreversible damage to pancreatic β cells and, 6-12 h after STZ administration, triggered intermittent hypoglycemia, followed by permanent hyperglycemia within 24 h after treatment. For this reason, streptozotocin is used as a very useful drug to induce an experimental model of diabetes in animals, such as mouse, rat, guinea pig, dog. The diabetes-inducing effect of STZ is thought to be more akin to juvenile onset diabetes in humans rather than to the alloxan-induced model of diabetes. [11, 12, 13]. Other aspects of streptoz otocin action include hypertriglyceridemia, muscle protease activity, altered lipid metabolism, microvascular disease, collagen metabolism or immunosupression [18, 21, 22].

In our previous experiments on biosynthesis of the sugar part of glycoproteins and glycolipids in STZ-diabetic rats, we observed profound changes in biochemical activity and ultrastructure of Golgi complexes, the main organelle responsible for biosynthesis and secretion of these compounds [14, 19, 20]. Then we decided to study the influence of STZ added to medium culture of the H35-19 cells line. The line is considered a very useful model for investigating hepatocytes, because it has been demonstrated to exhibit strong functional and morphological similarities to regeneration of normal hepatocytes [7].

## Material and Methods

### *Reagents*

DMEM, F12, glucose, L-glutamine, trypsin, tylosine, EDTA, albumin, penicillin, streptozotocin, streptomycin, neutral red, crystal violet (N-hexamethylpararosaniline), MTT [bromide 3-(4,5-dimetyltioazo-2)-2,5-diphenyltetrazole], glutaraldehyde 8% or 25% were obtained from Sigma Chemical Company, St Louis, USA; bovine serum and physiological buffered saline (PBS) came from WSS, Lublin, Poland; fetal bovine serum (FBS) was obtained from Biowest, South American Origin. For electron microscopy, Spurr epoxy resin from Pellco Co. and formaldehyde and osmium tetroxide were purchased from Polysciences Inc.; 15 and 50 ml test tubes, 2 ml Eppendorf tubes, 25 and 75 cm area Falcon bottles areas, 96-well plates, 10 cm plates, and sterilized filters with pore size 0.22 μm were obtained from Technoplastic Products AG, Switzerland. All the other reagents at analytical grade were provided by POChem, Gliwice, Poland.

### *Cell culture*

The H35-19 rat hepatoma cell line was obtained from the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The stock cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% FBS, 2 mM L-glutamine, 0,45% glucose, penicillin (100 units/ml) and streptomycin (100 μg/ml)] at 37 °C in atmosphere with saturated water vapor and 5% $CO_2$ in 25 $cm^2$ Falcon bottles. The cells were passaged two times per week, using 0,05% trypsin solution with 0,02% EDTA in buffered physiological salt (PBS) without Ca and Mg cations. The cell line was cultivated for a minimum of two passages after thawing prior to experimentation. To investigate proliferation or viability, the H35-19 cells were seeded at 96-well plates at a density of $1x10^4$ cells/well in 200 μl DMEM with 5% FBS and 2 mM glutaminę and 0.45%o glucose and antibiotics. After 24 h, the medium was replaced by serum-free DMEM/F12 (1:1) [supplemented with 5% albumin, 5 μg/ml transferrin, 0.3 mg/ml L-glutamine, 10 μg/ml tylosine, 2 ng/ml of sodium selenite and 100 units/ml of penicillin and 100 μg/ml of streptomycin] with streptozotocin (at the concentration within 0.5-500 μM or 1-10 mM). The experimental protocol defined the time of cell incubation with vanadium salts as 24 h, 48 h and 72 h. Triplicate independent experiments in six repetitions were used in all the experiments.

### *Estimation of proliferation rate by crystal violet and Bürker chamber counting*

a) Crystal violet (CV) staining by the modified method described by Gillies et al. [9]. Cells cultured in a defined DMEM/F12 medium without the addition of streptozotocin in six wells, immediately fixed with 80% methanol, served as the controls. After 48 h of incubation in 37°C, the "experimental cells" (i.e. the cells exposed to STZ) were fixed as previously described (80% methanol) and the control and experimental cells were dyed with 0.5% crystal violet. After 2 minutes, the dye was extracted with 1.1% sodium citrate in 50% methanol. Thirty minutes later, the absorbance was measured at 540 nm with a Spectra Fluor Plus (Tecan) plate counter. The results were monitored by the Magellan 3 program.
b) Bürker chamber counting (BC). Following planting of the H35-19 cells in 96-well plates and incubation with streptozotocin at appropriate concentrations, after 48 h, as described previously, the medium was discarded and 0.05% trypsin solution (0,02 ml/well) was added. Detachment of the cells was monitored under a light microscope and then counted in a Bürker chamber.

***Estimation of cells viability***

a) MTT staining by the method of Mosmann [16] (MTT). According to this method, the control was represented by cells cultured in a defined DMEM/F12 medium in six wells and immediately fixed with 80% methanol. After 48 h, the medium was replaced by 150 µl/well of MTT (at the concentration of 0.5 mg/ml DMEM), followed by 3 h of incubation with the dye to allow MTT to form formazon crystals by reacting with metabolically active cells. The formazon crystals were extracted and solubilized with concentrated isopropanol. The absorbance was measured at 570 nm with a Spectra Fluor Plus (Tecan) spectrofluorimeter. The percentage of survival was calculated using the formula:
% survival = [live cell number(test)] / [live cell number(control)] x 100.

b) Neutral red staining according to Borenfreund and Puerner [4] (NR). The amounts of neutral red transported by pinocytosis into the cells and stored in their lysosomes were proportional to the number of cells with normal physiology. Under the above-described standard conditions, after 48 h, the medium with STZ was discarded and 200 µl/well of neutral red in DMEM (at the concentration of 40 µg/ml) were added to each well. After 3 h of incubation, the cells were fixed with 1% CaCb in 0.5% formaldehyde for 1 min. Subsequently, the dye was extracted with 1% acetic acid in 50 % ethanol and after 15 min., the absorbance was measured at 540 nm with a Spectra Fluor Plus (Tecan) spectrofluorimeter.

***Electron microscopy examination***

After passaging, the H35-19 cells were planted in the amount of $5xl0^6$/plate on plates 10 cm in diameter, and 15 ml DMEM with 5% FBS and 2 mM glutaminę and 0.45 % of glucose, penicillin (100 units/ml) and streptomycin (100 µg/ml)] was added to each plate. After 24 h, the medium was replaced by a standard defined serum-free medium DMEM/F12 (1:1) at the investigated concentration values (from 1-10 mM) of STZ (see above). In the case of the control cells, the medium consisted solely of DMEM/F12 without the addition of the drug. After 48h in 37°C, 14 ml of the liquid was discarded from each plate; the cells were scraped away from the plate and placed in 2ml Eppendorf tubes with approximately 1 ml of the liquid. The tubes were centrifuged in a MPW-360 centrifuge at 1000 rpm for 2 min. After discarding the supernatant, the cells in the form of pellets were underlayered with 2 ml of 4% glutaraldehyde at 4°C and allowed to remain in the same temperature for approximately 60 min. Subsequently, the samples were transferred to the Chair of Pathomorphology, Collegium Medicum, Jagiellonian University. After centrifuging, all the samples were fixed for 120 min. according to the method of Karnovsky [10]. Subsequently, the samples were postfixed in 1% osmium tetroxide. After dehydration in graded concentrations of ethyl alcohol and propylene oxide, the cells were embedded in the Spurr medium. The samples were sectioned with a Reichert Ultracut S ultramicrotome using a diamond knife. Semi-thin sections were stained with methylene blue and ultra-thin sections with 8% uranyl acetate dissolved in 50% methanol, and then in lead citrate according to Venable and Coggeshal [23]. All the studies were performed under a Zeiss EM 900 electron microscope operating at 80 kV.

***Statistical analysis***

The statistical analysis of the data was performed with the STATISTICA version 7.0 package (Statsoft Inc., Tulsa, USA). All the data were expressed as the mean value ± SD. According to the t test, all the presented data were statistically significant as compared to the controls (the H35-19 cells incubated without STZ addition). Moreover, the data were analyzed using the Two-Way ANOVA, followed by the Benferoni post- hock test [17].

## Results

The inhibition of the H35-19 cell proliferation by streptozotocin was determined by two methods, i.e. staining with crystal violet (CV) and Biirker chamber (BC) counting. Their results showed that within the concentration range of 0.5-500 µM STZ improved the viability of the H35-19 cells in keeping with the known action of the drug as an antibiotic (data not shown). Within the concentration of 1-10 mM in the medium and the time of 24 h, 48 h, 72 h, STZ showed a time and concentration-dependent effect on the viability and inhibition of proliferation. After 48 h of incubation at the 5 mM STZ concentration in the culture medium, the differences between the control and experimental cells were 70-90% (Fig. 1). The inhibition of viability of these cells, determined by staining with neutral red (NR) or MTT was 50-70% of the controls (Fig. 2). The strongest effect was observed after 48 h and at 10 mM concentration of streptozotocin.

With the exception of crystal violet staining (when a decrease of cell growth inhibition with prolonged incubation time from 48 h to 72 h was observed), all the results obtained by other methods (BC, MTT and NR) showed no significant difference in STZ impact between 48 h and 72 h

time of incubation, according to the Benferoni post-hock test. The results obtained by MTT showed an extremely high statistical significance ($p<0.001$) of the concentration effect on the results, with a non-significant interaction ($p=0.2236$) and general time impact ($p=0.3600$). An extremely significant ($p<0.001$) interaction of the effect of time and concentration was observed in the results obtained by neutral red method, whereas a significant general time and concentration impact was also observed, but according to [17] it is difficult to explain. The results obtained by crystal violet staining showed a high statistical significance ($p<0.001$) in the effect of time and concentration exerted on the data, without a significant interaction between the above-mentioned factors ($p>0.05$). Cell counting in a Burker chamber proved a highly significant time and concentration effect on the results, but the interaction was moderately significant ($0.01<p<0.05$); the impact of time and concentration are said to be difficult to interpret.

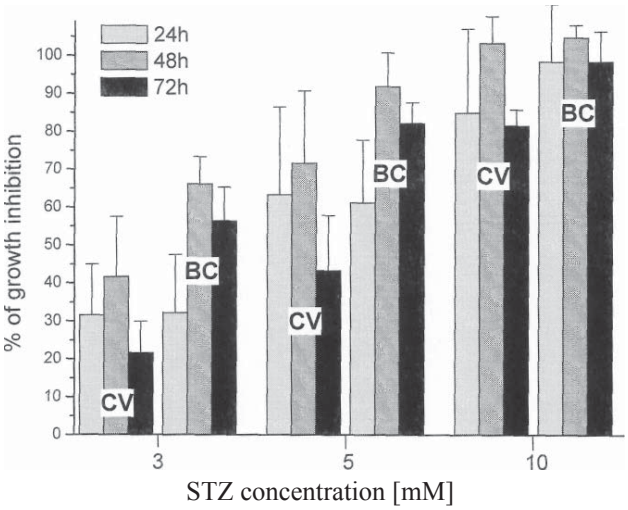


Fig. 1. Influence of streptozotocin on H35-19 hepatoma cell line growth inhibition after 24h, 48h and 72h of incubation in culture medium. Reaction with crystal violet (CV) or Bürker chamber counting (BC). The mean values + SD are given in the graph.

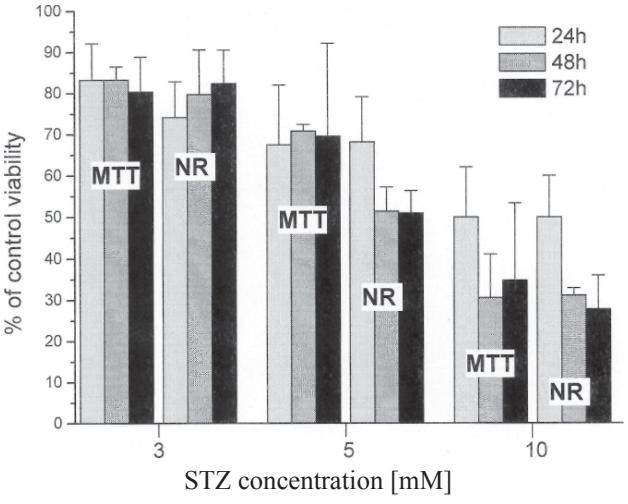


Fig. 2. Influence of streptozotocin (STZ) on control viability of H35-19 rat hepatoma cell line after 24h, 48h and 72h of incubation in culture medium. Study with MTT or neutral red (NR) reactions. The mean values + SD are given in the graph.

Morphological analysis was performed after 48 h and at the STZ concentration in the culture medium of 5 mM. For the above-mentioned reasons, these conditions of experiments were chosen, when not all cells were destroyed and the time of streptozotocin action was long enough to induce morphological alterations. Electron microscopy most often demonstrated changes within the nuclei. Usually, these structures were enlarged, almost entirely filled with euchromatin with a clearly visible nucleolus and numerous interchromatin and less frequent perichromatin granules. As a rule, the nuclei were oval or kidney-shaped. The cytoplasm was usually poor in organelles. The with endoplasmic reticulum tubules, with sparsely distributed ribosomes, being the most common observation. Not numerous, mitochondria were also noted. Such an appearance was characteristic both for the controls (non-STZ treated cells) (Fig. 3) and the experimental cells treated with 5 mM STZ (Fig. 4a). At times, cells richer in organelles were encountered, in which the investigators managed to note short and narrow smooth membrane canaliculi that formed small stacks. One might adopt the view that these struc-

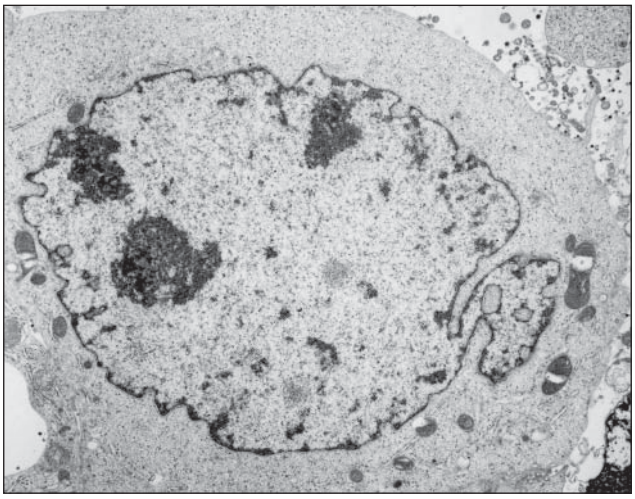

Fig. 3. A control cell. The nuclear cytoplasm is poor in organelles. Note endoplasmic reticulum tubules and scarce mitochondria. Magn. 3000x

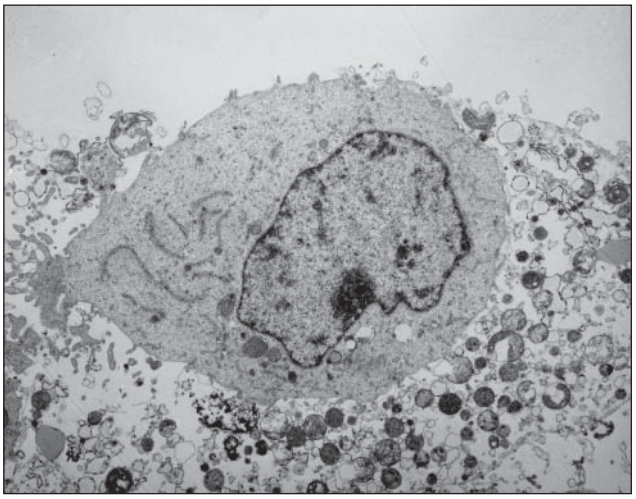

Fig. 4a. A poorly differentiated cell. The cytoplasm is poor in organelles. Note endoplasmic reticulum tubules and scarce mitochondria. Magn. 3000x

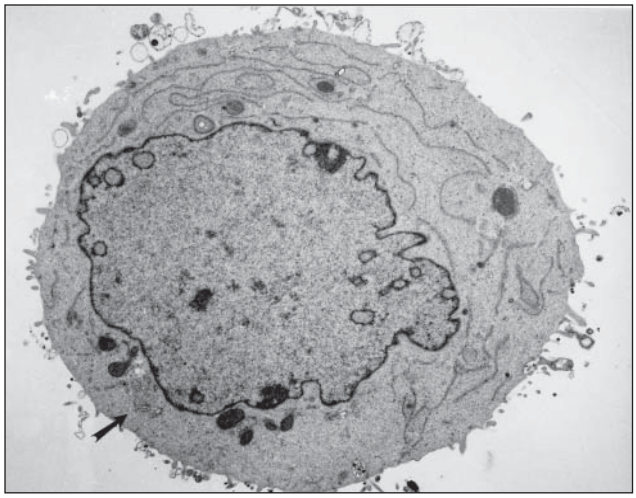

Fig. 4b. A cell that is somewhat richer in organelles, with short and narrow smooth membrane canaliculi that form small stacks, which form a Golgi complex (arrow). The nucleus demonstrates markedly uneven envelop with numerous indentations, which form pseudoinclusions. Magn. 3000x

tures represented nascent Golgi complexes. The nuclei of these cells had markedly uneven borders with numerous indentations, which formed pseudoinclusions (Fig. 4b). Finally, there were seen cells with a considerable number of mitochondria. The high percentage of these cells (20-30%) were already damaged. These cells often showed numerous small oval nuclei. In the intercellular space, in close vicinity to the cellular membrane, numerous cytoplasm-filled vesicles were seen; they were most likely detached from the cellular cytoplasm. The cytoplasm itself demonstrated

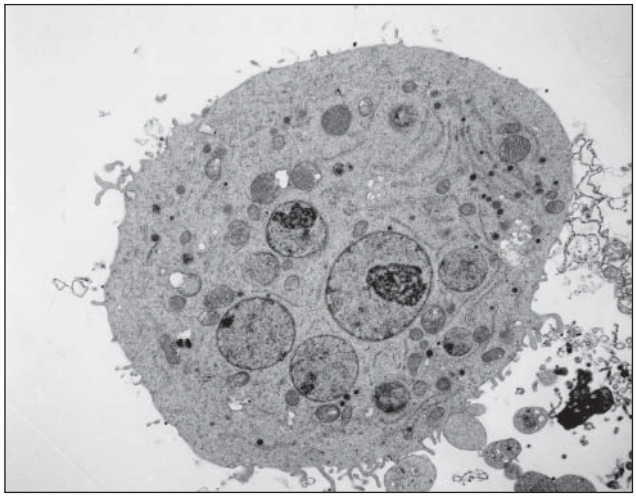

Fig. 4c. A cell with numerous small nuclei. Close to external surface of the cell, numerous cytoplasm-filled blebs, are seen. The cytoplasm itself demonstrates numerous coated granules with electron-dense contents, as well as small lysosome-like structures. Magn. 3000x

numerous coated granules with electron-dense contents, as well as small lysosome-like structures (Fig. 4c). A fairly high concentration of STZ combined with its prolonged effect on the cultured cells was manifested as the presence of a relatively high number of dying cells, usually showing necrotic changes (Fig.5a). However, shrunk cells were also encountered, with indentations both in the cellular membrane and nuclear envelop. Under the nuclear envelop at one pole, a typical caping of condensed heterochromatin was seen. This is a known morphological change in early apoptosis (Fig. 5b). Amidst cellular detritus observed in the vicinity of living cells, almost unchanged mitochondria were seen, as well as entire nuclei, almost totally filled with condensed heterochromatin, typical for apoptosis (Fig. 5c). Nevertheless, the frequency with which these pictures were noted was markedly lower as compared to cells dying through necrosis.

## Discussion

Streptozotocin [STZ] is commonly used in induction of experimental model of diabetes in animals [11, 12, 13]. As it has been mentioned above in Introduction, we used the drug administered intraperitoneally at the concentration of 60-75 μg/g of body weight of animal, and found drastic alterations in the biochemical activity and morphology of hepatocytes [14, 19, 20]. Additionally, our preliminary investigations [5, 15] showed the influence of various vanadium derivatives as the drugs with anti-dia-

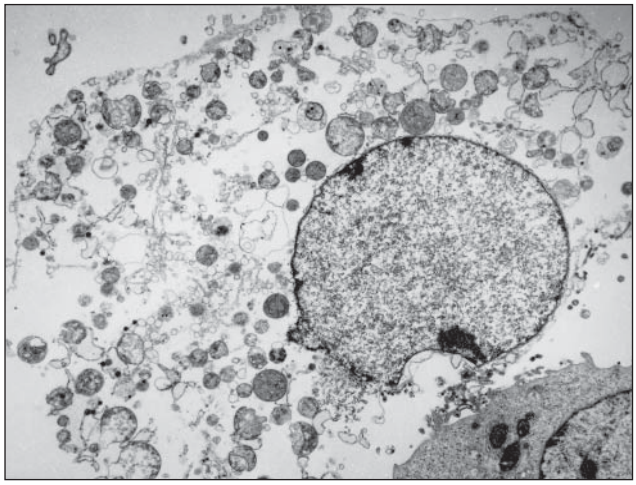

Fig. 5a. A dying cell. Necrosis. Magn. 3000x

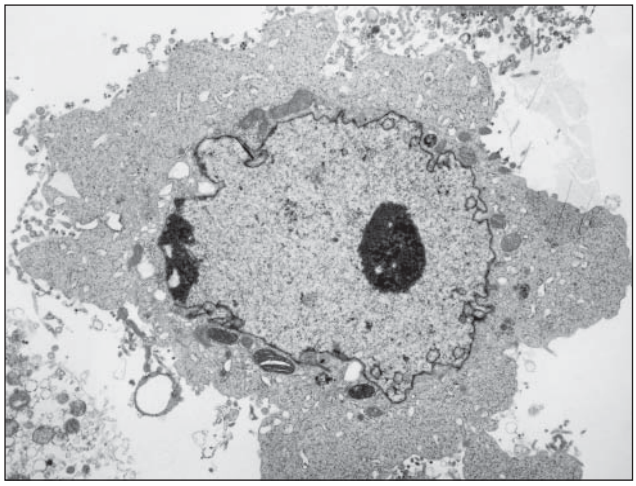

Fig. 5b. Shrunk cells with indentations both in the cellular membrane and nuclear envelop. Under the nuclear envelop, a cap of electron-dense heterochromatin is seen. Apoptosis. Magn. 3000x

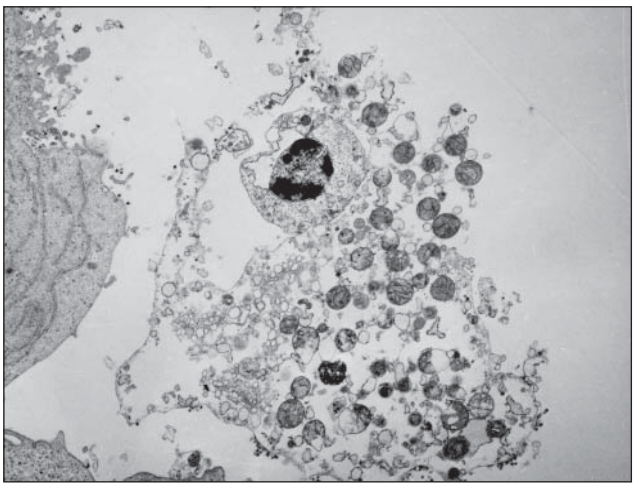

Fig. 5c. Cellular detritus observed in the vicinity of living cells. Note almost unchanged mitochondria, as well as a nucleus filled with condensed heterochromatin. A typical picture of apoptosis. Magn. 3000x

betic properties, on the H35-19 cells line as a very useful model to study regenerating rat hepatocytes [7]. In view of the above-mentioned, sometimes controversial, opinions on the effect of streptozotocin, we decided to study its action at various concentrations of STZ after three exposure times – 24 h, 48 h, and 72 h. The addition to the medium of streptozotocin at the concentration range of 0.5-10 μM, as well as 100-500 μM did not induce distinct metabolic alterations (measured by reactions with neutral red or crystal violet), but it caused improved cell growth up to 72 h after STZ application. The results obtained by MTT or neutral red staining, as well as by counting these cells in a Biirker chamber showed no significant differences in STZ impact between 48 h and 72 h of incubation (the Benferoni post-hock test). According to MTT, the impact of concentration showed a high statistical significance ($p<0.001$), while the effect of interaction and general time was non-significant. A similar, statistically significant interaction of time and concentration was observed in studies performed with the use of neutral red (unpublished, not shown data).

Streptozotocin concentration at the range of 1-10 mM, with the substance employed for 24 h, 48 h, and 72 h, showed some damage of the cells, especially mitochondrial destruction with an increased lysosomal activity, particularly pronounced with 5 mM of STZ in cell culture medium. The most valuable information will be obtained following an exact morphological analysis in electron microscopy.

In our previous investigations on a diabetic rat model, in addition to a change in galactosyltransferase activity, we observed a spectrum of ultrastructural changes in the hepatocytes [6]. We were interested in finding out in what way vanadium alone [5, 15] and STZ alone directly affected the cell. The experiments were carried out in the H35-19 cells line, whose properties are the most similar to those of hepatocytes of a regenerating liver. The presently observed ultrastructural changes in cellular nuclei are associated with the very nature of the neoplastic cell. They do not develop as the effect of STZ. This is also true in the case of cytoplasm that is poor in organelles. This property is characteristic of neoplastic cells, especially the rapidly growing ones. In rat liver hepatocytes treated with STZ, ring-like forms of Golgi complex were encountered very frequently [6]. In the H35-19 cells, Golgi complex is rarely seen, and – if it is noted – it is so small that it would be hard to expect it to appear in a cylindrical, twisted form. As it is suggested by our investigations, the H35-19 cells seem to differentiate under the influence of vanadium [5, 15]. In the H35-19 cells after neoplastic transformation, STZ does not trigger the formation of cylindrically twisted forms of Golgi complex, what was the

most common observation in the hepatocytes originating from the livers of live animals. The described-above cells containing a higher number of organelles are probably older, what may be supported by their morphology, especially some damaged organelles, as well as disintegration (gemmation) of the cytoplasm. Thus, STZ does not exert a normalizing effect on the H35-19 cells. However, as an antibiotic, it does have a protective effect, especially at STZ concentrations up to 3 mM, what results in a more rapid cell growth. Obviously, STZ also exerts a cytotoxic effect, what begins to be manifested at the concentration of 5 mM and leads to massive death of cultured cells at the concentration of 10 mM. Contrary to vanadium salts, STZ does not affect the incidence of apoptotic changes in the investigated cell population.

## Acknowledgements

The authors are grateful to Maria Kawecka MSc and Alicja Strzelbicka MSc for their excellent technical help during experiments.

## References

1. *Agarwal MK:* Streptozotocin: Mechanism of action. FEBS Letters 1980, 120, 1-3.
2. *Baba S, Kazumi T, Yoshino G, Utsumi M, Morita S:* Histochemical and biochemical characteristics of 91 islet cell tumors of the rat pancreas induced by streptozotocin In: Streptozotocin: fundamentals and therapy Agarwal, MK eds, Elsevier/North Holland Biomedical Press Amsterdam, New York, Oxford 1981, 275-285.
3. *Bolzan AD, Bianchi MS:* Genotoxicity of streptozotocin. Mutation Res. 2002, 512, 121-134.
4. *Borenfreund E, Puerner JA:* Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett 1985, 24, 119-124.
5. *Dąbroś W, Kajda B, Kordowiak AM:* The inhibitory effect of bis(2,2'-bipyridine)oxovanadium(IV) sulphate on the growth of rat hepatoma cell line H35-19. ELSO Proceedings Dresden, Germany 20-24 September 2003 abstr no 228, 79.
6. *Dąbroś W, Kordowiak AM:* Comparison of bipirydyl, maltol and kojic acid action as organic vanadium ligands on activity of galactosyltransferase (EC 2.4.1.38), some physiological parameters and ultrastructure of Golgi complexes in rat hepatocytes. Folia Histochem. Cytobiol. 2007, 45, 239-244.
7. *Essner E, Novikoff AB:* Cytological studies on two functional hapatomas. Interrelations of endoplasmic reticulum, Golgi apparatus and lysosomes J. Cell Biol. 1962, 15, 289-312.
8. *Ganda OP, Rossini AA, Like AA:* Studies on streptozotocin diabetes. Diabetes 1976, 25, 595-603.
9. *Gillies RJ, Didier N, Denton M:* Determination of cell number in monolayer cultures Anal Biochem. 1986,159,109--113.
10. *Karnovsky MJ:* A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 1965, 27,137A-138A.
11. *Karunanayake EH, Baker JRJ, Christian RA, Hearse DJ, Mellows G:* Autoradiagraphic study of the distribution and cellular uptake of ($^{14}$C )streptozotocin in the rat. Diabetologia 1976, 12, 123-128.
12. *Karunanayake EH, Hearse DJ, Mellows G:* Streptozotocin: Its excretion and metabolism in the rat. Diabetologia 1976, 12, 483-488.
13. *Karunanayake EH, Hearse DJ, Mellows G:* The synthesis of [$^{14}$C] streptozotocin and its distribution and excretion in the rat. Biochem. J. 1974, 142, 673-683.
14. *Kordowiak A, Turyna B, Kaczmarski F, Sarnecka-Keller M:* Comparison of rat plasma glycoprotein composition with biochemical activity and morphology of liver Golgi apparatus in streptozotocin-diabetes treated with insulin. Folia Histochem. Cytochem. 1981, 19, 179-186.
15. *Kordowiak AM, Klein A, Goc A, Dąbroś W:* Comparison of influence $VOSO_4$, $Na_3VO_4$ and $NaVO_3$ on proliferation, viability and morphology of H35-19 rat hepatoma cell line. Pol. J. Pathol. 2007, 58, 51-57.
16. *Mosmann T:* Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytoxicity assays. J Immunol Methods 1983, 65, 55-63.
17. *Motulsky W:* Analyzing Data with GraphPad Prism, 1999, GraphPad Software Inc., San Diego CA, www.graphpad.com.
18. *Reaven GM, Risser LR, Wieland D, Mondon CE:* Mechanism of hypertriglyceridemia in streptozotocin-induced insulin deficiency In: Streptozotocin: fundamentals and therapy Agarwal, MK eds, Elsevier/North Holland Biomedical Press Amsterdam, New York, Oxford 1981, 141-159.
19. *Sarnecka-Keller M, Kaczmarski F, Kordowiak A:* Morphology of the Golgi apparatus in streptozotocin-diabetic rat liver. Path. Res. Pract. 1980, 168, 126-133.
20. *Sarnecka-Keller M, Kordowiak A:* The activity of Golgi membrane galactosyltransferase EC 2.4.1.38.in the livers of streptozotocin diabetic rats Acta Biochim. Polon. 1977, 24, 179-185.
21. *Schneir M, Golub L:* The effect of streptozotocin-induced collagen catabolism In: Streptozotocin: fundamentals and therapy Agarwal, MK eds, Elsevier/North Holland Biomedical Press Amsterdam, New York, Oxford 1981, 159--182.
22. *Stearns SB, Benzo CA:* Effects of streptozotocin on aspects of carbohydrate and lipid metabolism in liver and muscle In: Streptozotocin: fundamentals and therapy Agarwal, MK eds, Elsevier/North Holland Biomedical Press Amsterdam, New York, Oxford 1981, 183-211.
23. *Venable JH, Coggeshall RA:* Simplified lead citrate stain for use in electron microscopy. J Cell Biol. 1965, 25, 407-408.
24. *Vessely DL:* The interrelationship of streptozotocin, its analogues and cyclic nucleotide metabolism In: Streptozotocin: fundamentals and therapy Agarwal, MK eds, Elsevier/North Holland Biomedical Press Amsterdam, New York, Oxford 1981, 245-260.
25. *Wiley PF:* Isolation and chemistry of streptozotocin In: Streptozotocin: fundamentals and therapy Agarwal, MK eds, Elsevier/North Holland Biomedical Press Amsterdam, New York, Oxford 1981, 1-15.

**Address for correspondence and reprint requests to:**
Wojciech Dąbroś
Department of Clinical and Experimenta l Pathomorphology
Faculty of Medicine, Jagiellonian University
ul. Grzegórzecka 16
31-531 Kraków
E-mail: mpdabros@cyf-kr.edu.pl