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The influence of a single dose of adriamycin on pregnant rat female liver; biochemical and ultrastructural evaluation. Assessment of Bcl-2 and Bax protein expression

Multidirectional thousands on thousands experimental animals proved that hepatocytes regenerate via single duplication. Dysfunction of that process as compared to other cells, for example fibroblasts, afteraction of different pharmacological substances, especially those with strong activity on human or animal organism could lead to decrease in the number of those cells, excess of production of collagen fibres and hepatic cirrhosis.

Many reports show that morphological changes in liver are caused especially by anticancer drugs. Among them is adriamycin, an antibiotic from antracyclines group, which inhibits DNA and RNA synthesis, upsetting processes of replication, transcription and protein synthesis (3). For cytotoxical activities of adriamycin are responsible free radicals, which arise in the process of transformation of that drug (13).

Multidirectional changes could be really visualized especially in ultrastructural picture. In this study there was evaluated Bax and Bcl-2 protein expression and were also assessed biochemical features of liver damage which were then statistically assessed.

The subject of the present study was an attempt to answer the question whether the pregnancy changes the picture of liver damaged by a single dose of adriamycin. From the literature (11) and own research it is known that adriamycin given in the dose 5 mg/kg of body weight develops in hepatocytes already after 4 weeks' changes typical of apoptosis.

MATERIAL AND METHODS

In the study there were used 24 female Wistar rats which were chosen randomly. Female rats were divided into 3 equal control group and two experimental groups. At the very beginning of the experiment the animals from experimental groups (APREG, A) were administered intraperitoneally adriamycin in the dose 5 mg/kg of body weight. In the control group animals were administered 0.5 ml of 0.5% NaCl intraperitoneally.

4 weeks after drug administration (or saline in the control group) female rats were matched with males. Then every day vaginal swab was taken and observed in light microscopy. The presence of vaginal plug or sperm cells in vaginal swab was treated as an effective copulation (fertilisation).

To the next part of experiment were chosen females which were fertilised between the second and fourth day after matching. Female rats from groups: K and APREG were decapitated on the 20^{th} day of pregnancy, and rats from experimental group A after similar time (after 48 days). For biochemical investigation there was taken blood from the heart to determine: bilirubin (mg/dl) Alat (u), Aspat (u).

From each animal after taking blood sections from the right lobe of liver were taken after macroscopical observation. Taken for investigation liver sections were then fixed in fixation fluid

containing 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M Sorensen phosphate buffer. Then the sections were treated with osmium tetraoxide, stained in uranyl acetate, dehydrated in increasing concentration of ethanol and embedded in Aralchit ACM Fliska resin. Preparations were cut into ultrathin slides 60 nm thick with ultramicrotom Reichert Ultracut S. The slides were stained with 8% solution of uranyl acetate and plumbic cytrate according to Raynolds. Documentation was performed using electron microscopy Tesla BS-500.

Blood results were showed as averages with standard deviation and statistically analysed with t-Student test. 5% risk of conclusion error and statistical significance differences with $p<0.005$ were admitted.

Evaluation of histopathological features was shown as an obershiption. The sections taken for immunohistochemical studies were fixed in 10% formalin, and then after dehydratation and embedding in paraffin were cut into 7 μm slides. To identify proteins Bax and Bcl-2, prepa-rations from both experimental and control groups were used. For each preparation negative control was performed (a slide without primary antibody).

The proteins expression level was evaluated with a standard three-step immunohistochemical procedure. Rabbit anti-Bcl-2 and anti-Bax antibodies were used as a primary antibody. Then biotynylated secondary antibody was added, and then horse-radish peroxidase conjugated with streptavidin. Because streptavidin has big affinity to biotin after adding a chromatogen (AEC), it places where primary antibody has caught the background, and a reddish colour appears. Expression of Bax and Bcl-2 proteins was assessed in preparations coming from 8 rats from the control group and 16 rats from the experimental groups (2 preparations from each individual: 16 control preparations and 32 experimental preparations).

An analysis of microscopic picture evaluating the expression of Bax and Bcl-2 proteins was conducted with microscope. In liver sections from each animal there were evaluated 100 hepatocytes on a randomly chosen field. We counted cells with positive reaction. Results were statistically analysed with ANOVA test and t-Student test. Statistical significance of differences was stated when $p<0.05$. Preparations with Bax (+) positive reactions were divided into 3 groups: + small number of Bax (+) cells (to 40/100); ++ medium number of Bax (+) cells (from 41 to 70/100); +++ big number of Bax (+) cells (above 71/100). Preparations with Bcl-2 sections were divided in the same way.

RESULTS

The body mass of female rats from the experimental groups was at the end of pregnancy significantly lower than the body mass of rats from the control group (ex. 364.5 g; con. 395.5 g). After 4 weeks from adriamycin administration almost all females were effectivelly fertilized after matching with males. Fertilization took place mostly on the 3rd and 4th day after matching with males (effectiveness of copulation was proved when sperm cells were present in vaginal swab.

Tab. 1. Mean AlAT, AspAT, bilirubin concentration in blood serum

	Bilirubin (mg/dl)			AlAT (j)			AspAT (j)		
Group	A	A PREG	K	A	A PREG	K	A	A PREG	K
Mean value	0.42	0.40	0.25	96,75	94.25	83.50	233,63	247.50	150.00
Standard deviation	+/-0.16	+/-0.20	+/-0.07	+/-16.93	+/-9.13	+/-9.29	+/-100.89	+/-85.27	+/-32.76
Statistical significance	A to K $p<0.05$	A to APREG $p>0.05$		A to K $p<0.05$	A to APREG $p>0.05$		A to K $p>0.05$	A to APREG $p>0.05$	

In the experimental groups (A, APREG) there was noticed an increased concentration of aminotranspherases (AlAT and AspAT) as compared to the control group. That increase was significant statistically. Between the experimental groups we did not notice statistically significant differences. The bilirubin concentration in blood serum of rats from the experimental groups increased much as compared to the control group, however that increase was not significant statistically ($p=0.08>0.05$) (Tab. 1).

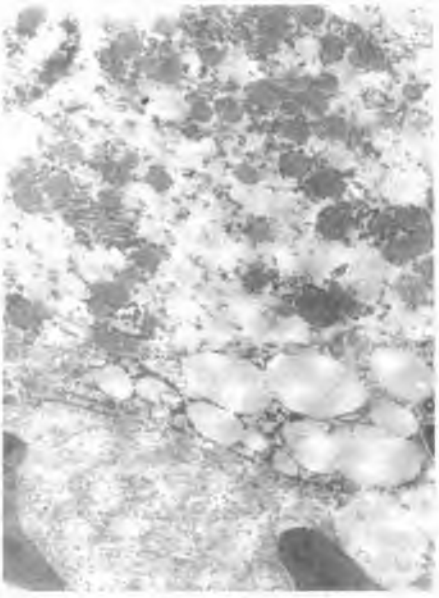

Fig. 1. Electronogram of part of the liver of rat from experimental group EXP-A (7 weeks after adriamycin administration). Features of steatosis (fat droplets), "rinsed cytoplasm", degranulation of rough endoplasmic reticulum. Magn. 5000x

The picture of liver of females from both experimental groups was similar. In liver parenchymal cells in the animals submitted to adriamycin there were observed features of large damage. In part of cells significant degree of steatosis was found – numerous fat droplets in cytoplasm (Fig. 2). In some cells was observed "rinsed" cytoplasm without organelles – cells empty inside (Fig. 2, 3). Cell lysis and indistinctness of cell structures was also present, which is a feature of necrosis. In cytoplasm was often visible microdroplet degeneration. Mitochondria were often swollen with brightened matrix and partial cristal destruction. Decreased number of mitochondria was observed. Destruction of some mitochondria was manifested by external membrane rupture, and even flowing out of their contents outside. Myelinic structures in damaged mitochondria were created.

Hepatocytes nuclei showed different shapes and size. In some cells there was observed picnotic nuclei with chromatin condensed circumpherentially, which were the evidence of cell apoptosis (Fig. 3). In part of nuclei was stated evident vacualisation of nucleolus (Fig. 3). Rough endoplasmic reticulum was often defragmentated, especially in sourrounding of mitochondria (Fig. 2). The number of glicogen granules decreased. Irregular dispersion and increased number of lysosomes was observed. Close to bile canaliculus numerous autophagosomes were noticed.

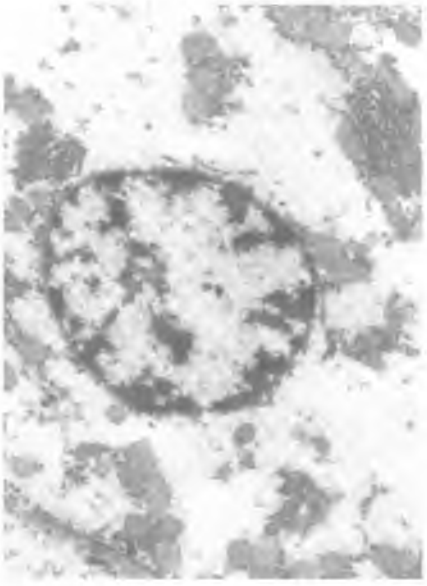

Fig. 2. Electronogram of part of the liver of rat from the experimental group EXP-A (7 weeks after adriamycin administration) "rinsed cytoplasm", slight presence of all organelles – mainly mitochondria and endoplasmic reticulum. Magn. 5000x

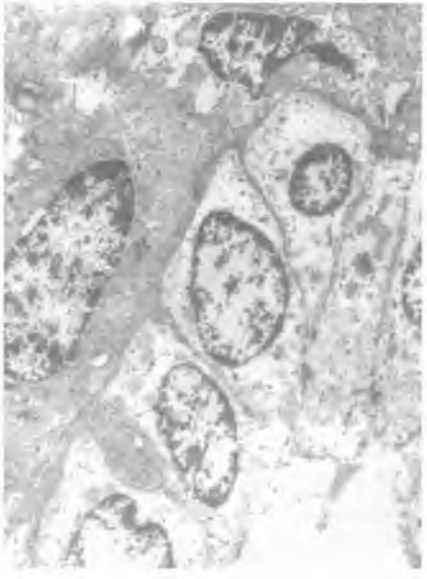

Fig. 3. Electronogram of part of the liver of rat from the experimental group EXP-A PREG (female pregnant rats on the 20th day of pregnancy, fertilized 4 weeks after adriamycin administration). Pseudoductular cells visible. Focal damage of cell membranes from the side of tubular lumen. Magn. 4000x

Comparatively to control group the number of peroxisomes in hepatocytes much increased. Endothelial cells damage was also observed. Perisinusal spaces were often widened and swollen with contents of damaged hepatic cytoplasm and with connective tissue proliferation, which was the evidence of cell membrane damage. The presence of erythrocytes in hepatic cytoplasm was the evidence of cell membranes damage.

Ductular cells observed in central vein region was characterised with bright cytoplasm comparativelly poor in cell organelles, big round nucleus, with chromatin condensed circumpherentially. In ductular sections was observed 5 to 8 cells. These cells were poor in glycogen granules, cuboidal or polyhydronic in shape and small number of mitochondria irregulary dispersed in cytoplasm (Fig. 4). In some cells, cell membrane damage from tubular lumen side was observed (Fig. 4).

In 16 preparations coming from 8 female rats from the control group no Bcl-2 positive reaction was observed. The same results were noted in the experimental groups (Tab. 2).

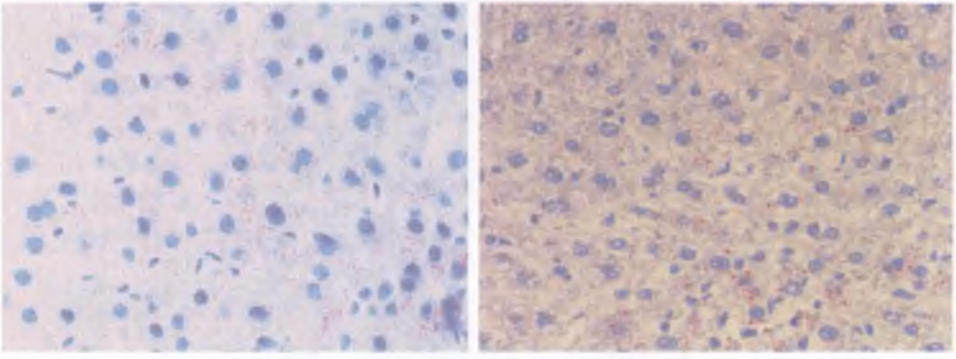

Fig. 4, 5. Immunohistochemical Bax (+)positive reaction in liver of female rat from the experimental group EXP-A (Fig. 4. Magn. 150x) and EXP-A PREG (Fig. 5. Magn. 100x). Staining with AEC +hematoxylin

Tab. 2 Bcl-2 protein expression in the control and experimental group

Group	Number of preparations	Number of individuals	Bcl-2 (-) negative cases	Bcl-2 (+) positive cases	% of Bcl-2 (+) positive cases	Mean percentage of Bcl-2 (+) positive cells (+/- standard deviation)
Control	16	8	16	0	0%	0.0(+\-0.0)
EXP. A	16	8	16	0	0%	0.0(+\-0.0)
EXP. A PREG	16	8	16	0	0%	0.0(+/-0.0)

In immunohistochemical investigations in Bax(+) positive cells granulomatous-diffused cytoplasatic reaction was observed. The cytoplasm staining was from pale to dark rose. In 16 preparations from the control group no Bax (+) positive reaction was observed.

In the experimental group (Exp A) in 14 from 16 preparations, Bax (+) positive reaction was observed (about 87%). In the experimental group EXP-A PREG Bax (+) positive reactions were observed in all preparations. Bax protein expression was visible with statistical significance in a bigger number of cells in preparations coming from experimental rats from group A as compared to controlled rats ($p<0.001$). The amount of cells with Bax granules in this experimental group was 72.34 (+/−22.24). In preparations from experimental group A with positive Bax reactions there was determined percentage of cells with Bax positive reaction and slides from this experimental

group were divided into several sections: 0–40 (+) positive (2 slides), positive (++) – 40–70 (6 slides) and strong positive (+++)–70–100 (8 slides) (Tab. 3).

In the experimental group EXP-A PREG Bax protein expression was visible in statistically significant bigger amount of cells than in the experimental group A ($p<0.0001$), and than in control group $p<0.0001$). The amount of cells with Bax (+) positive reaction was 88.20 (+/–10.9) in that group. Preparations with Bax (+) positive reaction were divided into the following groups: 40–70 (++) positive (7); 71–100 (+++) positive (9 preparations). Bcl-2/Bax index was in the experimental group EXP A 0/72.34, in experimental Group EXP –A PREG 0/88.20 and in control group 0/0 (Tab. 4).

Tab. 3. Bax protein expression in the experimental group

Group	Mean number Bax(+)/100 in division (standard deviation)	Number of BAX(+)cells/100 in division	Mean number of preparations in division
Experimental EXP-A	0 (+/–0.00)	(–)- do 40/100	2
	59.83(+\–8.18)	(++) od 41 do 70/100	6
	79.87(+\–6.24)	(+++) powyżej71/100	8
Experimental EXP-A PREG	0 (+/–0.00)	(–)- do 40/100	0
	61.71(+/–6.24)	(++) od 41 do 70/100	7
	88 (+/–6.16)	(+++) powyżej71/100	9

Tab. 4. Bax protein expression in the experimental and control group

Group	Number of prepara-tions	Number of individu-als	Number of Bax(+) positive reactions	Number of Bax(-) negative reactions	Mean number of cells with Bax(+) positive reaction in the group	Bcl-2/Bax Index of cells
Control	16	8	0	16	0.00(+/–0.0)	0/0
Experimental EXP-A	16	8	14	2	62.37(+/–26.07)	0/62.37
Experimental EXP-A PREG	16	8	16	0	76.5(+/–14.35)	0/76.5
T-Student test (stat. significance) K to EXP-A K to EXP APREG EXP-A to EXP-A PREG					P<0.001 P<0.001 P<0.001	

DISCUSSION

In the present study Bcl-2 and Bax protein expression was evaluated. These proteins belong to family of Bcl-2 proteins. Proteins from that family play a critical part in cell death regulation. Bcl-2 protein is connected with survival of cell. Over-expression of Bcl-2 has been shown to promote cell survival by suppressing apoptosis in a number of cells (6). In tissues where Bcl-2 protein is determined we have inhibited apoptosis process and excessive cells proliferation which is determined in several neoplasms.

Bax is proapoptotic protein. It has been shown to play a critical role in cytichrome C release from mitochondria and thus to initiate apoptosis (12). In this study cell proliferation was not observed. Bcl-2 protein was not determined in any group. Increased apoptosis took place in females after adriamycin administration and in pregnant females, which 4 weeks before planned pregnancy had adriamycin administered. Cell death was not observed also in liver of the controlled rats. It was confirmed also in the picture from the electron microscope.

Especially many changes in the experimental group showed mitochondria. Swollening of mitochondria with brightening of mitochondrial matrix, cristas destruction and creation of myelinic structures confirms the toxic influence of adriamycin on mitochondria, described also by other authors (1, 2, 4, 10). Swollening of a big number of mitochondria is a base, known from light microscopy parenchymatous degeneration. In experimental conditions swollening of mitochondria appears in starvation, oxigen insufficiency, after partial hepatectomy, after exposure to X-ray radiation, intoxication of phosphorus, ethionin, in viral hepatitis and cirrhosis of the liver.

If changes in swollen mitochondria's volume are not bigger than physiological border value – 20%, it is called low amplitude swollening, and if it is bigger than 20%, it is irreversible high amplitude swollening (lethal changes). Reversible low amplitude swollening is a way of adaptation of mitochondria to increasing energetic requirement.

Myelinic structures are according to Groniowski the evidence of starting of mitochondrial degeneration. Changes observed in the present study were also observed after action of different toxins or due to hypoxia or shock (5, 7, 8).

Plasmatic membranes damage including mitochondrial membrane, "rinsed, "empty" cytoplasm, without organelles, nuclei with chromatin condensed circumpherentially – these are the features of apoptosis. These features were observed in the present experimental groups .

An interesting phenomenon observed in the present study was proliferation of ductules. There could be distinguished two types of ductular proliferation: in the first, ductules have irregular course, appear in the whole portocholangial space, their lumen is not observed or hard to observe. That ductular cells are flattened and nuclei are strongly stained. That picture is visible in chronic hepatitis with massive lymphocytes infiltration.

The second part of proliferation is characterised by long and dilatated ductules with lumen present. Their longitudional sections are parallel to border lamina and are close to it. Ductular cells with bright cytoplasm and clear borders are bigger than in the first type. That type of ductules appears most often in extrahepatic cholestasis. That type was also observed in the present study. Biochemical results showed in the experiment (increased concentration of bilirubin and aminotranspherases) are the evidence of cholestasis and hepatic damage. In ultrastructural picture, ductular cells were characterised by bright cytoplasm poor in organelles and with a big round nucleus with chromatin condensed circumpherentially. The cells were poor in glycogen granules, numerous mitochondia were irregularly scattered in cytoplasm. In some cells there was observed cell membrane damage from the side of ductular lumen. In a section the ductule contained 5–8 cells, and comparatively to 4–5 cells in the section of not changed ductules (9).

The expression of proapoptotic Bax protein increased significantly after adriamycin and enlarged during pregnancy. The results indicate that pregnancy increases apoptosis.

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SUMMARY

The purpose of the study was ultrastructural evaluation of liver and evaluation of biochemical factors of liver damage (bilirubuin, Alat, Aspat) in female pregnant rats, which 4 weeks before planned pregnancy had adriamycin administered. Female rats were divided into 3 groups: 2 experimental (EXP A; EXP-A PREG) and one control (K). Females from the experimental groups were administered adriamycin in a dose 5 mg/kg of body weight intraperitoneally. Females from group EXP-A PREG were fertilized after 4 weeks. Liver sections were collected for investigations on the 20th day of pregnancy. Ultrastructural changes observed in liver in the experimental group included: numerous focal changes in hepatocytes typical of apoptosis, damage of blood vessel endothelial cells, proliferation of pseudoductules in the region of central veins and portocholangial spaces. Apoptosis in hepatocytes was assessed after observation of expression of proapoptotic protein Bax and antiapoptotic protein Bcl-2, and was much increased in group of pregnant rats as compared to non-pregnant female rats.

Wpływ jednej dawki adriamycyny na wątrobę ciężarnej samicy szczura. Ocena biochemiczna i ultrastrukturalna. Badanie expresji białka Bcl-2 i Bax

Celem pracy była ultrastrukturalna ocena wątroby oraz biochemicznych wykładników uszkodzenia wątroby (bilirubina, Alat, Aspat) u ciężarnych samic szczura, którym na 4 tygodnie przed planowaną ciążą podano adriamycynę. Samice podzielono na 3 grupy: dwie doświadczalne (EXP-A; EXP-A PREG) i jedną kontrolną (K). Samicom z grup doświadczalnych podano adriamycynę w dawce 5 mg/kg mc. dootrzewnowo. Samice z grupy EXP-A REG zapłodniono po 4 tygodniach. Wycinki wątroby do badań pobierano 20 dnia trwania ciąży. Na ultrastrukturalne zmiany obserwowane w wątrobie w obu doświadczalnych grupach składało się wiele ogniskowych zmian w hepatocytach typowych dla apoptozy, uszkodzone komórki śródbłonka naczyń, proliferacja pseudokanalików w okolicy żył centralnych. Apoptoza hepatocytów była oceniona również dzięki obserwacji ekspresji proapoptotycznego białka Bax i antyapoptotycznego białka Bcl-2. Ekspresja białka Bax znacznie wzrosła w grupie ciężarnych samic szczura w stosunku do ich niecięzarnych rówieśniczek.