Adriamycin activity's durational governance of different cell death types and zonality in rat liver acinus. Immunohistochemical studies

Agnieszka Pedrycz1, Zbigniew Boratyński2, Piotr Siermontowski3, Jacek Mendocha4, Marcin Orłowski5, Katarzyna Van Damme-Ostapowicz6

1Department of Histology and Embryology with Laboratory of Experimental Cytology, Medical University of Lublin, 20-080 Lublin, Poland, 2Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences Lublin, 20-950 Lublin, Poland, 3Department of Maritime and Hyperbaric Medicine, Military Institute of Medicine, 81-103 Gdynia, Poland, 4Lublin Oncology Centre, 20-090 Lublin, Poland, 5Centre for Diagnosis and Therapy of Digestive System Diseases, 80-210 Gdansk, Poland, 6Department of Integrated Medical Care Medical University of Bialystok, 15-096 Bialystok, Poland

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Abstract

The aim of this study was to develop and examine a model of apoptosis and necrosis of hepatocytes induced by a damaging factor – adriamycin, correlating time after its administration with cell death type, and to investigate the localisation within the liver acinus of hepatocytes dying in these two ways. The results obtained in the present and previous studies were compared in order to make a map of cell death localisation in the liver acinus, showing the effect of time in action and dose of adriamycin. The experiment was performed on 32 female Wistar rats, divided into four groups: I and II – experimental, and III and IV – control. Adriamycin (3 mg/kg b.w.) was administered intraperitoneally to rats in groups I and II, and the rats were decapitated after four (group I) and eight (group II) weeks. Animals in control groups III and IV were given 0.5 mL of 0.9% NaCl solution, and decapitated after four and eight weeks respectively. Sections of the liver were examined with a three-stage immunohistochemical method. This method allowed to examine hepatocytes qualitatively and quantitatively for the presence of proteins involved in three types of apoptosis: induced by the mitochondrial pathway (caspase 3, 9), the intrinsic pathway related to endoplasmic reticulum stress (caspase 3, 12), and the extrinsic pathway (caspase 3, 8). One of the inflammatory markers, caspase 1, was also examined. The zonal localisation of all three types of apoptosis was assessed in the liver tissue. More oxidated hepatocytes indicated only signs of the internal mitochondrial pathway, whereas less oxidated hepatocytes induced the internal reticular pathway and the external apoptotic pathway. The period between adriamycin administration and hepatic cell investigation was a main factor of the process. A longer period post insult resulted in a more pronounced effect of the activation of apoptosis. Sections explored eight weeks after treatment with different doses of the drug (3 and 5 mg/kg in the previous study) showed a similar intensity of apoptosis.

Key words: rats, liver acinus, adriamycin, apoptosis, necrosis.

Introduction

Adriamycin (doxorubicin) is one of the anthracycline antitumour antibiotics. Under natural conditions, it is produced by a mutant of the Streptomyces species S. coeruleorubidus (20). Adriamycin enters cells by a process of passive diffusion, which is proportional to the concentration of medicine in the blood serum. In cells, it accumulates in the nucleus and lysosomes (7).

All anthracycline antibiotics function in every phase of the cell cycle but they are the most effective during the S phase. Benzo-semiquinone, a free radical responsible for the cytotoxic effect of adriamycin, is
produced by NADPH reductase, with the participation of cytochrome P-450 in the process of the antibiotic metabolism in microsomes (13).

Studies of liver microcirculation have identified the liver acinus as the hepatic microcirculation unit. Two axes of a vessel are concerned: one lying around the lobules (branching from the perilobular artery) situated on the perimeter of the classic lobulus, and the other, the long axis, being a line connecting the central veins of neighbouring lobuli (15). Rappaport (15) believes that the liver lobulus is also the liver’s secretory unit, from which bile would drain into the bile duct in the portal space.

The following zones in the liver acinus were described:

I. - A zone of cells lying close to the perilobular artery (periportal zone), which covers the best oxygenated hepatocytes, with a small number of large mitochondria and a small number of membranes of smooth endoplasmic reticulum.

II. - An intermediate zone - transient.

III. - A zone of cells lying close to the central vein (centrolobular zone), which covers the worst oxygenated hepatocytes, with a large number of small mitochondria and a large number of membranes of smooth endoplasmic reticulum (ER) (19).

Apoptosis is a fundamental phenomenon, which affects the tissue’s kinetics. It is responsible for regression of mammary gland epithelial cells, follicular atrophy, and removal of T- cells from the thymus and B-cells from lymph nodes. It is also responsible for the removal of cells infected by viruses and cancer cells (8).

Characteristic features of apoptotic death include a change in the size and shape of the cell. Cytoplasm is condensed and local vacuolisation of the cell is observed. In the nucleus condensation and a marginal position of chromatin is noted (12). The organelles and cytoplasmic membranes retain their integrity for a longer period. At a later stage of apoptotic death, apoptotic cells are formed due to further condensation and fragmentation of the nucleus and cytoplasm (6).

Apoptosis leads through extrinsic pathway with activation of death receptor and pro caspase 8 and through intrinsic pathway with activation of pro caspase 9 or 12. Both pathways end on pro caspase 3, which is caspase of degradation phase.

A cell, which undergoes apoptotic death, participates actively in its self-destruction and annihilation by starting a cascade of events occurring at molecular and biochemical levels (10, 22). Thus, for histological purposes, apoptotic cell death is intentional (genetically programmed), controlled and happens in several stages, such as the decisive phase, the execution phase, and the degradation phase.

Studies have shown that apoptotic cell death is induced in hepatocytes after treatment with cytostatic drugs, such as doxorubicin (adriamycin) (1, 2, 3). It was demonstrated that liver cells exposed to free radicals, which are formed during the process of biodegradation of adriamycin, are responsible for oxygen shock, leading to apoptosis. The severity of these reactions depends on the location of hepatocytes within one of three liver acinus zones.

In our previous studies, the structure of hepatocytes in the liver acinus of the rats treated with adriamycin (5 mg/kg b.w.) was examined. It was concluded that apoptosis and necrosis occur in hepatocytes after adriamycin administration, and these processes are zonal and depend on the elapsed time since medicine administration (12).

In present study authors examined immunolocalisation of all caspases involved in extrinsic (caspases 8, 3) and intrinsic (caspases 9, 12, 3) pathways of apoptosis and caspase 1 marker of postinflammatory necrosis.

The aim of the study was to develop and examine a model of apoptosis and necrosis of hepatocytes induced by adriamycin, and to investigate the structure of hepatocytes undergoing death processes, as well as their location within the liver acinus. The results obtained in the present and previous studies were compared in order to make a map of different types of cell death in the liver acinus, showing time in action and dose of adriamycin.

The results of this study may be helpful in the development of a suitable anti-cancer therapy, and may provide information on the doses of adriamycin, which induce apoptosis in defined parts of the liver. Regulation of dose and time of action of adriamycin seems to be important for killing cancer cells with precision.

**Material and Methods**

Thirty-two white Wistar female rats, with initial weight of 200-250 g and age of 2.5-3 months, were used in the study. The rats were assigned randomly to the control and experimental groups. The animals received standard feed and water ad libitum. They were kept in 0.2m² metal cages, four individuals in each cage, at a temperature of 20 +/-2°C and humidity about 60%. The air temperature, lighting, and noise changed slightly during the day.

The animals were divided into four equal groups. Groups I and II received adriamycin. Groups III and IV (control groups) received 0.9% NaCl.

Hepatocyte death was induced by a single administration of 10 mg of adriamycin (Adriblastin, Farmitalia) dissolved in 5 mL of water for injection at a dose of 3 mg/kg b.w. The volume of the administered solution did not exceed 0.5 mL per rat. Each animal from the control groups received 0.5 mL of 0.9% NaCl.

Group I was decapitated after four weeks and group II after eight weeks. Groups III and IV were decapitated after four and eight weeks respectively. The
studies were approved by the local bioethics committee.

Two liver samples of each rat were collected for immunohistochemical examination. The specimens were fixed in 10% formalin, dehydrated in alcohol series, cleared in xylene, and embedded in paraffin blocks which were cut into 5 µm sections and placed on silanised glass.

Two sections from each animal were used in the study. Altogether, 64 tests for each antibody were performed in the experimental and control groups. The sections were deparaffinised in xylene and hydrated in decreasing concentrations of ethyl alcohol. Sections were subjected to heat treatment under acidic conditions (10 M citrate buffer, pH 6.0) in a microwave at 750 W and after a 5 min interval at 375 W. After cooling, the sections were rinsed with distilled water and then placed in Tris buffered saline (TBS buffer).

Endogenous peroxidase was blocked by incubating the sections in a 0.3% H₂O₂ solution (99 mL TBS buffer + 0.1 g of Na₂O₂ + 1 mL of 30% H₂O₂). Sections were washed in TBS buffer and incubated for 60 min at room temperature with rabbit primary antibodies (caspases 1, 3, 8, 9, 12) used at a dilution of 1:100 in buffer TBS/Bovine Serum Albumin (BSA) 1%. A set of ready-made staining reagents (Dako, formerly DakoCytomation) was used to obtain an immunohistochemical reaction, the set comprising biotinylated secondary antibody against rabbit antibodies (Biotinylated Link Universal), streptavidin merged with horseradish peroxidase (Streptavidin-HRP), and AECSubstrate as the reaction pigment with horseradish peroxidase (AEC Substrate Chromogen). After staining with chromatogen, the slides were stained by haematoxylin. For each tested sample, a negative control was used, which was a preparation with no added primary antibody. Photographic documentation was prepared using a colour video camera CCD-IRIS (Sony).

The results of the immunohistochemical examinations were assessed both qualitatively and quantitatively. In the qualitative assessment, the intensity of the colour reaction between antigen and antibody in the liver acinus of Rappaport (in experimental and control groups) was taken into account. In order to better visualise the changes in colour in the areas of the Rappaport’ sacinus, a computer programme (analySIS pro software, version 3, Olympus Soft Imaging System GmbH, Germany) was used to change pink/red into the more prominent green.

In the quantitative assessment, the sections were arrayed by liver acinus zone location and the number of positively stained sections out of the total number of sections was converted to a percentage.

For histological studies, liver slides were stained with haematoxylin and eosin (H+E). The results were presented in a descriptive form.

Immunohistochemical results were presented as mean values and standard deviation, and analysed statistically by dependency rate (%). The obtained results were compared to those from a previous study by one of the authors (12).

Results

In the control groups, no immunohistochemical staining for tested antibodies was observed in any of the evaluated samples. Equally, no immunohistochemical staining was noted in any of the negative control sections (the preparations with no primary antibody added).

Caspase 3 is direct evidence for apoptosis induction, regardless of the pathway of induction. In experimental group I, positive immunohistochemical staining was mainly present in zone 3 of the liver acinus four weeks after administration of adriamycin (Fig. 1a). In experimental group II, positive immunohistochemical staining was observed in zones 2 and 3 of the liver acinus eight weeks after adriamycin treatment (Fig. 1b, Table 1).

In order to confirm the histological observations, an immunohistochemical staining of caspase 1, one of the markers of inflammation associated with necrosis, was carried out. In experimental groups I and II, there was no positive immunohistochemical staining confirming the presence of caspase 1 (Figs 2a, 2b, Table 2).

Caspase 8 is a marker of the extrinsic apoptotic pathway. In experimental group I there was no positive immunohistochemical staining for caspase 8 four weeks after adriamycin treatment in the majority of preparations (Figs 3a, 3b, Table 3).

In experimental group II immunohistochemical staining was present only in zone 3 of the liver acinus eight weeks after administration of adriamycin in most preparations (Fig. 3b).

Caspase 9 is a marker of the internal pathway initiating the mitochondrial signal to apoptosis. In experimental group I there was positive immunohistochemical staining for caspase 9 in the liver acinus zone 3 four weeks after adriamycin treatment in the majority of the preparations (Fig. 4a). In experimental group II, positive immunohistochemical staining was found mainly in the liver acinus zones 2 and 3 eight weeks after administration of adriamycin (Fig. 4b, Table 4).

Caspase 12 is a marker of the intrinsic apoptotic pathway induced in the course of ER stress. In experimental group I was no positive immunohistochemical staining for caspase 12 four weeks after adriamycin treatment in the majority of preparations (Fig. 5a). In experimental group II, positive immunohistochemical staining was found mainly in the liver acinus zone 3 four weeks after administration of adriamycin (Fig. 5b, Table 5).
Fig. 1a. Rat liver. Caspase 3 stained red. Experimental group I. AEC + H staining

Fig. 1b. Rat liver. Caspase 3 stained red. Experimental group II. AEC + H staining

Fig. 2a. Rat liver. No reaction with antibodies against caspase 1. Experimental group I. AEC + H staining

Fig. 2b. Rat liver. No reaction with antibodies against caspase 1. Experimental group II. AEC + H staining. Scale bare 100 µm

Fig. 3a. Rat liver. No reaction with antibodies against caspase 8. Experimental group I. AEC + H staining. Scale bare 100 µm

Fig. 3b. Rat liver. Positive IHC staining with antibodies against caspase 8. Experimental group II. AEC + H staining. Scale bare 100 µm
Fig. 4a. Rat liver. Positive IHC staining with antibodies against caspase 9. Experimental group I. AEC + H staining

Fig. 5a. Rat liver. No reaction with antibodies against caspase 12. Experimental group I. AEC + H staining. Scale bare 200 µm

Fig. 5b. Rat liver. Positive IHC staining with antibodies against caspase 12. Experimental group II. AEC + H staining. Scale bare 100 µm

Fig. 6. Rat liver. Control group III. This tissue section shows the liver with the central vein and hepatic trabeculae composed of hepatocytes. These are hepatocytes with pink cytoplasm and one or two round, blue stained nuclei with one or two nucleoli. Erythrocytes are visible in sinuses. H+E staining

Fig. 7. Rat liver sample. Experimental group II. The photomicrograph shows focal abnormalities in the structure of the liver tissue, irregular hepatocytes, nuclei of various shape, colour, and size, as well as numerous erythrocytes in sinuses. H+E staining
Table 1. The number (%) of preparations where a positive immunohistochemical reaction for caspase 3 occurred in the liver acinus zones

<table>
<thead>
<tr>
<th>Zone 1</th>
<th>Zone 1+2</th>
<th>Zone 2</th>
<th>Zone 2+3</th>
<th>Zone 1+2+3</th>
<th>Zone 3</th>
<th>Preparations without reaction</th>
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Table 2. The number (%) of preparations where a positive immunohistochemical reaction for caspase 1 occurred in the liver acinus zones

<table>
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<th>Preparations without reaction</th>
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<td>0(0)</td>
<td>0(0)</td>
<td>0(0)</td>
<td>22(68.75)</td>
</tr>
<tr>
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Table 3. The number (%) of preparations where a positive immunohistochemical reaction for caspase 8 occurred in the liver acinus zones

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<th>Zone 2+3</th>
<th>Zone 1+2+3</th>
<th>Zone 3</th>
<th>Preparations without reaction</th>
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<td>0(0)</td>
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<td>22(68.75)</td>
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<td>22(68.75)</td>
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Table 4. The number (%) of preparations where a positive immunohistochemical reaction for caspase 9 occurred in the liver acinus zones

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<th>Zone 2</th>
<th>Zone 2+3</th>
<th>Zone 1+2+3</th>
<th>Zone 3</th>
<th>Preparations without reaction</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>0(0)</td>
<td>0(0)</td>
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Table 5. The number (%) of preparations where a positive immunohistochemical reaction for caspase 12 occurred in the liver acinus zones

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Table 6. Identified caspases

<table>
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<th>Experimental group</th>
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<tbody>
<tr>
<td>3 mg/kg b.w. – decapitation after four weeks</td>
<td>-</td>
<td>-</td>
<td>caspases 3, 9</td>
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<tr>
<td>3 mg/kg b.w. – decapitation after eight weeks</td>
<td>-</td>
<td>caspases 3, 9</td>
<td>caspases 3, 8, 9, 12</td>
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<td>5 mg/kg b.w. – decapitation after four weeks</td>
<td>-</td>
<td>caspases 3, 9</td>
<td>caspases 3, 8, 9, 12</td>
</tr>
<tr>
<td>5 mg/kg b.w. – decapitation after eight weeks</td>
<td>caspases 3, 9, 12</td>
<td>caspases 3, 9, 12</td>
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</table>
A comparison of the results obtained in the present study (adriamycin in a dose of 3 mg/kg b.w.) and in our previous study, in which adriamycin was used in a dose of 5 mg/kg b.w. (12), enabled us to prepare a map. It shows localisation in the liver acinus of hepatocytes with positive immunohistochemical reaction for caspases involved in adriamycin-induced apoptosis and necrosis. The dose of adriamycin and time in action (four and eight weeks) were considered (Table 6). A comparison of the results of the present study (adriamycin in a dose of 3 mg/kg b.w.) and our previous study (adriamycin in a dose of 5 mg/kg b.w.) is seen in Table 6 (12). It is a kind of map of hepatocytes with positive immunohistochemical reaction for caspases 1, 3, 8, and 9 in zones of the liver acinus. Dependence on time in action and dose of adriamycin is observed.

The liver of control groups did not differ from the normal structure of the organ (Fig. 6). The cytoplasm of hepatocytes was stained pink with visible basophilic granules evenly distributed. One or two bright violet nuclei - quite large, distinct, and round with the regular nuclear membrane and chromatin stroma, were visible in the cell centre. One or two nucleoli were clearly visible in the nucleus. The endothelium of sinuses showed hepatic macrophages and a small number of erythrocytes in their lumen.

The nature of the microscopic image of the liver in the experimental groups depended on the localisation of cells in one of the three zones of the liver acinus. In the first and second zones of the liver acinus in rats from experimental group I, and in the first zone of the liver of rats from experimental group II, the microscopic image of cells did not differ from that described in control groups. In the third zone of Rappaport acinus in experimental group I, and in the second and third zones of experimental group II, blurring of the membrane of parenchymal hepatic cells and focal disintegration of the architecture of hepatocytes concerning the shape and size were visible. Hepatic trabeculae were focally destroyed (Fig. 7). The characteristic different sizes, shapes, and colours of nuclei were more visible than in control groups. Chromatin showed the features of dispersion. Some nuclei revealed peripheral chromatin condensation. An increased number of nucleoli were often observed. The cytoplasm of hepatocytes was markedly brighter than in control groups (more acidophilic). Some cells had no cytoplasm, especially around the nuclei. Other cells had fine-granular, bright or completely dark cytoplasm without the nucleus or with one of reduced perimeter, changed shape and colour. The cytoplasm of some cells also showed numerous vacuoles (vacuolar degeneration).

The lumen of sinuses was focally reduced or markedly widened. The hepatic macrophages were significantly larger and more numerous than those in controls.

**Discussion**

Immunolocalisation of caspases activated during adriamycin-induced apoptosis (caspases 3, 8, 9, 12) and postinflammatory necrosis in the liver acinus were assessed in rats. In our previous study, necrosis was observed in three zones of the liver acinus eight weeks after adriamycin administration in a dose of 5 mg/kg b.w. (14). This dose stimulatated apoptosis after four weeks in zones 2 and 3 of the liver acinus. Programmed cell death was induced via the mitochondrial pathway (zone 2) and the mitochondrial, reticular, and extrinsic pathways (zone 3). The same results were noted in the present study eight weeks after administration of adriamycin in a dose of 3 mg/kg b.w.

A higher dose of adriamycin and longer time in action were responsible for necrosis in zone 3. A short time in action, or a small dose of adriamycin were responsible for firstly the mitochondrial pathway of apoptosis in zone 3, then the reticular pathway and next the extrinsic pathway in the same zone.

To achieve apoptosis in zone 1 of the liver acinus, a high dose (5 mg/kg b.w.) of adriamycin and a long time (eight weeks) in action were needed. Caspases 1, 4, 5, and 11, as proinflammatory caspases, are connected with the proteolytic proinflammatory cytokines processing pro-IL-1β and pro-IL-18. They play an important role in the induction of necrosis, which is a natural consequence of inflammation (4).

It has been reported that administration of adriamycin induces apoptosis in various organs of humans and experimental animals (1, 3). Free radicals released in large quantities in the process of biodegradation of adriamycin cause oxidative stress, which can lead to apoptotic cell death. However, the activation of this complex mechanism requires some time and may cause a delay in adriamycin reaction. Increased permeability of biological membranes (including mitochondrial membranes and ER caused by free radical activation results in the release of mitochondrial cytochrome c into the cytosol, and induction of the mitochondrial apoptotic pathway involving formation of apoptosome: Apaf-1, procaspase 9, and cytochrome c (16). In this experiment immunohistochemical staining of caspase 9 was significantly increased in adriamycin treated groups in comparison to controls, which confirmed the mechanism of this chemotherapeutic’s action.

Chibowska’s (2) research on the activity of the liver cells’ redox system after administration of adriamycin showed that not only free radicals but also some other mechanisms of toxicity must provoke this.

In the present study, the authors observed apoptosis of rats’ hepatocytes after administration of adriamycin. The intensity of apoptosis was the factor for differentiating cells by their liver acinus zonal location (Rappaport). The pathways of apoptosis activation within the liver tissue after treatment with
adriamycin were analysed. The study revealed that the mitochondrial pathway (internal), which involves cytochrome c release, formation of apoptosome, activation of caspase 9, and the executioner caspase 3, was the main apoptosis-inducing pathway after administration of adriamycin. Similar observations were made by Serafino et al. (17). The second apoptotic pathway, which was shown to be induced in the liver of rats treated with adriamycin was the intrinsic pathway connected with ER-stress and caspase 12 activation. Apoptosis activated by the destruction of ER was described by Yang et al. (21). The authors have noted that by the activation of caspase 12, the internal pathway is considerably more active than the other two (mitochondrial and receptor - external). Mehmet (11) found that if during the initiation of apoptosis caspase activation occurs at the cell membrane (outer pathway) or mitochondria (inner pathway - mitochondrial), caspase 12 will not be activated. In our experiment, it was not confirmed, as both pathways (mitochondrial and reticular) were active. The third, extrinsic pathway leading to apoptosis was far less pronounced.

The essence of this work was not only to obtain an experimental model of apoptosis after adriamycin administration but also to determine the pathway by which apoptosis is induced, and to display the location of apoptosis in hepatocytes in different zones of the liver acinus (Rappaport). Gumucio et al. (5) noted that the liver response to different drugs has a zonal character. For example, administration of phenobarbital (Luminal) increases the density of smooth endoplasmic reticulum firstly in zone III, and only later in zones II and I. Carbon tetrachloride with strong toxic properties firstly damages hepatocytes in zone III, and yellow phosphorus in zone I. Chibowska also observed the phenomenon of hypertrophy of smooth ER in hepatocytes localised closer to the central vein of lobules (liver acinus zone III) after administration of adriamycin. Furthermore, Chibowska’s analysis of oxygen uptake by the liver’ cells showed a significant increase in the uptake in cells localised around portal triads area (liver acinus zone I), while cells’ uptake localised near the lobule central vein was not changed (2). This data confirms the heterogeneity of hepatocytes, which was observed in this experiment. The liver cells in zone II, and especially zone III, were proved to be the most sensitive to the adriamycin administration. The observed apoptosis was initiated mainly by caspase 9 (mitochondrial pathway), and to a lesser extent by caspase 12 (endoplasmic reticulum pathway). The external pathway (through caspase 9) was much less pronounced. Apoptosis in cells of zone I occurred only when appropriate time elapsed between the administration of high doses of adriamycin and performance of the sample tests. Lower doses did not induce apoptosis in zone I. Similarly, when only a short time elapsed between administration and analyses, programmed cell death did not occur in zone II. It should finally be noted that the high doses of adriamycin connected with a long time in action resulted in necrosis in zone III of the liver acinus.

In this experiment, adriamycin was given at the dose of 3 mg/kg b.w. This dose has been shown to induce apoptosis but does not cause necrosis (18).

In order to induce apoptosis in various cells, the authors of publications administered adriamycin in doses ranging from 3 mg/kg to 10 mg/kg (9). Asakura et al. (1) found that caspase 3-dependent apoptosis was induced 6 h after adriamycin administration. Jang et al. (21) observed apoptosis 4 d after administration. Examining the effect of adriamycin on rat kidney cells, Skomra and Chibowski (18) showed the glomerulus dysfunction 3 h after adriamycin administration, despite the fact that the drug metabolites were excreted in much larger quantities in the liver than in the kidneys. However, morphological markers of toxicity, such as changes in the primary glomerular membrane were described four weeks after administration of adriamycin. After five weeks, proteinuria, as a signal of renal glomerular damage, was at its highest level (18).

In our previous study, adriamycin was administered to rats in a dose of 5 mg/kg b.w. In the present study, the results of both experiments were compared and a map was made of different types of cell death in the liver acinus showing the effect of time in action and dose of adriamycin (Table 6).

The types of induced apoptotic pathways were arranged in the more oxidated hepatocytes, whereas in less oxidated hepatocytes, the internal reticular and external pathways were observed.

References