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INFLUENCE OF PROLONGED TRIPTERELIN-INDUCED CENTRAL DEPRIVATION OF TESTOSTERONE SYNTHESIS ON MORPHOLOGICAL STRUCTURE OF RAT'S LIVER

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In recent years, the incidence of prostate cancer has increased worldwide. For example, in 2012, the GLOBOCAN project found that prostate cancer was the second most commonly diagnosed cancer and the fifth leading cause of cancer deaths among men worldwide. Insufficient testosterone can exacerbate liver damage caused by obesity. The aim of our study was to identify morphological changes in the liver of rats at the tissue and cellular levels, to study the processes of formation of reactive oxygen species and the intensity of lipid peroxidation during prolonged central deprivation of testosterone synthesis caused by triptereline. Triptorelin-induced central deprivation of testosterone synthesis leads to oxidative damage to hepatocytes due to increased production of reactive oxygen species and decreased activity of antioxidant enzymes. Oxidative damage to liver cells begins at the molecular and cellular levels and becomes apparent at the tissue level on day 180 of the central deprivation of testosterone synthesis.

Key words: liver, triptoreline embonate, NO-synthase, superoxide dismutase, malon dialdehyde, superoxide anion radical, rats.

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ВПЛИВ ПРОДОВЖЕНОГО ЦЕНТРАЛЬНОГО БЛОКУВАННЯ СИНТЕЗУ ТЕСТОСТЕРОНА ТРИПТЕРЕЛІНОМ НА МОРФОЛОГІЧНУ СТРУКТУРУ ПЕЧІНКИ ЩУРІВ

В останні роки рак простати збільшується у всьому світі. Наприклад, у 2012 році проект GLOBOCAN показав, що рак передміхурової залози був другим за частотою діагностики раком та п'ятою провідною причиною смертності від раку серед чоловіків у всьому світі. Недостатня кількість тестостерону може посилити пошкодження печінки, спричинені ожирінням. Метою нашого дослідження було виявлення морфологічних змін у печінці щурів, вивчення процесів утворення активних форм кисню та інтенсивності перекисного окислення ліпідів під час тривалої центральної депривації синтезу тестостерону. Індукована трипторелін центральна депривація синтезу тестостерону призводить до окислювального пошкодження гепатоцитів внаслідок збільшення виробництва активних форм кисню та зниження активності антиоксидантних ферментів. Окисне пошкодження клітин печінки починається на молекулярному та клітинному рівнях і стає помітним на рівні тканин на 180-й день центральної депривації синтезу тестостерону.

Ключові слова: печінка, трипторелінoвий ембонат, NO-синтаза, супероксиддисмутаза, малоновий диальдегід, SAR, щури.

The study is a fragment of the research project "Experimental morphological study of cryopreserved placenta transplants and diphereline, ethanol and 1% methacrylic acid action on the morphofunctional status in a number of internal organs", state registration No. 0119U102925.

Prostate cancer has been increasing worldwide in recent years. For instance, in 2012 GLOBOCAN project showed that prostate cancer was the second most frequently diagnosed cancer and the fifth leading cause of cancer mortality among men worldwide. This trend even affected Asian countries like Japan Korea, since the lifestyle of the population of these countries became similar to the Western World [10]. The increased prevalence of prostate cancer leads to economic loses even for the most developed countries. In Sweden, the total annual costs extrapolated to Sweden were calculated to be 281 000 000 € [4]. Therefore, prostate cancer treatment and diagnostic of prostate cancer is an important problem for modern medical science.

One of the approaches to the prostate cancer therapy is either chemical or surgical castration, since testosterone and other androgens are viewed as key risk factors of prostate cancer progression [5]. However, recent scientific discoveries provided a background for reconsideration of this paradigm [9]. The

reason for such shift in opinion being the damage organs and tissues suffer during testosterone deficiency on the background of simultaneous influence of external and internal pathogenic factors. Testosterone deficiency makes brain tissues vulnerable to oxidative damage caused by stress reaction [14]. Insufficient amount of testosterone may exacerbate liver damage caused by obesity [8]. In both abovementioned situations (stress reaction and obesity) the leading role in pathogenesis is taken by increased reactive oxygen species production and decrease in antioxidant defense. The reason for increased damage of tissues during testosterone deficiency may also lie in decreased activity of antioxidant enzymes such superoxide dismutase isoforms [2]. At the same time, it was shown in scientific literature that increased testosterone concentration leads to prostatic hyperplasia through activation of redox sensitive transcriptional factors and oxidative damage [12].

It is still a subject to much debate whether testosterone deficiency may induce pathologic changes in liver tissues without additional pathogenic influences.

The purpose of the study was to identify morphological changes in rat liver on tissue and cellular levels, study the processes of reactive oxygen species production and intensity of lipid peroxidation during prolonged tripterelin-induced central deprivation of testosterone synthesis.

Materials and methods. The experiments were carried out on 20 sexually mature male white rats of the Wistar line. Rats were divided into 2 groups: the control group (5) and the experimental group (15). Animals from the experimental group were injected subcutaneously with diphereline (Triptorelin embonate) at a dose of 0.3 mg of the active substance/ per kg of body weight for 180 days, while the control group received injection of saline [6]. Animals were kept in standard vivarium conditions of the Ukrainian Medical Stomatological Academy. Experimental animals were sacrificed in strict compliance with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes"; (Strasbourg, 1986), as well as with the "General Ethical Principles of Animal Experiments" adopted by the First National Congress on Bioethics (Kyiv, 2001). Animals from experimental group were removed from experiment on the 30^{th} day (n=5), on the 90^{th} day (n=5) and on the 180^{th} day of modelling of central deprivation of testosterone synthesis.

After an overdose of ketamine, the animals were decapitated, the prepared small pieces of the testes were fixed in a 2.5 % glutaraldehyde solution (pH=7.2–7.4). Postfixation of the material was carried out with 1 % solution of osmium (IV) oxide, followed by dehydration in propylene oxide and a sample was embedded into the epoxy resins mixture.

Using standard methods, the material was imbedded in paraffin blocks, of which sections 4 μm thick were made and stained with hematoxylin and eosin[1]. Histological preparations were examined using Biorex 3 light microscope with digital microfilter with software adapted for these studies (Serial No. 5604).

By using the method of standard areas, the images were photographed with magnitude of 400 and 1000, using microscope "Micromed" with the TSView software adapted for monitoring. The volume of the nuclei of hepatocytes was calculated out according to the formula of the ellips: $V = \pi/6\ D \bullet d^2 \bullet$ where V is the volume of the nucleus, d – the length of the small diameter of the nucleus, D – the length of the large diameter of the nucleus.

All biochemical studies were carried out in 10 % homogenate of testis tissue using Ulab 101 spectrophotometer.

Basic production of superoxide anion radical (SAR) was determined by the growth of diformazan concentration, formed in the reaction of SAR with nitro blue tetrazolium [3]. Superoxide dismutase (SOD) activity was determined by inhibition of adrenaline autooxidation, while catalase activity was determined by the amount of hydrogen peroxide, remained after its catalase-dependent reduction [3]. The concentration of free malondialdehyde (MDA) was determined by reaction with 1-methyl-2-phenylindole [11].

Statistical processing of the study results was carried out using the Microsoft Office Excel software and the Real Statistics 2019 extension to it. The nonparametric Mann-Whitney test was used to determine the statistical significance of differences between the groups. The difference was considered statistically significant at $p<0.05$.

Results of the study and their discussion. The reaction to central testosterone deprivation from the liver, and the hepatocytes itself, manifested itself already on the 30th day of the experiment (fig. 1). So, when studying semi-thin sections on the 30th day of the experiment, we found that in general the structure of the liver was preserved, but from the side of the stroma there was a slight increase in its thickness, in comparison with the control. Central veins are slightly dilated, full-blooded in comparison with the control group of animals, in the lumen of which erythrocytes and a small number of leukocytes were determined. Sinusoidal capillaries are mostly not widened, but there are capillaries with an increased inner diameter.

The balk structure is preserved, slightly thickened. The bile ducts are unchanged. From the side of the vessels of the hepatic triads, we did not find any statistically significant changes in comparison with the control group of animals.

From the side of the parenchyma, everything was ambiguous, as most of the hepatocytes were different in structural characteristics. Most of the nucleus cells with symptoms of karyopyknosis, with different density of the nucleus itself. The average value of the area of hepatocyte nuclei was determined as 59.03±1.02 μm^2, which in the control group it was 64.43±1.33 μm^2 (tab. 1). The number of binucleolar hepatocytes decreased in comparison with the control group of animals. Most cytoplasm had granularity. In a small part of hepatocytes, an increase in the volume of cytoplasm was determined in comparison with the control group of animals, which amounted to 10201.07±556.45 μm^3, in control group it was 8323.55±576.22 μm^3.

Table 1

Changes in morphometric parameters of rat liver cells during prolonged central deprivation of testosterone synthesis (M±m)

Parameters	Control group	30 days	90 days	180 days
Mean Area of nucleus, μm2	64.43±1.33	59.03±1.02*	54.31±1.12*	52.33±1.31*
Mean Volume of nucleus, μm3	3412.21±7.32	3082.12±25.33*	2711.98±22.23*	2440.45±20.11*
Mean Area of cytoplasm, μm2	394.66±3.49	441.08±14.33*	502.99±13.66*	518.19±16.33*
Mean Volume of cytoplasm, μm3	8323.55±576.22 *	10201.07±556.45*	12220.23±607.09*	13001.67±599.67*

* – indicates that data is statistically significantly different compared to control group ($p<0.05$).

The 90th day of the experiment was characterized by the fact that destruction of both the stroma and parenchyma was not observed by us. The balk structure is preserved, enlarged, thickened. The bile ducts are slightly dilated, but we did not find statistically significant difference with control. We did not reveal statistically significant changes from the side of the vessels of the hepatic triads. The central veins are slightly dilated, full-blooded in comparison with the control group of animals. Sinusoidal capillaries are slightly dilated (fig. 2).

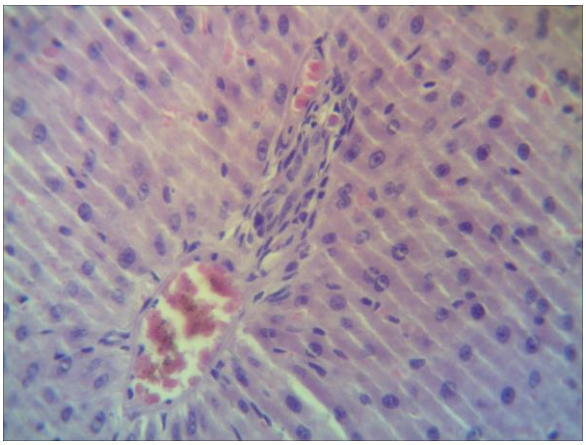

Fig. 1. Central vein of rat liver under conditions of prolonged central deprivation of testosterone synthesis. 30-days. Magnification: Lens x 40, Eyepiece x 10.

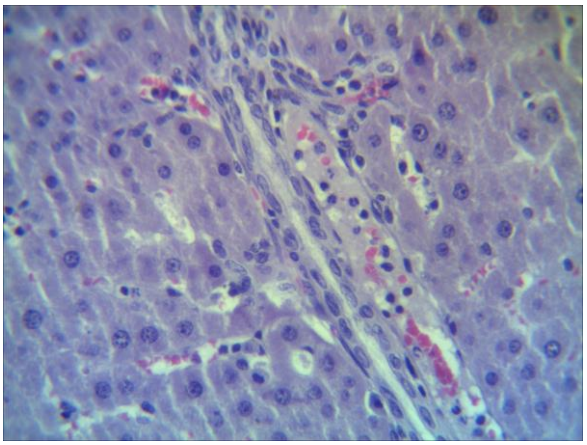

Fig. 2. Central vein of rat liver under conditions of prolonged central deprivation of testosterone synthesis. 90 – days. Magnification: Lens x 40, Eyepiece x 10.

From the side of the parenchyma, we observed that most of the hepatocytes were different in structural characteristics. Most cells had nucleus with symptoms of karyopyknosis, with different density of the nucleus itself. The average value of the area of hepatocyte nuclei was determined as 54.31±1.12 μm2, which, in comparison with the control group and the previous term (30 days) of the experiment, was statistically significant. An insignificant amount of binucleolar hepatocytes was determined in comparison with the control group of animals. The cytoplasm of most hepatocytes had granularity, the volume of which averaged 12220.23±607.09 μm^3.

The 180th day of the experiment was characterized by compaction and an increase in the diameter of the structures were traced in the structure of the stroma. The balk structure of the liver is enlarged, thickened. The bile ducts are preserved, no changes are found. The central veins are dilated, full-blooded in comparison with the control group of animals. Sinusoidal capillaries are dilated and full of blood (Fig. 3).

No parenchymal destruction was observed. The majority of hepatocytes have nuclei with symptoms of karyopycnosis, with different densities of the nucleus itself. The average size of the volume of nuclei decreased and amounted to 2440.45±20.11 μm^3. In all fields of vision, eosinophyia of the

cytoplasm was manifested, with areas of hepatocyte enlightenment with partial vacuolization of the cytoplasm. Most of the hepatocytes showed a significant decrease in the volume of the cytoplasm in comparison with the control group of animals (fig. 4).

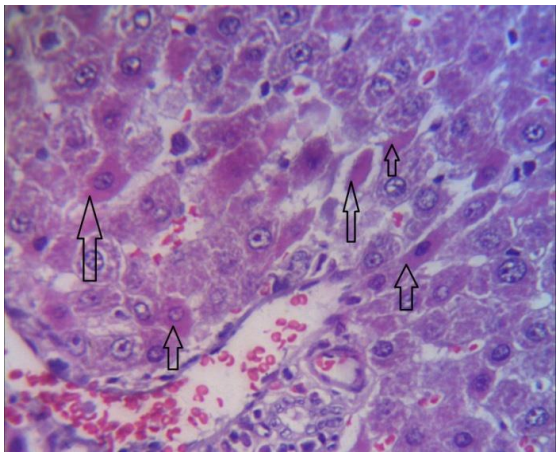

Fig. 3. Central vein of rat liver under conditions of prolonged central deprivation of testosterone synthesis. 180-days. Eosinophilic color of hepatocyte cytoplasm. Magnification: Lens x 40, Eyepiece x 10.

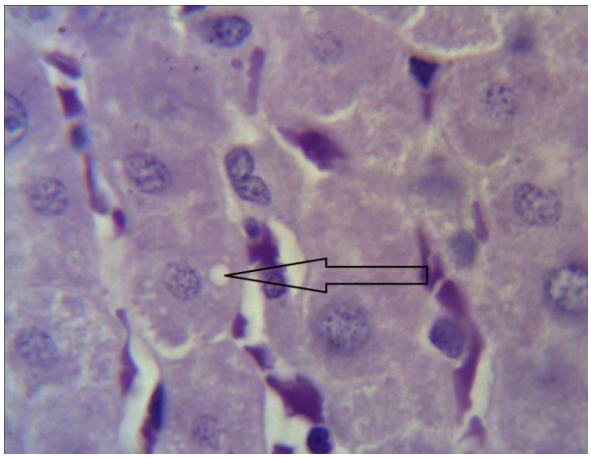

Fig. 4. Cytoplasm vacuolization on the 180th day of experiment. Magnification: Lens x 80, Eyepiece x 15.

Production of SAR increased on the 30th, 90th and 180th day of experimental central deprivation of testosterone synthesis by 19.88 %, 46.02 % and 104.54 %, respectively, compared to control group (tab. 2). SAR production was by 21.80 % higher on the 90th day of experiment compared to 30th day of experiment. On the 180th day SAR production was by 40.07 % higher compared to 90th day. This allowed us to see a tendency for increase of SAR production the longer the central deprivation lasts.

The SOD activity decreased on the 30th, 90th and 180th day of experimental central deprivation of testosterone synthesis by 74.11 %, 67.68 % and 61.96 % respectively compared to control group. There were no differences in SOD activity between the 30th, 90th and 180th day of central deprivation of testosterone synthesis.

Table 2

Changes of oxidative stress biomarkers in rat liver during prolonged central deprivation of testosterone synthesis (M±m)

Parameters	Control group	30 days	90 days	180 days
Production of SAR, nmol/s on g of tissue	1.76±0.05	2.11±0.04*	2.57±0.05*/**	3.60±0.10*/**
Activity of SOD, c.u.	10.12±1.54	2.62±0.61*	3.27±0.44*	3.85±0.36*
Activity of catalase, μkat/g of tissue	0.379±0.001	0.092±0.003*	0.166±0.001*/**	0.189±0.003*/**
Concentration of free MDA, μmol/g of tissue	14.04±0.20	16.77±0.17*	19.11±0.08*/**	24.66±0.20*/**

* – indicates that data is statistically significantly different compared to control group ($p<0.05$).

** – indicates that data is statistically significantly different compared to previous term of experiment ($p<0.05$).

Catalase activity was also decreased at all terms of experiment. On the 30th, 90th and 180th day of experiment its activity was lowered by 74.11 %, 67.68 % and 61.96 % respectively compared to control group. The lowest activity of catalase was observed on the 30th day of experiment. There was a clear tendency for increase of catalase activity with time.

The MDA concentration was increased on the 30th, 90th and 180th day of experimental central deprivation of testosterone synthesis by 74.11 %, 67.68 % and 61.96 % respectively compared to control group. We noticed a clear tendency for increase on lipid peroxidation processes in liver of rats with duration of central deprivation of testosterone synthesis.

Since balk structure of liver was not changed during fist terms of experiment (30th and 90th days) we can state, that changes in the liver during this period mostly occur on cellular and subcellular levels. This statement is proven by increased lipid peroxidation levels and elevated SAR production on the background of decrease of hepatocyte nucleus volume and area. The highest intensity of lipid peroxidation and SAR production was observed on 180th day of experiment, which coincides with the appearance of first signs of structural changes on tissue level. These changes were manifested by enlargement of balk structure and dilation of central vein.

The dilation of central vein observed on the 180th day of experiment may be seen as the result of disruption in nitric oxide (NO) cycle. Testosterone can positively modulate activities of endothelial and

neuronal nitric oxide synthases, therefore testosterone deficiency may decrease their activity [13, 15]. Decreased activities of constitutional isoforms of NO-synthases may lead to their uncoupling and be the reason for increased SAR production observed in our study.

At the same time, testosterone is a potent inhibitor of nuclear factor kappa B (NF-kB) activation [7]. Testosterone deficiency caused by triptorelin administration performed in our study may not be the cause of NF-kB activation directly. However, development of oxidative stress due to uncoupling of constitutive isoforms of NO-synthases may lead to NF-kB activation and increase the activity of inducible isoform of NO-synthase. Increased activity of inducible isoform of NO-synthase can explain the dilated blood vessels observed in our study. Further studies are necessary to evaluate role of NF-kB, inducible isoform of NO-synthase and to identify sources of SAR production in development of morphological and metabolic changes in liver during prolonged central deprivation of testosterone synthesis.

Conclusion

Triptorelin-induced central deprivation of testosterone synthesis leads to oxidative damage to hepatocytes due to increased reactive oxygen species production and decreased antioxidant enzymes activity. The oxidative damage to the liver cells begins on molecular and cellular levels and becomes visible on tissue level on the 180th day of central deprivation of testosterone synthesis.

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