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Morphological and Biochemical Studies of Daunorubicin Hepatotoxicity Including the Protective Effects of Tocopherol and Ascorbic Acid

Badania morfologiczne i biochemiczne hepatotoksycznego działania daunorubicyny z uwzględnieniem ochronnego wpływu tokoferolu i kwasu askorbinowego

INTRODUCTION

Performing the major function in the xenobiotic detoxication, the liver is also exposed to the toxic action of anthracyclines.

The anthracycline-induced liver disfunction in humans was reported by the authors of only a few papers (9, 12). More numerous studies concern the hepatotoxic effects of anthracyclines in the experimental animals (1, 2, 5, 7, 8, 11, 18).

The comparative studies of the activities of the oxidation system in the rat liver microsomes following the daunorubicin administration showed that the disorders of the oxygen consumption in the microsomes of the liver cells were so significant that there must have been some other than the free radicals toxic mechanisms resulting in the daunorubicin and doxorubicin-induced toxicity (13). The studies of the adriamycin effect on the electron transport in the rat liver mitochondria showed that adriamycin decreased the tissue respiration, oxidative phosphorylation and the activity of mitochondrial ATP. The authors believe that the changes are caused by the disorders of the physicochemical condition of the mitochondrial membranes (3).

The studies concerning the doxorubicin effects on the level of liver cytochrome, P 450 and the mixed function oxidase revealed that doxorubicin reduced the P 450 contents and the oxidase activity proportionally to its doses (17).

The studies showing that the animals with low antioxidant levels present high activities of peroxide dismutase (18) are indicative of the lipid peroxidation role in the hepatotoxic mechanism. It was proved that doxorubicin reduced retinol and retinol acetyltransferase in the liver by approximately 35% (17).

The studies of the doxorubicin toxicity and pharmacokinetics in the old and young rats showed that the former (24 months) died after the intravenous administration of doxorubicin in the dose of 2.5 mg/kg while the latter (6 weeks) after twice as big dosed (5).

On the basis of the literature one can assume that the hepatotoxic effects of anthracyclines while having less practical significance than the cardiotoxic effects, may result in hepatomegaly and the increased AspAT and AlAT levels.

MATERIAL AND METHODS

The examinations were performed in 40 male Wistar rats with the initial body weight ranging from 200 to 240 g, aged 2.5—3 months. The animals were fed with standard granulated LSM. The feeding stuffs and drinking water were kept in the constant environmental conditions. They were divided into the following groups:

AI — 6 rats which were given i. p. daunorubicin (Cerubidine Lab. Roger Bellon) dissolved in 0.9% NaCl 3 times a week for 5 weeks in the dose of 1.65 mg/kg of body weight up to the peak dose of 25 mg/kg of body weight. The animals were killed 24 hrs after the last dose administration.

AII — The experimental group consisting of 6 rats treated with daunorubicin in the identical way as AI but killed 3 weeks after the last dose administration.

BI — The experimental group consisting of 6 rats in which daunorubicin was given in the same way as in AI and AII but followed the administration of intragastric vitamin E in the dose of 40 mg/kg of body weight up to the total dose of 600 mg/kg of body weight and i.m. vitamin C in the dose of 35 mg/kg of body weight up to the total dose of 500 mg/kg of body weight. The animals were killed 24 hrs after the last doses.

BII — The experimental group consisting of 6 rats treated with daunorubicin, vitamin E and C in the same way as the animals in BI. The animals were killed 3 weeks after the administration of the last doses.

CI — the control group consisting of 2 rats which were given intragastric vitamin E in the dose of 40 mg/kg of body weight three times a week for 5 weeks up to the total dose of 600 mg/kg of body weight. The animals were killed 24 hrs after the last dose administration.

CII — the control group composed of 2 rats treated with intragastric vitamin E in the same way as in CI. The animals were killed 3 weeks after the last dose administration.

DI — the control group consisting of 2 rats treated with intragastric vitamin E in the dose of 40 mg/kg of body weight (total 600 mg/kg) and i.m. vitamin C in the dose of 35 mg/kg of body weight (total 500 mg/kg) three times a week for 5 weeks. The animals were killed 24 hrs after the last dose administration.

DII — the control group composed of 2 rats treated with vitamin E and C in the same way as in DI. The animals were killed 3 weeks after the last dose administration.

EI — the control group composed of 2 rats treated with i.m. vitamin C in the dose of 35 mg/kg of body weight three times a week for 5 weeks (total 500 mg/kg). The animals were killed 24 hrs after the last dose administration.

EII — the control group consisting of 2 rats in which vitamin C was administered in the same way as in EI. The animals were killed 3 weeks after the last dose administration.

FI — the control group consisting of 2 rats treated with i.p. 0.9% NaCl in volume corresponding to the volume used for daunorubicin dissolution. The injections were given three times a week for 5 weeks. The animals were killed 24 hrs after the last dose administration.

FII — the control group composed of 2 rats treated with 0.9 NaCl in the same way as in FI. The animals were killed 3 weeks after the last dose administration.

The animals were decapitated and then exsanguinated. For the biochemical studies the blood was collected from the cervical vessels. Directly after the killing, the liver was dissected, weighted and macroscopically evaluated. Then the specimens were collected for the examinations under the light microscope. The specimens for the histological examinations were fixed in 10% phosphatic buffer formalin and embedded in paraffin. Next they were cut into the 10 μm thick-sections and stained with hematoxylin and eosin.

The histoenzymatic specimens were fixed in the Backer's fluid at 4°C and after the 24 hrs fixation on freezing microtome cut into the 10 μm thick-sections and the histoenzymatic reactions for the presence of acid phosphatase and alkaline phosphatase (AP) (the Gomori method with Vorbrott modification) and for adenosinetriphosphatase (ATP) (according to Wachstein and Meisel) were performed. Moreover, the fats were stained with Sudan IV.

The histological and histoenzymatic photographic documentation was carried out by means of Axioplan microscope with the automatic microphotographic attachment, MC 80 (Opton). The biochemical examinations were performed in AI, BI, AII and BII. In the remaining groups (CI, CII, DI, DII, EI, EII, FI, FII) treated as the controls the same examinations were carried out. The serum levels of asparate transaminase (AspAT), alanine transaminase (AlAT), alkaline phosphatase, acid phosphatase, bilirubin and total protein were determined. The examinations were performed in the Central Laboratory of the Teaching Hospital No. 4 in Lublin. In the statistical analysis of the results concerning the serum levels of enzymes, bilirubin and total protein in the control group C and the experimental AI and BI the following basic values were calculated: arithmetical mean, standard deviation and confidence intervals. The values are compiled in Table 1.

Table 1. Analysis of biochemical results

Parameter studied	Group type	Mean value ± standard deviation	Confidence intervals	Values of test function F	Significance
AlAT	K	73.0 ±2.3	24.3—121.7	0.596	$p=0.564$
	AI	108.2 ±33.2	59.5—156.9		
	BI	88.3 ±21.4	39.6—137.0		
AspAT	K	161.3 ±12.7	52.2—270.5	5.736	$p=0.141$
	AI	400.8 ±52.0	291.7—510.0		
	BI	326.8 ±70.7	217.7—436.0		
Alkaline phosphatase	K	22.7 ±1.1	19.6—25.8	42.485	$p=0.000$
	AI	5.22 ±0.7	2.2—8.3		
	BI	8.15 ±2.1	5.1—11.2		
Acid phosphatase	K	8.53 ±0.7	6.8—10.3	0.548	$p=0.592$
	AI	7.4 ±1.2	5.3—9.5		
	BI	7.5 ±0.9	5.6—9.4		
Total protein	K	6.4 ±0.6	5.9—6.9	6.579	$p=0.008$
	AI	5.3 ±0.3	4.8—5.7		
	BI	5.6 ±0.3	5.1—6.1		
Bilirubin	K	0.15 ±0.09	0.07—0.37	4.570	$p=0.028$
	AI	0.6 ±0.07	0.37—0.82		
	BI	0.33 ±0.14	0.1—0.56		

The statistical conclusions were based on the variance analysis in the single classification and on the group comparisons taking into account the confidence intervals. The difference significance was determined taking account the level $p < 0.05$ (86). The calculations were performed on IBM PC computer in the Department of Mathematics and Medical Biostatistics, Medical Academy in Lublin.

RESULTS

During the studies the control animals were environment reactive and presented normal appetite. At the end of examinations the animals in the experimental groups AI, AII and BI, BII became drowsy and indifferent, reacted more poorly to the external feeding stuffs than the controls. Contrary to the controls, the experimental animals showed the subcutaneous tissue oedema and the transudates to the pericardial sec, the pleural and peritoneal cavities. Most common were the transucdates to the peritoneal cavity.

The livers of the control and the experimental rats showed no significant differences in the microscopic picture and in all groups were of similar reddish-brownish colour and smooth, shiny capsule. The liver mass was characterized by the slight individual variation within the norm limits, statistically insignificant changes between the individual experimental groups and the control groups. Since the morphologic liver pictures in the animals receiving vitamin E, vitamin C or vitamin E with C or i.p. 0.9% NaCl were similar both in rats killed 24 hrs and 3 weeks after the last dose of daunorubicin, all groups will be discussed together.

In each control group the lobule structure was well preserved, the hepatocytes were clearly contoured and formed quite regular trabeculas. The hepatocytes nuclei were rather big with regular contours and quite regularly distributed chromatin, often with 1 or 2 nucleoli. Most hepatocytes presented one nucleus, sometimes two or more. The cell cytoplasm showed thick basophilic granules regularly located in all zones of the liver lobule (Fig. 1).

In the sinus lumen a small number of erythrocytes was found. The ependymal cells were flattened and Browicz-Kuffer cells poorly visible. The staining with Sudan IV showed some fine fatty drops.

Group AI — The general architectonics of the lobules was well preserved, multilateral parenchymal cells in the liver showed slight contour blurring but formed regular trabeculas. In most hepatocytes one or more nuclei were found, bigger than the nuclei in the control groups with clearly scattered chromatin (Fig. 3). The increased nucleolus number was often observed. The hepatocyte cytoplasm in H + E staining was distinctly acidophilic and microgranular in all lobule zones (Fig. 2). The sinus lumen was narrowed, the ependymal cells flattened and Browicz-Kuffer cell enlarged and indented to the sinus lumen. Occasionally, the dead cell remains were found in the sinus lumen.

Group BI — The microscopic picture of the liver in rats treated with daunorubicin and vitamin E and C was basically similar to the pictures observed in AI. Only the cytoplasm acidophilia was weaker, especially in zone I where occasionally some thick basophilic granules corresponding to the unchanged intraplasmatic rough net were found.

Group AII — The picture was not essentially different from the picture in AI. The basophilic granules only rarely filled the hepatocyte cytoplasm. The nuclei were enlarged and "activated". The number of Browicz-Kuffer cells was increased.

Group BII — The picture was similar to the one observed in control groups except for the slight nucleus enlarged and the increased number of Browicz-Kuffer cells both in the central and the peripheral zone (Fig. 4).

The histoenzymatic results in all control groups were very similar. In controls the activity of acid phosphatase was observed near the biliary canaliculi and in Browicz-Kuffer cells. The single granules showing the positive reaction were also found in some other parts of the hepatocyte cytoplasm, however the reaction was significantly less intensified. The reaction intensity was most strongly marked in the lobule peripheral (Fig. 5), least-in the central zone.

In AI smaller number of the acid phosphatase positive granules was found, especially in the hepatocytes of the lobule central zone. Moreover, the decreased intensity of the acid phosphatase reaction was observed in Browicz-Kuffer cells (Fig. 6).

In BI the picture was similar to that in AI. However, it was slightly more intensive.

Group AII — the activity normalization of acid phosphatase reaction was visible in the picture which did not differ from the pictures in the control groups.

Group BII — the activity normalization of the acid phosphatase reaction was not found which was more significant than in AII (Fig. 7).

In controls the alkaline phosphatase activity was localization in the biliary canaliculi, its intensity being the highest in the lobule peripheral zone. The most intensive reactions were observed in the epithelial cells of the biliary canaliculi. Compared to the control groups no significant changes in the AP reaction intensity or its localization were found in each of the experimental groups. Similarly to the AP reaction, the ATP reaction was observed in the epithelial cells of the canaliculus hepatocyte surface in all control groups. This reaction in AI and BI was slightly increased, especially in the peripheral lobule zone which in AII aud BII was not different from the picture in the control groups.

The statistical analysis concerning the serum levels of AspAT proves the significantly higher values in the above mentioned groups which is mostly affected by the high levels in group AI. On the basis of the confidence intervals no significant differences between the enzyme levels in AI and BI were found.

However, significantly higher levels were observed in group BI compared to the control group. The analysis of the results concerning alanine transaminase (AlAT) does not reveal any significant differences. The highest AlAT level was found in AI, the lowest in the control group. However, the increased results both in AI and BI do not allow to conclude about the proper rise of these levels in the experimental groups compared to the control group.

The calculations concerning the alkaline phosphatase levels in serum allow to prove the significantly lower levels of this enzyme in AI and BI ($p < 0.001$) in comparison with the control group. However, there is no significant difference between AI and BI.

The variance analysis applied in the examinations of the differences in the serum acid phosphatase levels shows no relevant changes. The highest level was found in the control group, slightly lower in the groups A and B.

Compared to the control group the total protein levels are significantly reduced both in AI and BI ($p < 0.01$). The difference between AI and BI is significant. However, it can be noticed that the total protein level in AI is slightly lower than in BI, i.e. slightly higher reduction is achieved when nothing but daunorubicin is administered.

Analysing the values of the bilirubin levels the significant increase of these levels ($p < 0.05$) is observed in AI compared to the control group, although comparing BI and the control group no significant differences are found in the increased bilirubin levels. However, the significantly higher bilirubin level is observed in AI compared to BI ($p < 0.05$). Considering the average bilirubin levels we find twice higher average bilirubin level in AI in comparison with BI and about 4 × higher in comparison with the control group. The average values are 0.6, 0.33 and 0.15 mg%, respectively. The detailed results are presented in Table 1.

Compared to AI the decreased bilirubin levels to the average value of 0.4 mg%, the AspAT levels to 300 u, AlAT — to 80 u and total protein — to 5.22 g% were found in AII animals while the levels of phosphatases were slightly higher — acid phosphatase 7.5 u, alkaline phosphatase 15.2 u. In BII animals some further normalization of the biochemical results was found. The average level of bilirubin was 0.25 mg%, alkaline phosphatase — 19.5 u, acid phosphatase — 8.0 u and were similar to the levels in the control group. The average levels of AspAT were 290 u, of AlAT 76 u and of protein 5.43 g%.

DISCUSSION

The simultaneous administration of tocopherol and ascorbic acid resulted from the observations concerning the synergistic actions of both vitamins in the process inhibiting the peroxidation of the lipids which are the components of the membraneous cell structures (6, 10). Moreover, the report showing that vitamin

C, thanks to its high oxidation-reduction potential causes the constant tocopherol transfer to its reduced i.e. active form, was of great interest (6, 15). The morphological changes revealed under the light microscope in the animals treated with daunorubicin alone and with daunorubicin administered with vitamin E and C were characterized by the increase of the nucleus size and the number of nucleoli, by the cytoplasm acidophilia and the activation of Browicz-Kuffer cells which indented into the sinus lumen. The changes observed under the light microscope show the damage of the intraplasmatic rough loss of cytoplasm basophilia was found. The basophilia was replaced by the intensive acidophilia with numerous fine granules corresponding to the increased mitochondria. The degranulation of the intraplasmatic rough net according to Popper (14) is connected with the decreased synthesis and secretion of the protein produced by hepatocytes, including enzymes with which, in turn, the decrease of the acid and alkaline phosphatase levels observed in the animals treated with daunorubicin is related.

The liver sinus region was also the place where the daunorubicin-induced morphological changes appeared. The swollen and stimulated Browicz-Kuffer cells are of great significance in the protective reactions of the organisms which were most often observed in AI. The intensity decrease of acid phosphatase reaction found in AI animals may be the evidence of the decreased synthesis of this enzyme caused by daunorubicin.

The partial normalization of the biochemical results in BI compared to AI speaks for the profitable effects of tocopherol and ascorbic acid on the daunorubicin-induced hepatotoxicity. Further normalization of the biochemical parameters in AII and especially in BII allows to assume that the observed changes which in BII three weeks after the last dose of daunorubicin were morphologically similar to the changes in the control groups, are adaptative and reversible in character.

The maintenance of almost unchanged AspAT levels in all experimental groups proves the cardiotoxic effects of daunorubicin (4, 16) while the decrease in the serum total protein level speaks for the appearance of drug-induced nephrotic syndrome. The biochemical examinations showed the daunorubicin effect on the enzyme action in hepatocytes as well as in blood serum. The morphological changes observed in hepatocytes are characterized by: the changes in nucleus which are most probably the result of the complexes formed by daunorubicin with DNA (2) and the significant mitochondrium damage often exceeding the limits of the reversible changes. Moreover, there were changes in the intraplasmatic rough net showing the disorders in the protein synthesis and secretion expressed in the basophilia loss in cytoplasm hepatocytes. The less intensive morphological changes in the animals treated with vitamin C and E allow to assume that daunorubicin not only disturbs the nuclein acid synthesis but also damages the structural lipids of hepatocytes.

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EXPLANATION TO FIGURES

Fig. 1. The control group. The central zone of hepatic lobule. Basophil granules of intraplasmatic rough net clearly visible. H + E staining. Magn. 200 ×.

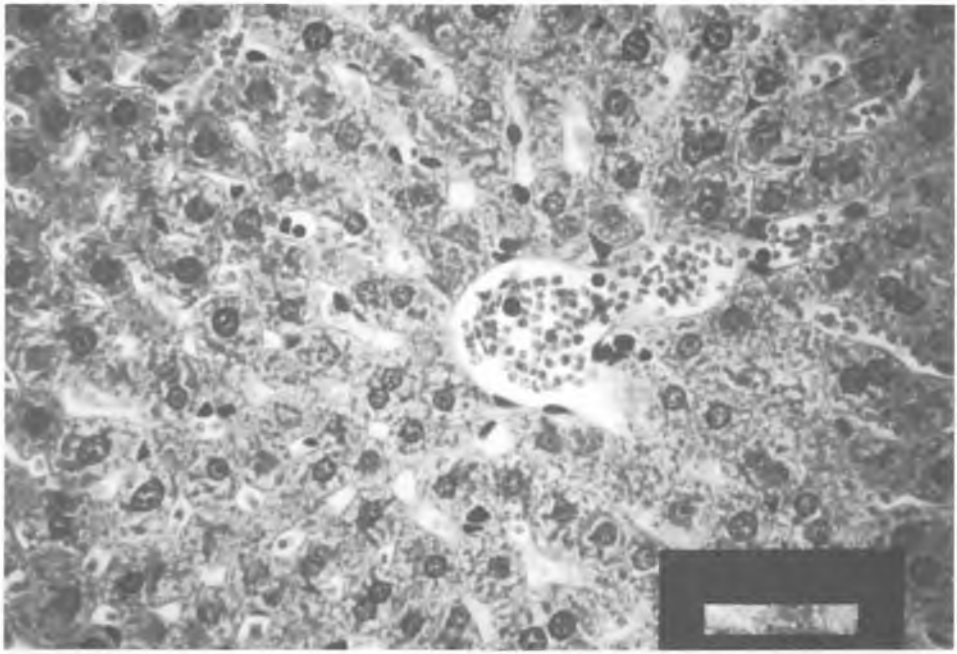

Fig. 1

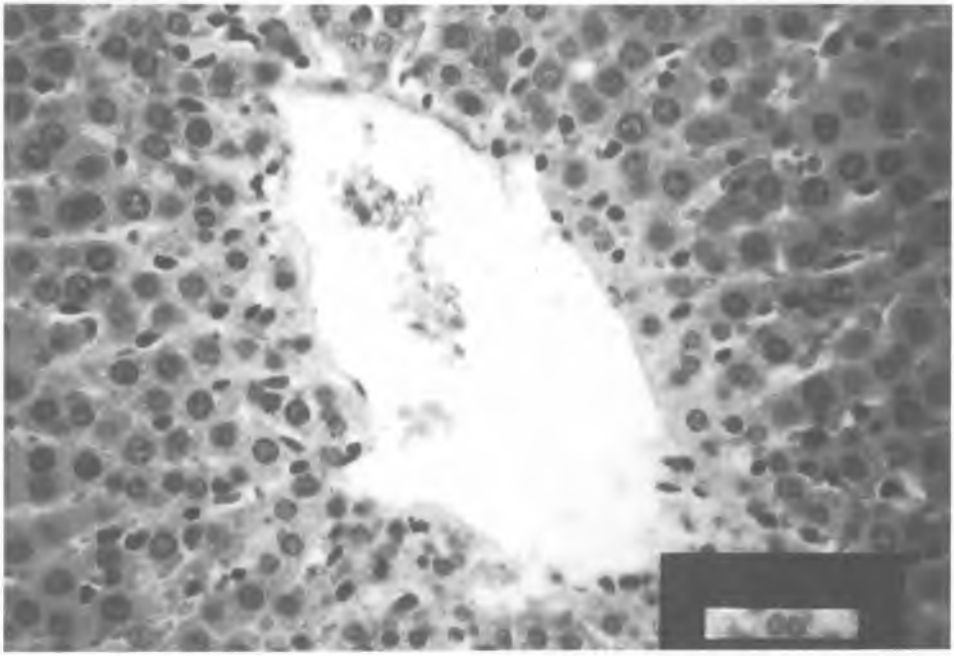

Fig. 2

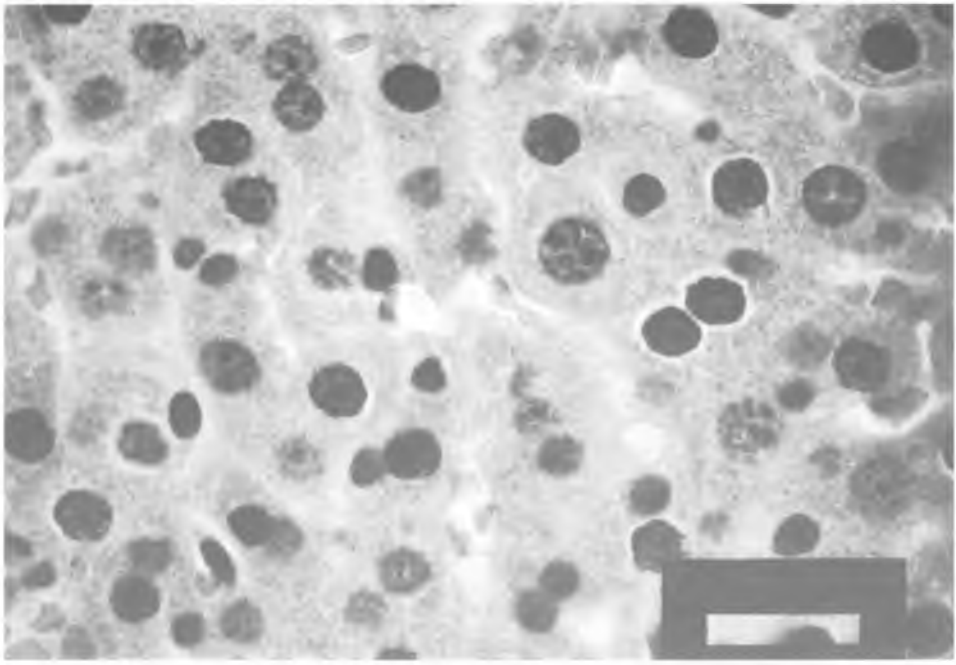

Fig. 3

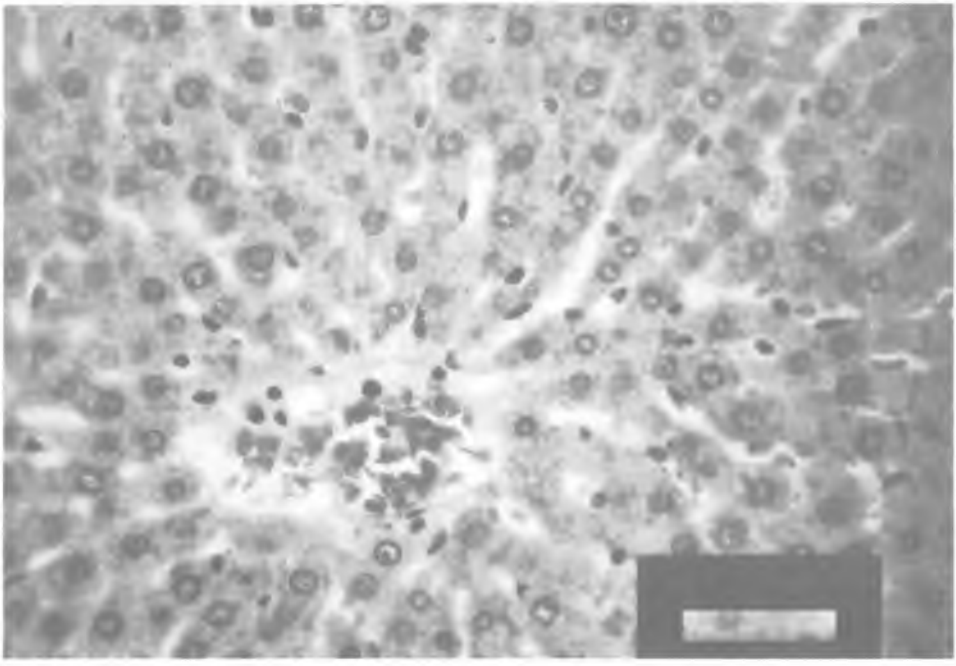

Fig. 4

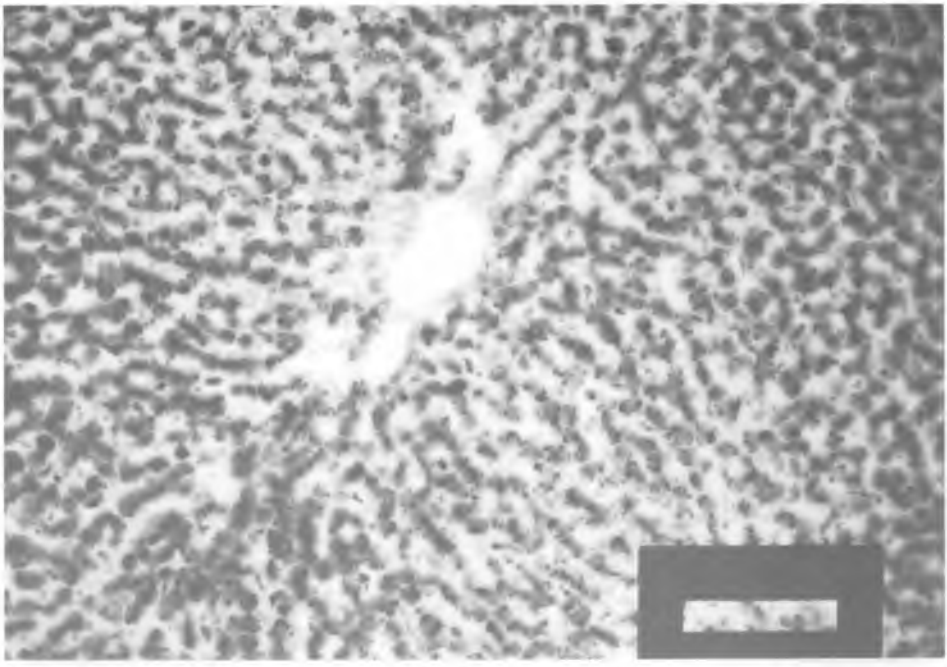

Fig. 5

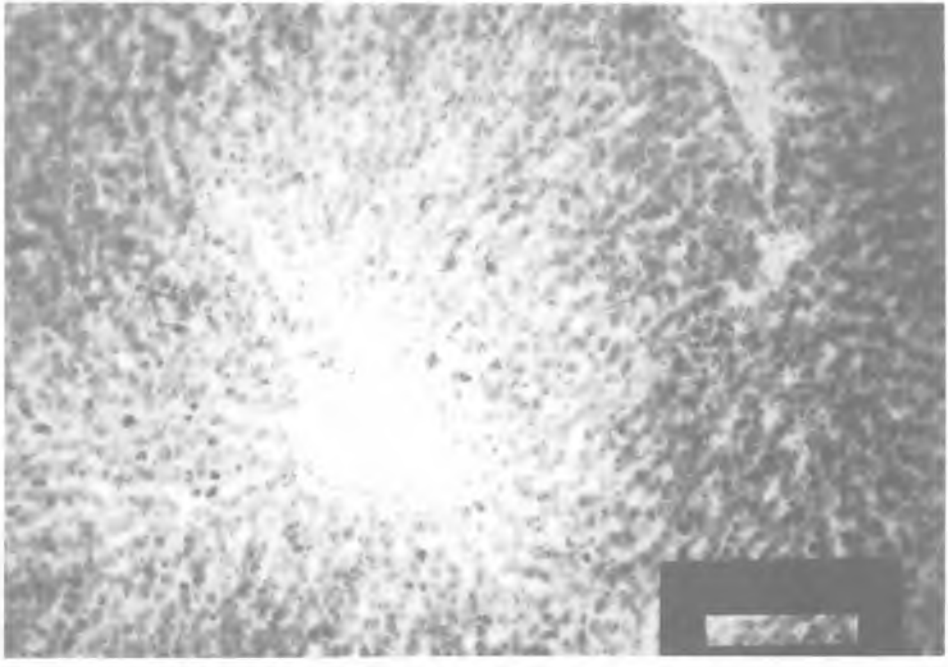

Fig. 6

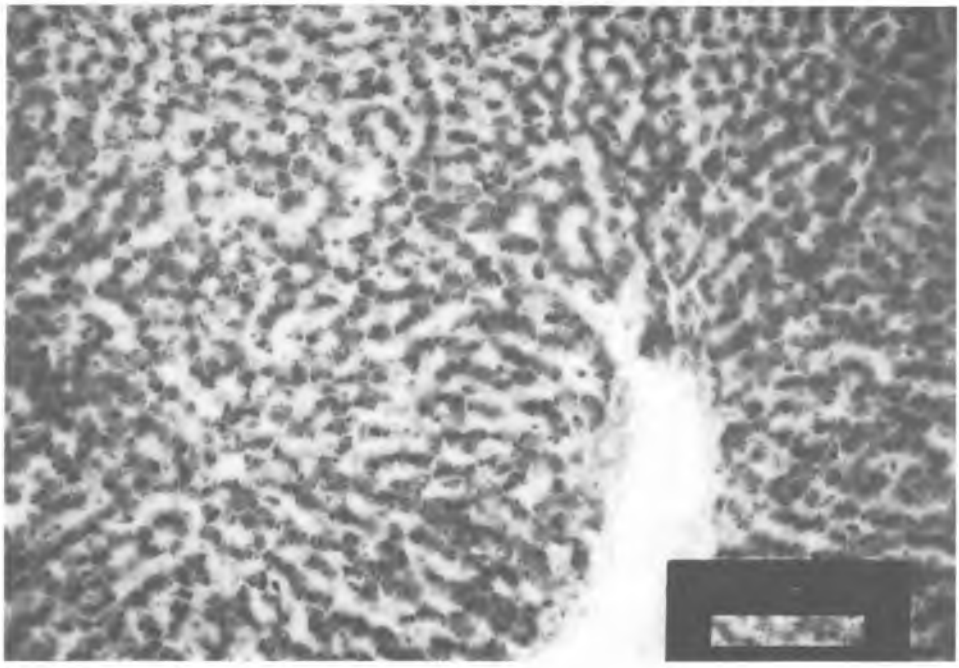

Fig. 7

Fig. 2. The experimental group AI. The central zone of hepatic lobule. Complete lack of intraplasmatic rough net. The hepatocyte nuclei of various size, narrowed lumen of sinus vessels. H + E staining. Magn. 200 ×.

Fig. 3. The experimental group AI. The central zone of hepatic lobule. Numerous acidopil granules in hepatocyte cytoplasm and variously shaped nuclei. H + E staining. Magn. 400 ×.

Fig. 4. The experimental group BII. The central zone of hepatic lobule. Numerous basophil granules of intraplasmatic rough net. Normalization of the sinus vessel lumen. H + E staining. Magn. 200 ×.

Fig. 5. The control group. The central zone of hepatic lobule. Distinctly positive reaction near the biliary capillaries and Borowicz-Kupffer cells. The acid phosphatase reaction. Magn. 200 ×.

Fig. 6. The experimental group AI. The central zone of hepatic lobule. Significant reaction decrease near the biliary capillaries, especially in the central lobule zone and the preserved reaction intensity Borowicz-Kupffer cells and party in the peripheral zone. The acid phosphatase reaction. Magn. 100 ×.

Fig. 7. The experimental group BII. The central zone of hepatic lobule. Positive reaction near the biliary capillaries and Borowicz-Kupffer cells of the intensity similar to the one observed in the control group. The acid phosphatase reaction. Magn. 100 ×.

STRESZCZENIE

Badania wykonano na 40 szczurach szczepu Wistar, którym podawano daunorubicynę w dawce odpowiadającej przeciętnej dawce stosowanej u ludzi. Analizowano zmiany histologiczne i histochemiczne w hepatocytach oraz badano wpływ daunorubicyny na poziom w surowicy krwi transaminaz: alaninowej i asparaginowej, fosfataz: zasadowej i kwaśnej oraz bilirubiny i białka całkowitego. Analizowano także działanie ochronne tokoferolu (witaminy E) i kwasu askorbinowego (witaminy C) na powstałe zmiany morfologiczne i biochemiczne. Przeprowadzone badania umożliwiły wyciągnięcie wniosków. Zauważono że nasilenie wykładników hepatotoksyczności daunorubicyny jest mniejsze u zwierząt, które równocześnie otrzymywały witaminę E i witaminę C. Pozwala to przypuszczać, że te antyoksydanty wywierają pewien ochronny wpływ na hepatocyty. Natomiast normalizacja zmian po 3 tygodniach od podania ostatniej dawki leku przemawia za tym, że większość zmian ma charakter odwracalny i nie prowadzi do przewlekłego uszkodzenia wątroby.