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Influence of interferon-alpha2b on hepatocytes regeneration in periportal and perivenular zones of liver lobules after partial hepatectomy in rats

Wpływ interferonu alfa-2b na regenerację hepatocytów w regionie wrotnym i okołocentralnym u szczurów, po częściowej hepatektomii

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Key words
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Słowa kluczowe
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Summary

Introduction. The liver is one of few organs in mammals with the ability to regenerate. However, there are morphological and functional differences and also different susceptibility of hepatocytes to harmful factors depending on their location in relation to blood vessels. Interferons (IFNs) are antagonists of growth factors for somatic and hematopoietic cells. The system of interactions among particular morphotic elements and chemical factors is very complex and that is why it is impossible to use in vivo pattern of the process of regeneration under in vitro conditions.The aim of the study is the evaluation of the long-term influence of two successive doses of IFN on proliferating hepatocytes in heterogenic perivenous and periportal areas of liver lobules in rats.

Aim. The aim of the study was the evaluation of the long-term interferon influence on proliferating hepatocytes with respect to periportal and perivenular lobular zone in rats.

Material and methods. Sixty male Wistar rats, three-month-old were divided into three groups of 20 animals each. The first group (group A) was injected with normal saline, 24 hours later 70% hepatectomy was performed and after the next 24 hours the second dose of normal saline was administered. A similar schedule was applied in the second group (group B) injected with interferon (INF)-α2b instead. The third group (group C) was injected with IFN-α2b and sham operated. Then the rats were killed at 48, 72, 96 and 120 hours after the surgery. The liver sections were stained with hematoxylin and eosin in order to perform a morphological and morphometric assessment and silver-stained for nucleolar organizer regions (AgNORs). Nucleolar organizer regions are segments of DNA that transcribe to ribosomal RNA and are situated on short arms of the acrocentric chromosomes 13, 14, 15, 21 and 22. The number of NORs is related to the cell cycle and the quantity of interphase NORs increases in cycling cells from the early G1 phase to the late S phase.

Results. The results showed the inhibitory influence of interferon on proliferating hepatocytes after partial hepatectomy, with the maximum after 48 hours. Analysis of mean values of the hepatocyte nuclear area for cells shows larger nuclei in periportal cells at 48 hours after PH, but larger nuclei in perivenous cells at 96 hours after PH. Higher values of the total area of AgNOR per nucleus were shown in periportal hepatocytes in all time intervals for proliferating cells and compared to non-proliferating cells.

Conclusions. Interferon α inhibits the proliferative activity of hepatocytes up to 72 hours after PH. The reaction of perivenous hepatocytes to interferon is delayed by 48 hours compared to periportal hepatocytes.

Streszczenie

Wstęp. Wątroba jest jednym z niewielu narządów ssaków zdolnym do regeneracji. Stwierdzono, że w zależności od położenia hepatocytów względem naczyń krwionośnych

istnieją istotne różnice morfologiczne i funkcjonalne oraz różna wrażliwość tych komórek na działanie czynników toksycznych. Interferony (IFN) są cytokinami o działaniu antagonistycznym w stosunku do czynników wzrostu dla komórek somatycznych i krwiotwórczych. Interakcje między poszczególnymi elementami morfotycznymi oraz wpływ czynników chemicznych na regenerujące komórki są na tyle skomplikowane, że nie można użyć modelu in vitro do badania procesu regeneracji. Celem pracy jest ocena wpływu dwóch kolejnych dawek IFN na proliferujące hepatocyty w heterogennych obszarach okołonaczyniowych i okołowrotnych regenerującej wątroby szczura.

Cel pracy. Celem pracy była ocena wpływu interferonu podawanego w dłuższym okresie na proliferację hepatocytów przestrzeni okołowrotnej i okołocentralnej regenerującej wątroby szczura.

Materiał i metody. Sześćdziesiąt samców szczura Wistar podzielono na trzy grupy po 20 zwierząt. Zwierzętom grupy A podano 0,9% NaCl, po 24 h przeprowadzono hepatektomię, a po kolejnych 24 h podano kolejną dawkę NaCl. W grupie B postąpiono analogicznie, podając jednak interferon (IFN)-a2b. W grupie C podano IFN-a2b i przeprowadzono zabieg pozorowany. Następnie zwierzęta wszystkich grup były uśmiercane w czasie 48, 72, 96 i 120 h po zabiegu. Preparaty wybarwiono hematoksyliną i eozyną w celu przeprowadzenia oceny morfologicznej, morfometrycznej oraz poddano procedurze srebrzenia białek argyrofilnych (AgNOR).

Wyniki. Wykazano hamujący wpływ interferonu na regenerujące hepatocyty po zabiegu częściowej hepatektomii. Efekt ten jest najsilniej wyrażony po 48 godzinach. Analiza średnich wartości powierzchni jąder hepatocytów wykazuje większe jądra w komórkach przestrzeni okołowrotnej w 48 godzinie po hepatektomii, w kolejnych przedziałach czasowych (w 96 godzinie) większe jądra obserwowano w komórkach okołocentralnych. Zanotowano wyższe wartości sumarycznej powierzchni AgNOR jąder komórek proliferujących przestrzeni okołowrotnej we wszystkich przedziałach czasowych w porównaniu z komórkami nieproliferującymi.

Wnioski. Interferon α hamuje aktywność proliferacyjną hepatocytów do 72 godzin po PH. Regeneracja hepatocytów strefy okołocentralnej pod wpływem interferonu jest opóźniona o 48 godzin w porównaniu do hepatocytów strefy okołowrotnej.

INTRODUCTION

The liver is one of few organs in mammals with the ability to regenerate. However, there are morphological and functional differences and also different susceptibility of hepatocytes to harmful factors depending on their location in relation to blood vessels. The process of regeneration starts in periportal regions and then gradually spreads towards perivenous hepatocytes.

The system of interactions among cytokines, growth factors, hormones, hepatocytes and non-parenchymal cells (1-3) is very complex and that is why it is impossible to use *in vivo* pattern of the process of regeneration under *in vitro* conditions. A partial hepatectomy (PH) by the method of Higgins and Anderson is a pattern of regeneration, which is universally recognized and used in the empirical studies. A removal of 70% of the liver stimulates the process of regeneration and one-third of the liver that is left after PH is sufficient to sustain life after the surgery. In the empirical studies it is assumed as a normally regenerating liver. The system of interactions among particular morphotic elements and chemical factors is very complex and that is why it is impossible to use *in vivo* pattern of the process of regeneration under *in vitro* conditions (4).

Interferons (IFNs) are antagonists of growth factors for somatic and hematopoietic cells. In cell cultures exposed to INF, disturbances in the mitotic cycle are observed – a longer time of transition from G0 and G1 to S phase and prolongation of S phase. A complete inhibition of mitosis was also seen, resulting from inhibition of ornithine decarboxylase synthesis, which is a key enzyme for polyamine and DNA synthesis (3, 4). Administration of two doses of interferon prior to and during PH influences cell proliferation through inhibition of polyamine and liver enzyme synthesis (e.g. ornithine decarboxylase), which are key factors in the process of regeneration (1, 2).

Taking above into consideration, in our study we divided liver cells according to their location in the liver lobule, treating periportal and perivenous hepatocytes as two different population of cells.

AIM

The aim of the study is the evaluation of the long-term influence of two successive doses of IFN on proliferating hepatocytes in heterogenic perivenous and periportal areas of liver lobules in rats.

MATERIAL AND METHODS

The study was performed on 60 three-month-old Wistar rats of mean weight 300 g. The animals were randomly assigned to one of 3 groups, 20 rats each. The rats were maintained on rat chow and water under standard conditions. During PH the left and middle lobe of the liver were removed. The study protocol was approved by the Local Ethics Committee for Animal Experiments of the Medical University of Silesia (No. 1/02; 19.02.2002). In all groups the animals were killed in subgroups of 5 at 48, 72, 96 and 120 hours after the surgery or sham operations (fig. 1).

Group A1 (Hepatectomy + NS)

Each rat was injected subcutaneously with normal saline (NS) in the shoulder girdle region. After 24 hours PH under general anaesthesia was performed. After the next 24 hours the second dose of normal saline was administered. Liver tissue samples were collected during PH and at autopsy.

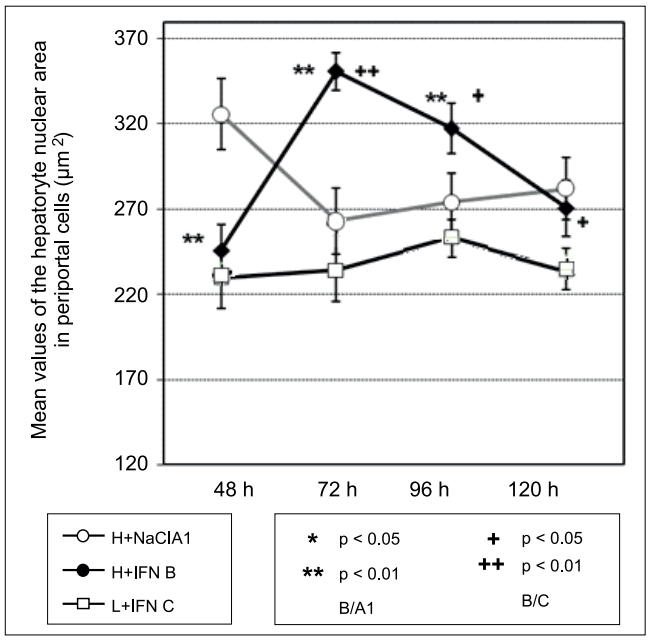

Fig. 1. Values of the hepatocyte nucleat area in periportal cells.

Group B (Hepatectomy + Interferon α)

The animals were injected subcutaneously with the first dose of interferon (Intron A, Shering-Plough, 5 MU/100 ml NS) in the shoulder girdle region. After 24 hours PH under general anaesthesia was performed. After the next 24 hours the second dose of interferon was administered.

Group C (Laparotomy + Interferon α)

The animals were injected subcutaneously with the first dose of interferon (Intron A, Shering-Plough, 5 MU/100 ml NS) in the shoulder girdle region. After 24 hours the rats were anaesthetised with ketamine and sham operated. Sham operations consisted of midline laparotomy and gentle manipulation of the liver in the same manner as resected animals. After the next 24 hours the second dose of interferon was administered.

60 liver tissue samples obtained at autopsy and 40 samples obtained during PH were fixed in 4% formaldehyde solution and embedded in paraffin. At least 6 slides, each containing 6 sections, were prepared from each paraffin block. The slides were stained with hematoxylin and eosin in order to perform a morphological and morphometric assessment by evaluating the area of cells and cell nuclei. The obtained sections underwent also one-step silver-staining for nucleolar organizer regions (AgNOR) by Ploton and colleagues with modifications recommended by the Committee on AgNOR Quantitation within the European Society of Pathology (5). NORs are segments of DNA that encode ribosomal RNA directly related to protein synthesis and cellular proliferation. Active NORs are associated with a subset of specific proteins (C23 and B23), that reacts with silver nitrate (AgNORs), appearing as secondary constrictions in acrocentric chromosomes during mitosis or as black dots in interphase nuclei. Being more numerous in actively proliferating cells, AgNORs quantity increases during G1 phase to reach a maximum during S phase. The amount of AgNOR proteins can be a marker of proliferation, because this amount is related to cell cycle phases the quantification of the nucleolar organizer regions (NORs) reflecting the proliferative capacity of cells or indicating the degree of malignancy.

The data were collected in MS Excel 2000 and analyzed using Statistica 7.1 PL software pack. For each parameter, measures of descriptive statistics were calculated (mean, standard deviation). Then, the Shapiro-Wilk test for normality was performed. Comparison between groups of parameters with normal distribution and parameters with non-normal distribution were performed using Student's t-test and Mann-Whitney U test, respectively.

A comparison of frequency of occurrence of the given classes between groups was performed using a chi-square test. When the assumptions of chi-square test were not met, comparison of contingency tables was performed using Fisher's exact test.

RESULTS

Testing the significance of differences between mean values of measured parameters for perivenous and periportal cells, higher values of the surface area of hepatocytes regenerating under the influence of interferon were found in perivenous cells compared to periportal cells at 48, 72 and 96 hours after the surgery (fig. 1, 2).

The analysis of mean values of the hepatocyte nuclear area in perivenous and periportal cells shows a significant difference between these two groups.

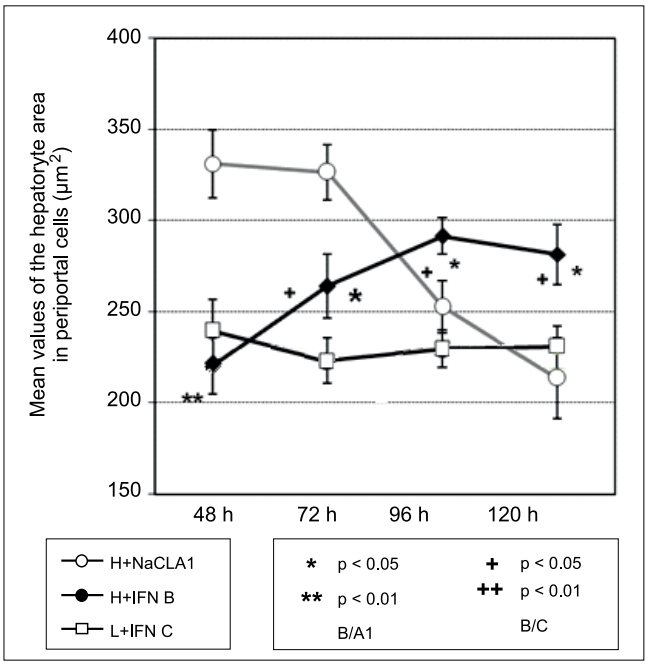

Fig. 2. Values of the hepatocyte area in periportal cells.

Perivenous cells in group A have smaller nuclei at 48, 72 and 96 hours after PH compared to periportal cells. Comparison of the mean values of the estimated parameter for cells in group B shows larger nuclei in periportal cells at 48 hours after PH, but larger nuclei in perivenous cells at 96 hours after PH (fig. 3, 4).

Higher values of the total area of AgNOR per nucleus were shown in periportal hepatocytes in all time intervals for proliferating cells in group A and B and compared to non-proliferating cells in group A0 and C. No differences between non-proliferating cells were found (fig. 5, 6).

DISCUSSION

The results of the observation of liver regeneration suggest that the process of cell division in regenerating liver depends not only on time but also on the location of cells in the liver. A higher proliferative capacity is observed in zone 1 compared to zone 3 of the liver acinus (6). Studies by Segal et al. and by Arber suggest constant proliferation of cells located where the canals of Hering connect with bile ductules. The authors used tritiated thymidine to prove that cells migrate toward the central vein and simultaneously differentiate into mature

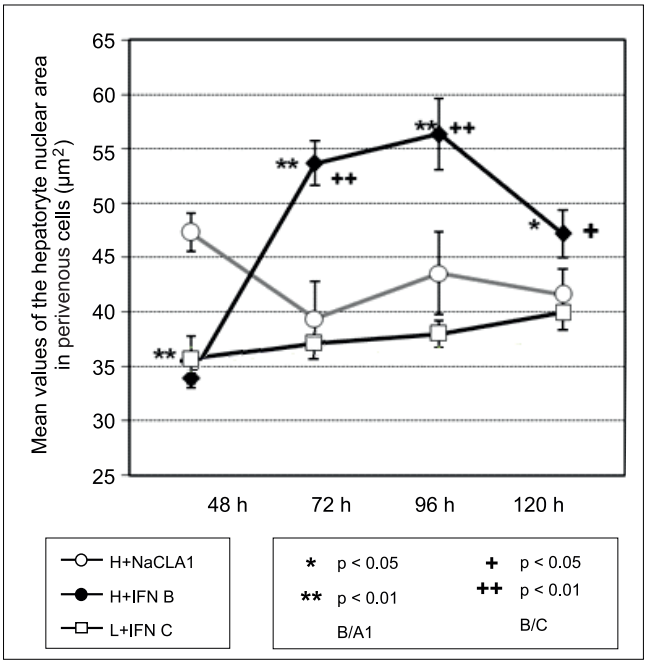

Fig. 3. Values of the hepatocyte nucleat area in perivenous cells.

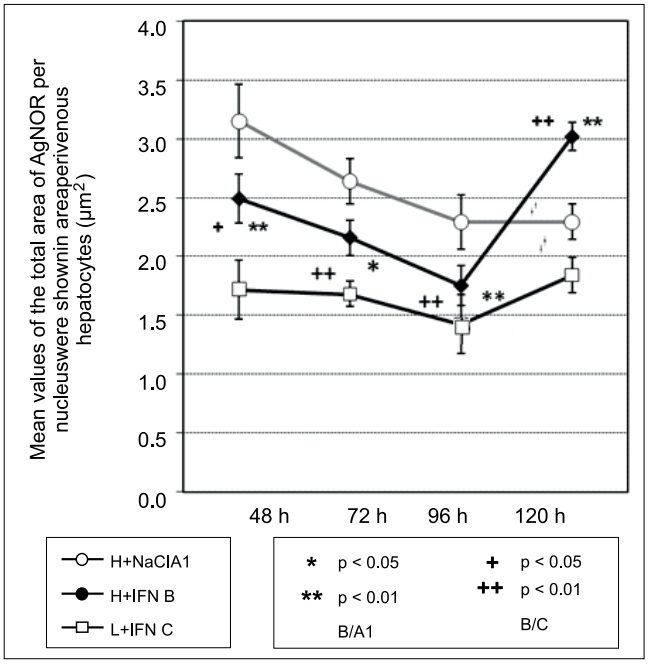

Fig. 5. Values of the total area of AgNOR per nucleus were shown in perivenous.

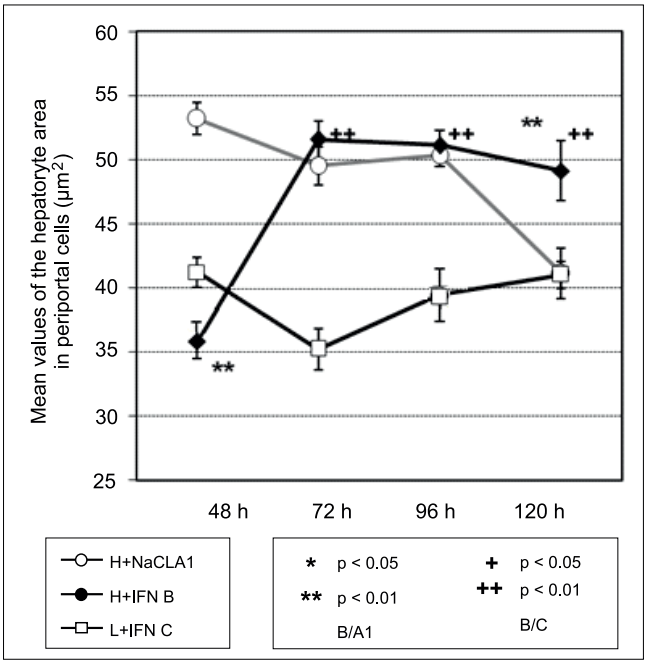

Fig. 4. Values of the hepatocyte nucleat area in periportal cells.

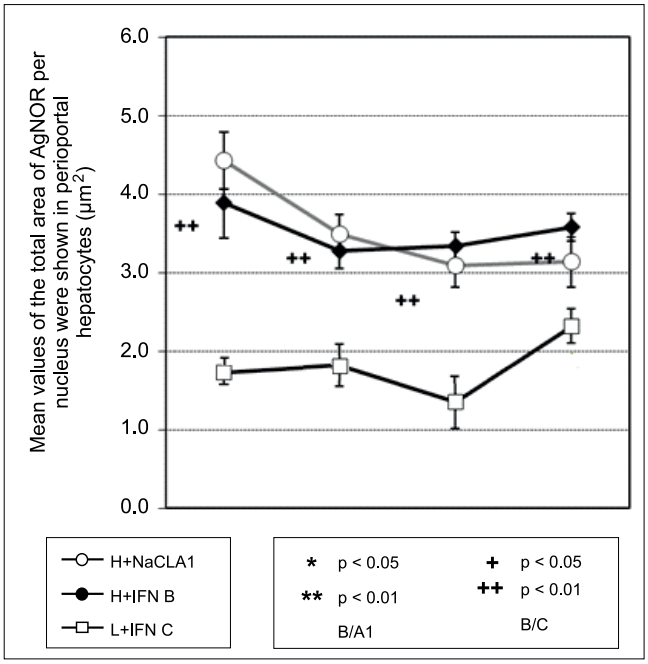

Fig. 6. Values of the total area of AgNOR per nucleus in periportal hepatocytes.

hepatocytes or cholangiocytes (6, 7). Availability of nutritional and regulatory substances like growth factors, hormones and cytokines, is an additional factor differentiating regenerative capacity of liver cells in particular zones of the liver acinus (8, 9). Taking the above into consideration, in our study we divided liver cells depending on their location, treating periportal and perivenous hepatocytes as 2 different population of cells.

The use of interferon in the treatment of chronic viral hepatitis is an efficient method limiting viral replication. The influence of interferon on proliferating hepatocytes depends on the number of doses and time of administration in relation to PH. In some studies only one dose of interferon was administered and the medication was injected before, during or after PH (1, 10-12). In the studies by Wong and Theocharis no influence of interferon on DNA synthesis, polyamide concentration and ornithine decarboxylase activity was observed if the medication was administered prior to or during PH (2, 12). In another study, Theocharis et al. (11) evaluated the influence of interferon injected at 2 or 12 hours after PH on DNA synthesis and observed a significant inhibition of DNA synthesis at 24 hours after the surgery (13). However, in the next time intervals (at 36 and 48 hours after PH) DNA synthesis increased to the levels observed in the control group. It is estimated that administration of one dose of interferon prior to or during PH does not influence DNA synthesis. Injecting the medication in a short time interval after the surgery inhibits DNA synthesis at 24 hours after PH, but does not influence this process in the next time intervals.

The influence of administration of 2 doses of interferon on the protein synthesis was also widely investigated. Interferon was administered in various schedules. Kocić et al. injected the first dose during PH and the next one at 24 hours after the surgery (1, 4). The influence of the medication on the activity of enzymes involved in adenosine metabolism (adenosine deaminase and 5'-nucleotidase) was observed at 48 hours after PH. A significant decrease in adenosine deaminase and an increase in 5'-nucleotidase activity was seen, suggesting antiproliferative effect of interferon. In the study by Favre et al. the first dose of interferon was administered 16 hours prior to the surgery and the second one simultaneously with PH (1). No influence of the medication on the DNA synthesis at 24 hours after the surgery was observed. However, inhibitory influence of interferon on the ornithine decarboxylase expression, an inhibition of total protein synthesis and a decrease in polyamine content in the analyzed tissue were found (1).

The above reports show a significant relationship between the time of administration of interferon in relation to PH and the antiproliferative effect of the medication. Injecting a single dose of interferon prior to or simultaneously with the surgery does not significantly influence DNA synthesis at 24 hours after PH. Administration of two doses of interferon, prior to and during the surgery, influences cell proliferation through inhibition of synthesis of polyamines and liver enzymes, such as ornithine decarboxylase, which are essential in the process of regeneration (1, 2). Administration of two doses of the medication in the above schedule resulted in a significant antiproliferative effect at 48 hours after PH (1, 13). Taking above into consideration, it may be concluded that the number of doses, time of administration of each dose and time intervals in which the observation is done are variables which significantly influence the results of the observation. The optimal schedule of administration of the medication has not been defined. Considering that pegylated interferon is used in the therapy of chronic hepatitis and that its half-life is considerably longer than that of recombinant interferon, it seems that at least 2 doses of the medication in longer time intervals should be administered and that the observation should be longer than 48 hours. The purpose of administration of the first dose of interferon prior to PH is to influence hepatocytes in the G0 phase. The second dose was injected when the process of regeneration had already started. Administration of interferon at 24 hours after PH when the proliferative activity of hepatocytes is the highest suggests the maximal effect of the medication on proliferating liver cells (12, 14). That is why the schedule with 2 doses of interferon was chosen, so that the medication would act on hepatocytes in the G0 phase and on cells that had already started the process of regeneration.

The evaluation of the surface area of hepatocytes showed a significant difference between regenerating cells and hepatocytes in the control group. Differences in cell size between hepatocytes in group B and A1 were also seen. A decrease in the cell size in group B is observed at 48 hours after the surgery and in the next time intervals there is an increase in the cell size in regenerating hepatocytes. In perivenous regions hepatocytes are larger than cells in group A1 already at 72 hours after the surgery, while in periportal regions this process is seen only at 96 hours after PH. These observations are consistent with studies by other authors. The studies by Murray et al. (15) showed a decrease in the cell volume at 14 hours after PH. The authors claim that this is a result of a decrease in the glycogen content in liver cells and of the osmotic changes due to an increased demand for glucose caused by stress after the surgery. Among many other ultrastructural changes, the authors emphasize an increase in the lysosome volume and increase in the number of mitochondria with a decrease in their volume. Other authors confirm a variable size of hepatocytes during normal liver regeneration with a significant increase in the cell volume between 24 and 48 hours after the surgery (16). Studies evaluating the influence of interferon on the size of hepatocytes showed a smaller cell volume in treatment groups than in control groups (17). Authors observed a decrease in the cell volume of regenerating hepatocytes after administration of interferon at 12 hours after PH with a subsequent increase after 24 hours. According to the authors an increase in the cell volume occurs between 6 and 12 hours after the surgery and an increase by 130 to 200% is observed at 24 hours

after PH. On the basis of the results of our study it may be concluded that interferon inhibits the increase in the size of hepatocytes up to 120 hours after PH.

The analysis of the hepatocyte nuclear area in group B in our study shows changes of this parameter compared to the cells in group A1 and A0. The values of the nuclear area in group B were lower at 48 hours after the surgery and they were comparable to those of group C and A0. In the next time intervals there is an increase in the nuclear area of hepatocytes in group B, reaching the maximum at 72 hours in periportal regions and at 96 hours in perivenous regions. These observations are coherent with studies by other authors. Vizzotto et al. showed an increase in the nuclear volume of regenerating hepatocytes at 12 hours after the surgery, with the maximum after 24 hours of regeneration (16). Changes in the morphological structure of nuclei of regenerating hepatocytes are explained by numerous physicochemical processes in cell nuclei. A reduction in the nuclear membrane fluidity resulting from an increase in the sphingomyelin content and a decrease in the phosphatidylcholine concentration in the nuclear membrane was observed already at 12 hours after PH. The nuclear membrane fluidity changes with the cell cycle and is connected with DNA replication. In the next time intervals a gradual increase in the nuclear membrane fluidity is seen as a result of a decrease in the lipid content, mainly cholesterol and sphingomyelin. This process enables the acceleration of mRNA transport from the karyoplasm to cytoplasm. The highest nuclear membrane fluidity is observed between 24 to 30 hours after PH, i.e. just before transition from S to G2 phase. An increase in the nuclear membrane fluidity results in the increased permeability to electrolytes and substrates for DNA replication and therefore an increase in the nuclear volume of replicating cells (16, 17). After administration of interferon a decrease in the nuclear volume of regenerating hepatocytes was seen at 12 hours after the surgery with a subsequent increase in the next time intervals. The highest values were noted at 36 and 72 hours after PH (17). The authors explain it by a decrease in the metabolic activity of the nucleus as a result of the administration of interferon. In our study no differences in the size of non-proliferating cells were found, irrespective of the administration of interferon. By comparing the morphometric parameters of perivenous and periportal cells, we can see a smaller size and higher values of the nuclear area of regenerating cells in periportal regions at 48, 72 and 96 hours after the surgery. In the literature we can find descriptions of the process of the extensive replication of cells in periportal regions without an increase in the extracellular matrix content during the first 3 days after the surgery. During this time lower values of nuclear-cytoplasmic ratio in regenerating hepatocytes is observed. Hepatocytes in periportal regions are smaller than in perivenous regions, whereas only small differences in the nuclear volume of these cells were found. The above results show that the administration of interferon changes the nuclear-cytoplasmic ratio of regenerating hepatocytes up to 120 hours after PH.

Many studies proved that evaluation of AgNOR expression is an excellent indicator of the proliferative activity of the analyzed cells. The number of AgNOR rises together with the increase in the biosynthetic activity of ribosomes. In normal, non-proliferating cells very low rRNA synthesis is observed. It is connected with the presence of a few interphase AgNOR. On the other hand, an increased rRNA synthesis correlates with the presence of a high number of AgNOR. The number of AgNOR dots correlates directly with parameters which are used for the evaluation of the cellular kinetics, like Ki-67, PCNA and the amount of incorporated thymidine or bromodeoxyuridine (18, 19).

In our study the evaluation of the total AgNOR area shows differences between cells in group A and B. Differences between perivenous and periportal regions are also observed. Lower values of AgNOR sum area of regenerating cells after administration of interferon are observed in perivenous regions. An increase in the AgNOR sum area of the cells in group B compared to group A was noted at 120 hours after the surgery. In periportal regions the increase in the AgNOR sum area of the cells in group B compared to group A is seen already at 96 hours. No study using the AgNOR technique for evaluating the influence of interferon on regenerating hepatocytes has been found in the literature. Most studies concern the influence of interferon on the cirrhotic liver and neoplastic processes in the analyzed organs. Azzaroli et al. evaluated the influence of the therapy with interferon on the AgNOR content in the liver in patients treated for chronic viral hepatitis and showed a decrease in the number of AgNOR after the end of treatment (20). The authors explain it by the inhibitory influence of interferon on the cell replication as well as by the inhibition of the inflammatory process caused by a chronic HCV infection (20-22).

Many authors analyze which parameter correlates the most with the proliferative activity of the analyzed cells: the number of AgNOR dots, the area of AgNOR or the mean number of AgNOR dots per nucleus. The study by Skonieczka et al. analyzes the usefulness of the number and area of AgNOR dots in the evaluation of proliferative activity of blastic cells (23). A higher NOR activity in cells stimulated to divide was seen. In cultures of cells which were not stimulated to divide only a small amount of NOR was observed. The next parameter analyzed by the authors was the mean area of AgNOR per nucleus. A distribution of this parameter is similar to that of the number of AgNOR dots. The authors emphasize also the significance of the area of a single AgNOR dot. In other studies higher values of the area of a single AgNOR dot correlated with the higher replication activity of cells (24). In many studies authors prove the usefulness of the evaluation of AgNOR in the analysis of the regeneration of hepatocytes and the influence of various factors on the observed

process. In the studies by Martin et al. (25) AgNOR dots in the oval cells of regenerating mouse liver were counted and compared to the bromodeoxyuridine (BrdU) content and a strong correlation between these parameters was found. Taking the above into consideration, the authors concluded that the number of AgNOR dots reflects the replication activity of hepatocytes.

On the basis of the results of our study and the reports found in the literature, it may be concluded that the evaluation of the AgNOR content in hepatocyte nuclei (the analysis of the total area of AgNOR) reflects the inhibitory influence of interferon on the proliferative activity of liver cells. The influence varies depending on the analyzed region. In periportal regions it is seen in the early phase of the regeneration up to 48 hours after the surgery, while in perivenous regions it is observed in the next time intervals. This phenomenon can be explained based on the studies by Segal et al. The authors proved the proliferation of liver cells in zone 1 of liver acinus where canals of Hering connect with bile ductules and described the migration of the daughter cells toward zone 3 of the liver acinus (7). Zajicek et al. proved with the use of tritiated thymidine that this process has only one direction – from zone 1 toward zone 3 of the liver acinus. The authors suggest that the differences observed between hepatocytes in zone 1 and zone 3 of the liver acinus do not reflect different conditions of microenvironment, but the level of the differentiation and the age of cells (7). The results explain also the higher values of the number and size of AgNOR dots in hepatocytes in the control group and after administration of interferon in periportal regions compared to perivenous regions.

CONCLUSIONS

Interferon α inhibits the proliferative activity of hepatocytes up to 72 hours after PH. The reaction of perivenous hepatocytes to interferon is delayed by 48 hours compared to periportal hepatocytes.

The most common use of IFN is in the treatment of chronic viral hepatitis. IFN type I can improve the underlying liver pathology and reduce the risk of HCC in patients with HBV or HCV infection. However, the effect of IFN in the management of HCC is still controversial, and no clear recommendations have been proposed.

Adjuvant interferon therapy may benefit patients independently of a sustained virological response and further randomised prospective studies would be reassuring to confirm this in different human models.

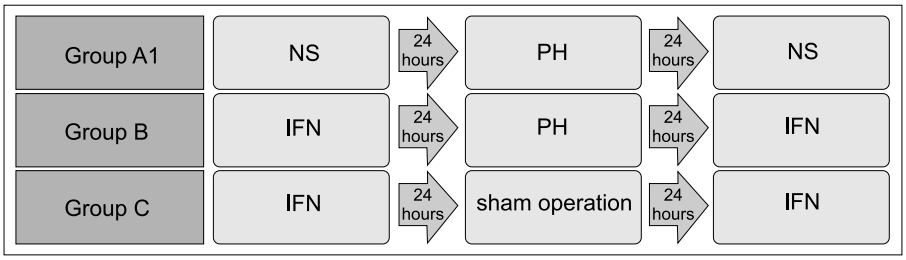

Fig. 7. Animal experiment schedule.
IFN – interferon, NS – normal saline, PH – partial hepatectomy

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