APOPTOSIS OF FOETAL RENAL TUBULAR EPITHELIAL CELLS AS A LATE EFFECT OF ADRIAMYCIN ACTION. IMMUNOHISTOCHEMICAL ASSESSMENT OF CASPASE 3 EXPRESSION

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Abstract

In previous papers, we noticed that adriamycin given to female rats before their planned pregnancy has a delaying effect under the form of apoptosis for foetal hepatocytes. The purpose of present study was for a quantitative assessment of foetal renal tubular epithelial cells, as an effect means of delaying adriamycin action (apoptotic index). Expression of effector caspase 3 was also assessed. In the investigations, a standard three-step immunohistochemical method was used. The area covered by positive caspase 3 reaction was examined. In the kidneys of foetuses from the experimental group, we noticed an increase in the apoptotic index; furthermore, immunohistochemical reaction for caspase 3 covered a statistically significantly larger range as compared to the control group. The delayed effect of adriamycin, which was given to female rats before pregnancy, was an increase in apoptosis in foetal renal tubular epithelial cells.

Key words: rats, apoptosis, caspase 3, foetal kidneys, apoptotic index, adriamycin.

Cell apoptosis, suicidal death determining the life of a whole multicellular organism, is the mechanism naturally balancing the cell proliferation. The normal cell constantly receives life signals, which are transmitted by other cells through hormones and growth factors (cytokines).

The factors, which induce the cell apoptosis, include (16) direct DNA damage due to various stress-inducing factors (hypoxia, UV and γ-radiation, free radicals, abnormal amount of growth factors) (12, 18).

In previous studies, we noticed that adriamycin, antineoplastic antibiotic from the anthracycline group (11), given to female rats before planned pregnancy has a delaying effect under the form of apoptosis for foetal hepatocytes (14).

We also reported that this drug caused increased HSP70 (sensitive marker of cellular stress) reaction in renal cells of rat offspring, whose mothers were administered with adriamycin before pregnancy. (13) In these offspring, we described increased apoptosis of the cells. (15).

The purpose of present study was the quantitative assessment of foetal renal tubular epithelial cells as an effect means of delaying adriamycin action (apoptotic index). Expression of effector caspase 3, which is the terminal part of all apoptosis signal transduction pathways, was also assessed immunohistochemically in foetal kidneys.

Material and Methods

Sixteen female Wistar rats were used in the experiment. The rats were divided into two equal groups; experimental and control. Female rats from the experimental group were administered intraperitoneally adriamycin (Adriblastin; Farmitalia, Carlo Erba, Italy) at the dose of 5 mg/kg of body weight. Female rats from the control group were given intraperitoneally 0.5 ml of 0.9% NaCl. After 4 weeks, the female rats were mated.

At the end of pregnancy (day 20), the rats were decapitated. Two randomly chosen foetuses were taken from each of the pregnant rats. The foetuses were decapitated and their kidneys were collected for immunohistochemical examination. The kidneys were fixed in 10% formalin and paraffin sections were prepared.

To identify the caspase 3 protein and to evaluate its expression level, a standard three-step
immunohistochemical procedure was applied. Rabbit caspase 3 antibodies (Lab Vision-USA) were used as a primary antibody (in dilution 1/100). Then, biotinylated secondary antibody was added, and then horseradish peroxidase conjugated with streptavidin (DakoCytomation, USA). Because streptavidin has a close resemblance to biotin, after adding a chromatogen – AEC (DakoCytomation, USA), it binds to the places where primary antibodies match the background, and a reddish colour appears. For each preparation, negative control was performed (a slide without primary antibody).

The expression of caspase 3 was evaluated in preparations from 14 foetuses from the control group and 12 foetuses from the experimental group (two preparations from each individual).

The analysis of a microscopic picture (125x), and assessing the expression of the protein were performed using the computer programme Analysis-Pro 3rd version (Soft Imaging System GmbH, Germany). From each slide, three randomly selected places within a range of 781 193.35 µm² were assessed. All fields of the sectioned surface of cells with positive reactions were measured. The colour assessed by the computer as positive was an intensively red colour. The colours that were not assessed as positive were red-pink or pink.

The degree of apoptosis in the specimens collected from the kidney was determined quantitatively using the apoptotic index (AI). Three specimens from each individual were examined. The nuclei stained dark with haematoxylin with reduced perimeter and diameter were accepted as the pyknotic ones, i.e. being one of possible manifestations of apoptosis. In the haematoxylin and eosin stained specimens magnified about 1 000 x (linear areas: 985 x, square spaces: about x 1000 000 x), the number of pyknotic nuclei was counted in 100 cells in a particular experimental group, and compared with the respective control group. The results were presented as the percentage. A projection microscope was used.

The results were statistically analysed using an ANOVA test and a Student’s t-test. Means and standard deviations of positive reactions in the examined tissue fields were determined. The differences were considered statistically significant when P<0.05

### Results

From the 2nd to 4th d after mating, pregnancy was noted in 7 females from the control group and 6 from the experimental group. For further studies, 14 foetuses from the control group and 12 foetuses from the experimental group were used.

In immunohistochemical examinations of the kidney cells, the reaction for capase 3 was diffused, focally granular, and was localised in the cytoplasm (Fig. No 1).

The cytoplasm staining was from bright pink to red. The intensity of caspase 3 (+) positive cytoplasm staining in cells from the experimental group (Fig. 1a) was much greater than that in the cells from the control group (Fig. 1b).

The mean field with caspase 3 (+) positive reaction in experimental group covered 55.38 µm², which was statistically significantly higher than the mean field with caspase 3 (+) positive reaction in control group – 0.51 µm² (P<0.001). (Table 1)

A statistically significant increase in the number of apoptotic cells (apoptotic index) was observed in the experimental group compared to control (P<0.001) (Table 1).

### Discussion

In the present study, increased apoptosis of renal tubular epithelial cells was noticed in experimental group foetuses, whose mothers were administered adriamycin before their planned pregnancy. Increased apoptotic index was the evidence of this. The increased expression of caspase 3 was also noticed. The relevant elements of the executing phase of apoptosis are cysteine proteases from the interleukin-1-β-converting enzyme (ICE) family called the caspase family (8). The caspases degrade proteins behind asparaginian residue using one of their cysteine residues (17), hence their name – cysteine-dependent asparaginian specific proteases. These enzymes are present in the cell in their inactive form (proenzymes, zymogenes, pro-caspases), and are activated during apoptosis. They activate one another and other enzymes (5).

<table>
<thead>
<tr>
<th>Mean area covered by caspase 3 reaction (µm²)</th>
<th>Mean number of apoptotic cells</th>
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<tbody>
<tr>
<td>Control group</td>
<td>0.51±2.32</td>
</tr>
<tr>
<td>Experimental group</td>
<td>55.38±22.02*</td>
</tr>
</tbody>
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*P<0.001; ±SD
To date, 14 caspases have been discovered and described (7). They have been divided into initiating, effector, and proinflammatory caspases. The initiating caspases start the caspase cascade, which leads to the activation of effector caspases. The effector caspases (including caspase 3) are involved in/or initiate the destruction of cellular DNA, which leads to cell destruction. Caspase 3/CPP32/Yama/apopain (Cysteinyl aspartic acid-protease-3) activates CAD endonuclease. A caspase-activated DNase in mouse and rat (6, 10), inactivates DNA-repairing enzymes (4), and cleaves the cytoskeleton proteins (actin, spectrin, lamine) (9). It is capable in inducing cell death by itself (1).

Asakura et al. (2) noticed in investigations with adult individuals that adriamycin activated caspase 3, which compares to the present study. Cheng et al. (3) made similar observations in renal tubular epithelial cells of adriamycin–treated rats. They reported that adriamycin in these cells induced apoptosis and stimulated activities of caspase 3.
References