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Does Interferon α2b administration exert an effect on DNA content difference in proliferating hepatocytes of perivenular and periportal zones in rats after partial hepatectomy?

Czy interferon α2b wywiera wpływ na różnicę zawartości DNA w proliferujących hepatocytach strefy okołocentralnej i okołowrotnej u szczurów po częściowym usunięciu wątroby?

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Key words

interferon, liver regeneration, integrated optical density, periportal area, perivenular area

Słowa kluczowe

interferon, regeneracja wątroby, zintegrowana gęstość optyczna IOD, obszar okołocentralny, obszar okołowrotny

Summary

Introduction. The liver is almost unique amongst the tissues of the body in its capacity for regeneration. Multiple studies have been conducted to study the liver regeneration in rodents. The experimental animal model for the study of liver regeneration is a 70% partial hepatectomy in the rat. In the study periportal and perivenular zones are observed separately. The Feulgen reaction is generally accepted as a stoichiometric DNA staining method.

Aim. The aim of the study is the evaluation of the long-term influence of two successive doses of IFN on DNA content in nuclei of replicating hepatocytes and cells under the influence of interferon in periportal zones compared to perivenular zones.

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. All Feulgen-stained sections were divided into anatomical zones: perivenular and periportal. Using morphometric technique, in sequential pairs of sections around each central vein and each portal triad, the area of around 200 000 μm^2 was determined, which defined perivenular and periportal zones, respectively.

Results. This study revealed a higher DNA content in nuclei of replicating hepatocytes and cells under the influence of interferon in periportal zones compared to perivenular zones. The same phenomenon is observed in non-replicating cells.

Conclusions. This study revealed a higher DNA content in nuclei of replicating hepatocytes and cells under the influence of interferon in periportal zones compared to perivenular zones. It may affect some of the liver functions while on interferon therapy as well as increase toxicity of drugs used during antiviral treatment which are metabolised by cytochrome P-45. It is particularly important in cirrhotic patients with the small volume of functional liver tissue.

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Streszczenie

Wstęp. W związku z możliwością naprawy wątroba jest unikalnym narządem organizmu ssaków. Eksperymentalnym modelem zwierzęcym do badania regeneracji wątroby jest zabieg częściowej hepatektomii u szczura. Model ten pozwala także na odrębną ocenę komórek przestrzeni okołowrotnej i okołocentralnej. Barwienie tkanek metodą Feulgena jest powszechnie przyjętym procesem w celu oceny DNA.

Cel pracy. Celem pracy jest ocena długotrwałego wpływu IFN na zawartości DNA w jądrach hepatocytów i replikację komórek pod wpływem interferonu w heterogennych obszarach okołowrotnym i okołocentralnym.

Materiał i metody. Sześćdziesiąt samców szczurów rasy Wistar, zostało podzielonych na trzy grupy, po 20 zwierząt każda. Zwierzętom pierwszej grupy (A) wstrzyknięto roztwór soli fizjologicznej, po 24 godzinach przeprowadzono 70% hepatektomię, po następnych 24 godzinach podano drugą dawkę soli fizjologicznej. Podobny schemat zastosowano w drugiej grupie (B), jednak zamiast soli fizjologicznej podano interferon (INF)-α2b. Trzecią grupę (grupa C) poddano zabiegowi pozorowanemu i podano IFN. Następnie szczury zabijano po 48, 72, 96 i 120 godzinach od zabiegu. Wszystkie skrawki barwiono metodą Feulgena i oceniano uwzględniając położenie hepatocytów względem naczyń (strefa okołowrotna i okołocentralna).

Wyniki. Uzyskane wyniki wykazały wyższą zawartość DNA w jądrach hepatocytów replikujących pod wpływem interferonu w strefie okołowrotnej w porównaniu do komórek strefy okołocentralnej. Takie samo zjawisko obserwowano w komórkach niereplikujących.

Wnioski. Wyższa zawartość DNA w jądrach hepatocytów strefy okołowrotnej replikujących pod wpływem interferonu w stosunku do komórek strefy okołocentralnej może mieć wpływ na niektóre funkcje wątroby podczas terapii interferonem oraz zwiększenie toksyczności leków stosowanych podczas leczenia przeciwwirusowego, które są metabolizowane przez cytochrom P-450. Może to odgrywać szczególną rolę w przypadku zaawansowanej choroby wątroby i małej objętości funkcjonalnej tkanki.

INTRODUCTION

The liver is almost unique amongst the tissues of the body in its capacity for regeneration. It is likely to believe that studies on liver regeneration will contribute to our understanding of pathways governing mammalian organogenesis. The hepatocytes of adult liver divide only rarely under normal conditions. The loss of liver functional mass, leads to rapid proliferation and restoration of functional liver tissue (1). Not only is this knowledge crucial for clinical medicine, but also for the use of interferon and stem cells for therapy of liver disorders. In addition, an increased understanding of the liver regeneration cascade in humans could lead to improved therapies for the treatment of patients with acute or chronic liver pathologies, where the ability to specifically stimulate liver cells would be valuable. These patients suffer from profound liver insufficiency because of the small volume of residual or transplanted liver tissue. Since Higgins and Anderson pioneered the experimental model for the study of liver regeneration in 1931, multiple studies have been conducted to study the liver regeneration in rodents (2). The experimental animal model for the study of liver regeneration is a 70% partial hepatectomy in the rat, because more than 90% of the hepatocytes in the remnant 30% cells are in the process of dividing and the liver mass if fully restored some 7 to 10 days later.

From a metabolic perspective, the functional unit is the hepatic acinus, each of which is centered on the line connecting two portal triads and extends outwards to the two adjacent central veins. The blood flows directly from the portal triad towards the central vein, while in between the portal triads and the central vein, rows of hepatocytes are arranged in single lines with a sinusoid on one side, and a bile canaliculi on the other. Bile is emptied from the hepatocytes into the bile canaliculi and flows in the opposite direction of the blood, i.e. towards the portal bile duct for drainage. The periportal zone I is nearest to the entering vascular supply and receives the most oxygenated blood, making it least sensitive to ischemic injury while making it very susceptible to viral hepatitis. Conversely, the centrilobular zone III has the poorest oxygenation, and will be most affected during a time of ischemia.

Functionally, zone I hepatocytes are specialized for oxidative liver functions such as gluconeogenesis, β-oxidation of fatty acids and cholesterol synthesis, while zone III cells are more important for glycolysis, lipogenesis and cytochrome P-450 and thus most sensitive to toxicity (3). New hepatocytes arise in the periportal area, and then gradually migrate towards the perivenular area. In the study periportal area and perivenular area are observed separately.

Several methods have been employed to quantify nuclear DNA. Some of the studies involved bulk biochemical DNA extraction techniques to estimate the total DNA content of a preparation.

The Feulgen reaction is generally accepted as a stoichiometric DNA staining method, although the chemical processes are only partly understood. Feulgen densitometry relies on the simple premise that the amount of stain bound is directly proportional to the amount of DNA present. The quantity of stain is itself determined based on the amount of light it absorbs. In brief, in a first step, the DNA is submitted to mild acid hydrolysis to split off the purine bases from the double-stranded DNA lobes (4). The result is an apurinic acid presenting reactable aldehyde groups at the C1-position. In a second step, a Schiff's base binds stoichiometrically to these aldehyde groups and produces a reddish to blueish-violet colour with an absorption maximum of 545 nm (5, 6).

The preparations stained DNA content by Feulgen calculated the integrated optical density (IOD) nuclei. The optical density of the transmitted material is a physical quantity equal to the logarithm ratio of the intensity of light falling on the test material (Ip) to the intensity of light after passing the material (Ik) otherwise called absorbance:

$$Dn = \backslash \log (I\_p1/I\_k1).$$

This volume is characterized by the amount of radiation passing through the transmittance material (slide

or negative) or reflected from the material is not transmittance (photography, copy printing). The higher the optical density D, the more absorbing material. In order to measure the optical density of the cell nuclei stained by the Feulgen further assess the background optical density (laboratory slides):

$$Dt = \backslash \log (I\_p2/I\_k2).$$

Calculating the difference between the optical density of cell nuclei studied (Dn) and background (Dt), we obtain the value of the integrated optical density of nuclei:

$$Do = Dn\text{-}Dt.$$

Instead, absorbance (optical density – OD) must be calculated indirectly from measurements of the amount of light passing through the object (transmittance – T). Transmittance, in turn, is measured as the difference between the intensity of incident light entering the object and that of the transmitted light leaving it. In Feulgen DNA densitometry, measurements are taken both within the nucleus and outside the nucleus in a clear area of the slide. The difference in light intensity between the two areas represents the transmittance. It is necessary to take a series of point densities covering the entire nuclear area. The sum of these individual optical densities is the integrated optical density (IOD) of the nucleus.

Feulgen reaction is fully stoichiometric for pure DNA. In the cell nucleus the course of this reaction is conditioned by additional variables such as the morphological structure of chromatin. It is necessary to calibrate the reference amount of DNA contained in the nuclei of control cells. You can calibrate the system using external or internal calibration. External calibration system can be applied to rat hepatocytes or other cells of known DNA content. Internal calibration system are unaffected own cells test subject (5).

For DNA measurements, the Feulgen staining technique was applied as previously described. The nuclei of Feulgen-stained cells were evaluated for DNA ploidy using a Nikon Eclipse E600 microscope (Nikon, Japan) connected with a Panasonic (GP-KR222E) video camera cell measurement software. The nuclear boundaries were defined by the operator by establishing a threshold grey level for each image. A total of 200-300 nuclei were analyzed in each tissue sample. The optical density for each nuclear pixel was measured, and the integrated optical density calculated for every whole nucleus. All images were corrected for the optical density of the background, ensuring stable and reproducible measurements. Cytometry measurements were performed with a magnification of ×100 and calculated automatically according to the algorithms described previously by measuring the nuclear integrated optical density (IOD), representing the cytometrical equivalent of DNA content. The procedure was performed for all nuclei, and the overall mean represents DNA content (6).

AIM

The aim of the study is the evaluation of the long-term influence of two successive doses of IFN on DNA content in nuclei of replicating hepatocytes and cells in periportal zones compared to perivenular zones.

MATERIAL AND METHODS

Sixty three-month-old male Wistar rats were maintained on rat chow and water under standard conditions. The animals were randomly assigned to three main groups, 20 rats each. The first group of animals (A) was injected subcutaneously with normal saline (NS) 0.5 ml. After 24 h, first? (70%) partial hepatectomy was performed according to Higgins and Anderson under anesthesia (ketamine intraperitoneally, 50 mg/kg) by removing the median and left lateral lobes (7). After next 24 h, the second NS was administered. The second group of animals (B) was injected subcutaneously with 0.5 ml IFN-α2b (Intron A, Shering-Plough, 5 MU/100 ml NS). After 24 h, 70%) partial hepatectomy was performed. After next 24 h, the second IFN-α2b dose was administered. The third group (IFN/O) was injected with IFN-α2b and sham operated (laparotomy, gentle manipulation of the liver). In all groups the animals were killed in subgroups of 5 at 48, 72, 96 and 120 hours after the surgery or sham operation.

The excised livers samples were frozen and stored at -80°C. 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).

The thick tissue sections were deparaffinized. Acid hydrolysis was performed in 5 M HCl at 22°C (room temperature) for 60 minutes. Slides were then rinsed under running tap water, stained in Schiff's reagent for 2 hours at room temperature in darkness, rinsed again, and put 3 times into freshly prepared sulfite washing solution (10 ml Na, S, O, 10 ml 1 M HC1, 180 ml distilled water) for a total of 30 minutes, rinsed again (first in running tap water and then in distilled water), finally dehydrated in rising alcohol concentrations followed by xylene, and covered with a coverslip (7, 8).

The image of the nucleus is digitized by a video camera, and the digitized information is relayed to the computer. For each pixel, the amount of light reaching the camera is measured, and this value is converted to OD units, thus assigning an optical density for each pixel.

All Feulgen-stained sections were divided into anatomical zones: perivenular and periportal. Using morphometric technique, in sequential pairs of sections around each central vein and each portal triad, the area of around 200 000 μm^2 was determined, which defined perivenular and periportal zones, respectively.

Each region of interest (perivenular and periportal) was divided into 4 regions: right upper, right lower, left upper and left lower and saved in bmp format (Windows Bitmap Format) to avoid changes in conditions of histological examination (consistent lighting) and viewing the same area a few times.

Statistical analysis

All analyses were performed at a significance level of with the use of the statistical package Statistica 8.0. Descriptive statistics were calculated and data were tested for normality of distribution and homogeneity of variance. For independent samples, differences between groups the analysis of variance and post hoc tests were performed, while for dependent variables t tests for dependent samples were used, with $p < 0.05$ considered significant (10).

The analysis of IOD values of the hepatocyte nuclei in group B perivenular cells shows a decrease at 48 and 72 hours after the surgery compared to group A cells. An increase and the maximum IOD value was noted at 96 hours (fig. 1).

Lower IOD values for group B cells compared to group A cells in periportal zones were also observed at 48 hours after the surgery. However, in the next time intervals an increase in IOD of the cell nuclei was found with the maximum at 96 hours after the surgery. No significant differences in successive time intervals for cell nuclei in non-proliferating hepatocytes (group C) were observed (fig. 2 and 3).

Testing the significance of differences between mean IOD values for hepatocyte nuclei, higher values for group A periportal cells compared to perivenular cells at 48 hours after the surgery were found. In group B lower values of the assessed parameter for periportal hepatocytes compared to perivenular cells were observed. In the next time intervals higher IOD values for periportal zones were noted. Analysing the variance for the assessed parameter in non-proliferating cells, higher values for periportal hepatocytes were found in all time intervals (fig. 2 and 3).

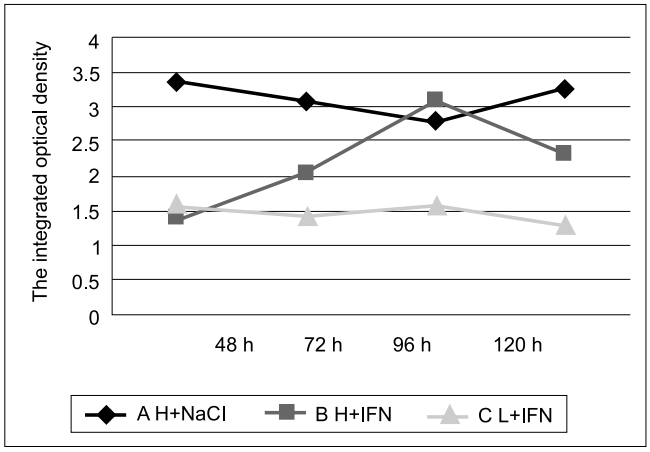

Fig. 2. The integrated optical density (IOD) of nucleus perivenular cells.

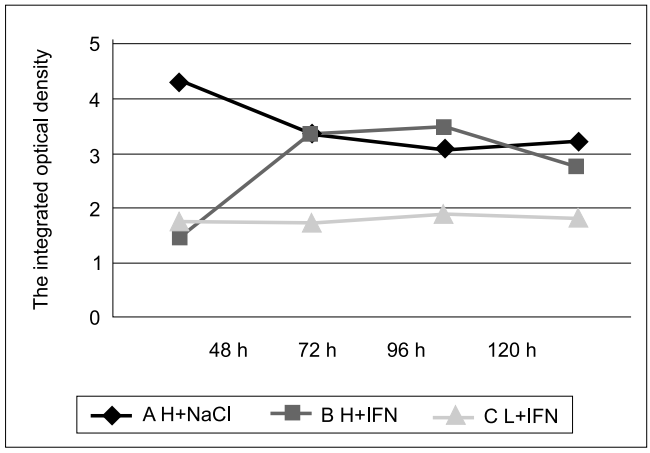

Fig. 3. The integrated optical density (IOD) of nucleus periportal cells.

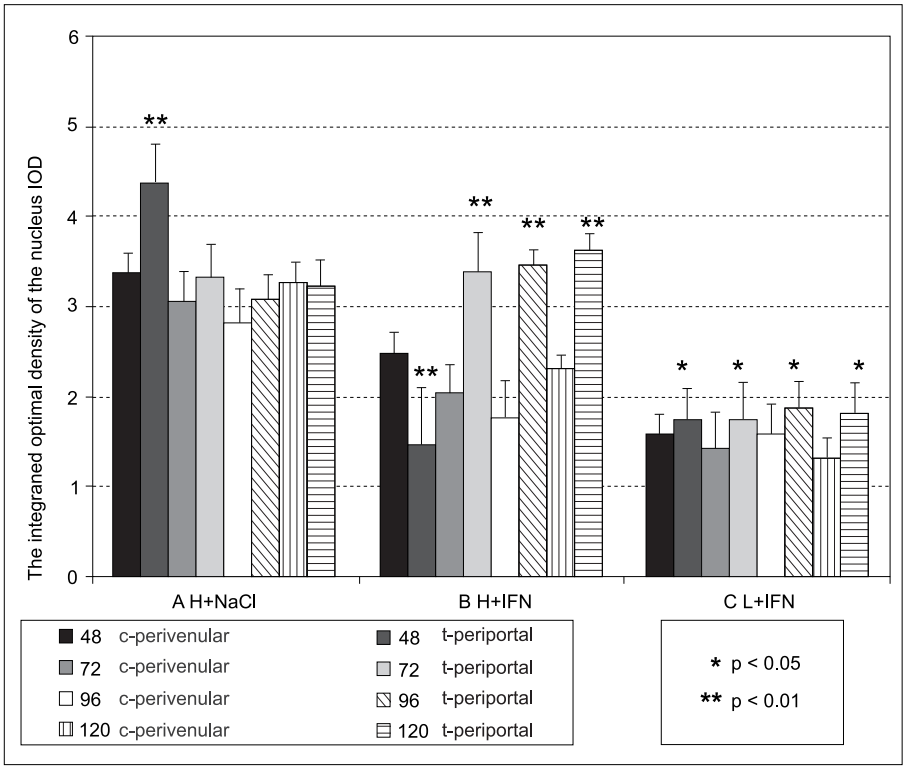

Fig. 1. The integrated optical density (IOD) of the nucleus periportal and perivenular cells.

RESULTS

The analysis of the DNA content in replicating hepatocytes in group A showed higher IOD values in periportal cells only at 48 hours after the surgery. The influence of interferon is noticeable particularly at 48 hours in periportal cells. In the next time intervals it is also observed in perivenular hepatocytes.

DISCUSSION

The analysis of the results of our study shows a decrease in IOD value for perivenular hepatocyte nuclei at 48 and 72 hours after the surgery compared to regenerating cells of group A. At 96 hours an increase and the maximum IOD was noted. Higher values of the assessed parameter were observed in periportal zones compared to perivenular cells. Lower IOD values for the examined group cells compared to proliferating hepatocytes at 48 hours after the surgery were also found. However, in the next time intervals an increase in IOD of the cell nuclei in the examined group was found with the maximum at 96 hours after the surgery. Taking into consideration reports by other authors and the results of our study it may be concluded that interferon administered as described above inhibits DNA synthesis at 48 hours after the surgery (1). In the next time intervals the DNA content in the examined group is increasing to the values found in the control group. No studies comparing DNA content in regenerating hepatocytes in heterogeneous areas of the liver were found. Our study revealed a higher DNA content in nuclei of replicating hepatocytes and cells under the influence of interferon in periportal zones compared to perivenular zones. The same phenomenon is observed in non-replicating cells.

The analysis of the DNA content in replicating hepatocytes in group A showed higher IOD values in periportal cells only at 48 hours after the surgery, so as we can see interferon affects DNA synthesis in regenerating hepatocytes. The influence of interferon is noticeable particularly at 48 hours in periportal cells. In the next time intervals it is also observed in perivenular hepatocytes. It may affect some of the liver functions while on interferon therapy as well as increase toxicity of drugs used during antiviral treatment which are metabolised by cytochrome P-450 (e.g. paracetamol). It is particularly important when using many drugs metabolised by the same cytochrome, especially in cirrhotic patients with the small volume of functional liver tissue.

CONCLUSIONS

Higher DNA content in nuclei of replicating hepatocytes and cells under the influence of interferon in periportal zones compared to perivenular zones may affect some of the liver functions while on interferon therapy as well as increase toxicity of drugs used during antiviral treatment which are metabolised by cytochrome P-450, especially in cirrhotic patients.

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