HEALTH, ENVIRONMENT, DEVELOPMENT

MICROSCOPIC ARCHITECTURE OF THE LIVER OF EXPERIMENTAL RATS AFTER CANNABIDIOL OIL APPLICATIONS

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Summary

Cannabidiol (CBD), found in Cannabis sativa (hemp), is a non-psychoactive phytochemical substance that has gained considerable popularity over the past decade. Cannabidiol is the main phytocannabinoid, its share in the plant extract can reach 40%. The aim of the study was to study and compare the microscopic architecture of the liver in normal and after 2 weeks cannabidiol oil applications in experimental rats. We conducted an experimental study of the effect of CBD on the liver 2 weeks after its use as a dietary supplement. The main group consisted of 18 rats to which 5 drops (3 mg 10%) of cannabidiol oil were added to the main feed once a day for 2 weeks. The control group consisted of 6 sexually mature white male rats, which were provided with water and food without any restrictions. Our research results showed that the use of CBD oil as a food supplement did not have a toxic effect on the liver, did not cause any damage.

Key words: experimental research, cannabidiol oil, liver vessels, histology, morphometric studies.

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1. Introduction

Cannabidiol (CBD) is found in Cannabis sativa, is the main phytocannabinoid, and its fraction in the plant extract can reach 40%. Cannabidiol does not have any psychoactive properties that tetrahydrocannabinol (THC). Cannabidiol has become very popular in recent years *(Boggs Douglas L. et al., 2018)*.

In Ukraine, cannabidiol is a 100% legal substance because, as a substance, it is excluded from the list of narcotic and psychotropic substances (CMU Resolution No. 770 dated 05.06.2000, CMU Resolution No. 324 dated April 7, 2021) *(zakon.rada/324-2021).*

Today, multicenter clinical studies of the use of cannabidiol in the treatment of anxiety, disorders of the motor apparatus, and depression are being conducted. But the potential use of cannabidiol in medicine is question of long debate and research *(Cassano, 2020; Breijyeh, 2021)*. Cannabidiol has a complex mechanism of action, including anti-apoptotic, antioxidant and anti-inflammatory properties *(Pacher, 2020; Pertwee, 2010; Pisanti, 2017)*. In addition, cannabidiol is used in large quantities in cosmetics, nutritional supplements, and skin oils.

Advances in the field of pharmacology have made it possible to synthesize many compounds that target various structures of the endocannabinoid system – agonists and antagonists of cannabinoid receptors, anandamide uptake blockers, and potent selective inhibitors of endocannabinoid degradation. These new tools have made it possible to investigate the physiological role of endocannabinoids and have opened up new strategies in the treatment of patients with pain, obesity, neurological diseases including multiple sclerosis, psychiatric disorders, including anxiety disorders and addiction to psychoactive substances *(Chevallier, 1994; Crippa, 2013; Devinsky, 2017)*.

Despite the achievements, the literature emphasizes that further research is needed to determine the mode of administration and dose, possible side effects with long-term use, including the impact on cognitive and mental functions, motor activity, as well as the possibility of addiction *(Gamble, 2018; Millar, 2019; Pisanti, 2017; Schonhofen, 2015; Thiele, 2018).*

In addition to its purported therapeutic effects, accumulating evidence from preclinical in vivo studies and large-scale clinical trials suggests that cannabidiol may cause potentially negative health effects. In particular, numerous reports have demonstrated neurological, cardiovascular, and reproductive toxicity after the use of cannabidiol *(Carvalho, 2018; Jadoon, 2017; Schonhofen, 2015).*

Of particular concern is the risk of hepatotoxicity caused by cannabidiol *(Marx, 2018).* Animal studies reported increased liver weight in rhesus macaques (macaca mulatta) and elevated liver enzymes in dogs when cannabidiol was administered at doses as low as 2 mg/kg body weight *(Gamble, 2018; Rosenkrantz, 1981).* In relatively recent clinical trials, elevated liver enzymes were observed in 5–20% of patients treated with cannabidiol, and several patients were excluded due to the threat of fulminant liver failure *(Devinsky, 2017; Thiele, 2018).*

Given that liver damage is possible with cannabidiol, experimental studies are needed to investigate the hepatotoxicity potential of cannabidiol. The research results will provide important information for both industry and regulatory authorities regarding the short-term toxicity of cannabidiol. In addition, the results of the studies will help in the selection of appropriate models and doses for long-term studies (ie, subchronic and chronic toxicity studies). At the same time, experimental studies are needed to further study the mechanisms of action, the features of the pathohistological changes of the liver, with an emphasis on the nature and severity of possible damage to the liver vessels, the features of hemodynamics, at the level of the microcirculatory bed, and the features of the ultrastructure to determine the safety of the use of cannabidiol *(Fouad, 2011).*

The aim of the study was to study and compare the microscopic architecture of the liver in normal and after 2 weeks cannabidiol oil applications in experimental rats.

2. Material and methods of research

We conducted experimental studies to simulate the effect of cannabidiol oil on the liver, determine the nature and expressiveness of possible histological changes in the liver at the light-optical level, and hemodynamics at the level of the microhemocirculatory channel.

The experimental research protocols were approved by the bioethics committee of Danylo Halytsky Lviv National Medical University (protocol No. 7 dated August 29, 2022). Experiments were conducted in compliance with moral and ethical norms in accordance with the provisions of the European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes *(Strasbourg, 1986)*, Council of Europe Directive 2010/63/EU, Law of Ukraine No. 3447-IV "On protection of animals from cruel treatment".

Experimental studies were performed on 24 sexually mature white male rats, weighing 180-230 g, aged 5–7 months at the beginning of the experiment. All animals were housed in the vivarium of the Lviv National Medical University named after Danylo Halytsky. The rats were housed in separate special cages, in a heated room with a temperature regime of 20±1°C and ventilation, with a 12/12-hour light/dark cycle, and had free access to food and water – ad libitum. During the experiment, daily observations were made of the appearance, behavior, feed intake and general condition of the animals. The main group consisted of 18 rats to which 5 drops (3 mg 10%) of cannabidiol oil were added to the main feed once a day for 2 weeks. The control group consisted of 6 sexually mature white male rats, which were provided with water and food without any restrictions. Collection of biological material was carried out after euthanasia using diethyl ether. Liver samples were fixed in 10% buffered formalin. Then, according to the protocol, dehydration was carried out in alcohols of increasing concentration, embedded in paraffin according to the standard method. Histological sections with a thickness of 5±1 μm were made from paraffin blocks with liver tissue samples, which were applied to glass slides with a special adhesive coating. Deparaffined histological sections were stained according to the standard method with hematoxylin-eosin. Histochemical studies were conducted to detect neutral lipids (Sudan III), special methods of connective tissue staining (Van Gieson, Masson's trichrome) were used. Histological examination of liver sections was carried out.

Visualization and microphotography were performed using a Leica DM 2500 light microscope (Leica Microsystems GmbH, Germany) with a Leica DFC450 C digital camera (Germany) and Leica Application Suit Version 3.8 software. We conducted a morphometric study of the central veins, vessels of the portal tract and sinusoids. For this, a series of microphotographs was made of histological preparations at different magnifications of the microscope. Photomicrographs at a magnification of x400 were used to measure the inner diameter of blood vessels and wall thickness, and at a magnification of x1000 the diameter of sinusoids was measured. Measurements of the lumen of the central veins (longitudinal, transverse, diagonal), wall thickness, diameter of sinusoids in different fields of view were performed using the Aperio ImageScope v12.3.3 software (Leica biosystems, Wetzlar, Germany).

The obtained results were processed by the method of variational statistics using the Microsoft Excel program. The probability assessment of statistical studies was carried out using the Student's t-test, differences between groups were considered significant at a value of $p<0.05$.

3. Results of the research and their discussion

3.1. Microscopic architecture of the liver in normal (literature review and own research)

The liver of a sexually mature white rat under normal conditions is characterized by a balanced structural homeostasis, which is achieved due to stromal-parenchymal interactions. There is very little connective tissue in the liver, it is mainly visualized around the blood vessels, so the liver lobes are weakly expressed (Fig. 1). The connective tissue capsule of the liver is thin, and the connective apparatus is also weakly expressed.

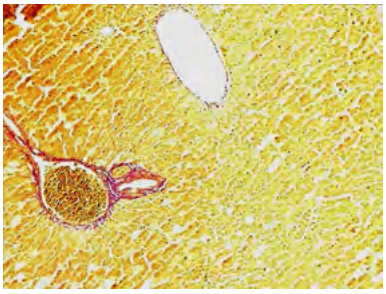

Fig. 1. Microstructural organization of the liver of an experimental white rat is normal (own research). The presence of layers of loose connective tissue in which the components of the portal tract are located. The thin wall of the central vein. Staining with picrofuchsin according to Van Gieson, x200. Collagen is red, other tissue elements, including erythrocytes, are yellow

The liver is a vital organ with a complex structure, which is characterized by three closely interconnected compartments, which include hepatocytes that make up the liver parenchyma, the biliary system, and the vascular system. Liver parenchyma is the main tissue that makes up the organ. Normal liver parenchyma is uniform in structure and has a low density *(Roskams, 2007).*

Hepatic lobules are microscopic structural and functional units (Fig. 2). Exactly this structural and functional organization of the liver makes it possible to assess possible damage, which can be both diffuse and focal in individual lobes, according to scientific research Elizabeth M Brunt *(Brunt, 2014).*

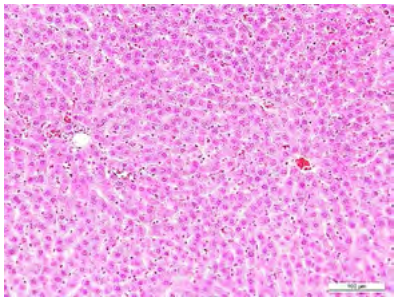

Fig. 2. Structural and functional organization of the liver. Two central veins of classic hepatic lobules. (one of them full of blood, one empty, uneven hyperemia (own research, control group). Staining with hematoxylin and eosin. x200

The classic hepatic lobule, which was described by Kiernan F. back in 1833, is considered the first or traditional type of lobule and is what is meant when the term "hepatic lobule" is used *(Kiernan, 1833).* It is a regular hexagon, in the center of which is the central vein. In each of the six corners, that is, on the periphery, there are portal tracts. Different types of organisms are characterized by a different number of liver lobules. According to Rezania et al. *(Rezania, 2016)* the liver of an experimental rat contains approximately 50-70 thousand particles, which is 20 times less than in a human liver and 10 times more than in a mouse liver *(Lamers, 1999; Wagenaar, 1994).* According to Ruijter et al. the centro-portal distance is 300–350 μm in rats and 211 μm in mice *(Ruijter, 2004).*

According to Suriawinata A.A. et al. *(Suriawinata, 2007)* and Crawford A.R. et al. *(Crawford, 1998)* microscopic structure is conceptualized in several ways, the two most common being acinus and lobule. An acinus is a unit that contains a small portal tract in the center and central veins on the periphery. This is the smallest functional unit, which is divided into three zones (1, 2 and 3). Zone 1 surrounds the portal tract and zone 3 surrounds the central vein. Blood from the portal tract flows through these zones to the central vein with a decreasing gradient of oxygen and nutrients. Zone 2 is located midway between zone 1 and zone 3. Alternatively, the traditional lobule concept can also be used, in which the central structure is the central vein, and the periphery corresponds to the portal tracts. Acinar zones 1, 2 and 3 correspond to the periportal, middle and pericentral zones of the lobule, respectively *(Crawford, 1998, Suriawinata, 2007).* In addition, the division of the liver lobe into zones along the central-portal axis is described. The centrolobular zone is located around the central vein (zone 3), the mesolobular zone is located between the centrolobular and periportal zones (zone 2), and the periportal zone is located around the portal tract (zone 1).

Microscopically, on a histological section, the acinus resembles a rhombus, and its tops converge to the central veins. The acinus includes the parenchyma of the liver, which is partially located in two different classic lobules. Parenchyma cells, which are closest to the main vascular trunks, have the best blood supply compared to other parts of it. The liver parenchyma, which surrounds zone 1, has almost circular contours on the histological section. This zone is designated as zone 2. After zone 1, zone 2 has a better blood supply than other parts of the acinus. The outer part of the acinus, which has an irregular shape and reaches the central veins, is called zone 3. This zone receives less blood than any other zone (Fig. 3). Today there is evidence that the metabolic processes that occur in each of the three zones of the acinus are somewhat different in nature. This explains the fact that some toxins or a deficiency of one or another nutrient in the diet affect different areas to a different degree. Understanding the structure of the acinus helps to understand why some parts of the liver lobes are affected in different conditions more than others, and why the degree of damage is different *(Rappaport, 1957; 1966; 1973).*

Classic liver lobules in the form of a regular hexagon are not always located side surfaces next to each other. They can be arranged in such a way that one particle is directed in one direction, and the second particle is in the other direction. Another difficulty of histological diagnosis is that, theoretically, it is easy to imagine that all 6 portal tracts belong to each lobule, but even with an ideal location, each portal tract interacts with the three lobules between which it is located and, accordingly, belongs to them *(Bruni, 1965; Burkel, 1970).*

With a small magnification of the microscope, the cells of the liver parenchyma – hepatocytes are usually arranged in the form of threads that are one or two cells thick, separated by sinusoids and radially moving from the central vein to the periphery of the classic lobule. Hepatocytes are polygonal, with an average size of 20 μm to 30 μm, with eosinophilic cytoplasm and a centrally located round or oval nucleus. But we previously showed that hepatocytes

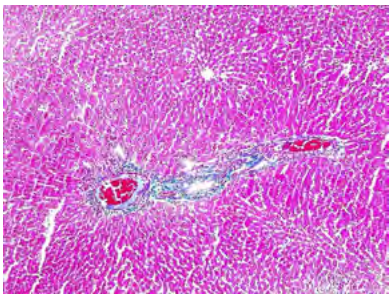

Fig. 3. Part of the liver acinus includes the portal tract and the central vein (own research, control group). On a red background, collagen fibers are colored blue, and nuclei are colored black. Staining according to Masson. x200

in the subcapsular zone and in the depth of the liver parenchyma differ in their morphology. Thus, it was established that in the subcapsular zone, compared to areas in the depth of the parenchyma, hepatocytes were characterized by pronounced morphological heterogeneity without zonal regularity. In addition, in the subcapsular zone, hepatocytes with clear cytoplasm, round and giant cells with a diameter of 30-35 μm were more common *(Shevchuk, 2022).*

In addition, indicators of tissue and cellular homeostasis were the absence of cells in the stage of mitosis, the presence of well-formed marginal liver plates and the presence of binucleate hepatocytes, which were more often found in groups of 2–3 near vessels, single binucleate cells could also be observed. Histologically, dark hepatocytes were diagnosed, which also gathered in small groups of 3–5 hepatocytes and were located near the portal tracts.

An important morphological structure of the liver are sinusoids, that is, fenestrated capillaries that do not have a basement membrane and are located along the course from the portal tracts to the centrilobular veins. Sinusoidal capillaries are wide and lined with a discontinuous layer of endotheliocytes. The lining of the hepatic sinusoids differs from the lining of ordinary capillaries in that it is formed by two different types of cells. Cells of the same type are relatively thin and flattened, resembling endothelial cells of ordinary capillaries. Cells of the second type are much larger. On histological sections, they are basophilic and often have a stellate appearance, hence the name Kupffer stellate cells. Kupffer cells belong to the phagocytic system of macrophages, they are in the sinusoidal lumen and form part of the lining, as they are located between endothelial cells (Fig. 4). Kupffer cell phenotypes and functions differ along the portal-central gradient.

According to the authors Saxena R. et al. *(Saxena, 1999)* and Wisse E. et al. *(Wisse, 1996)* found liver-associated lymphocytes or foveal cells in the lumen of the sinusoids. It is a resident population of large granular lymphocytes or NK cells that contact endothelial cells or Kupffer cells *(Saxena, 1999, Wisse, 1996).* Pituitary cells are also present in the space of Disse and have a T-lymphocyte or natural killer cell phenotype.

Endothelial cells in sinusoids can overlap, but when overlapping, they do not have intercellular junctions.

The conducted morphometric study of sinusoidal hemocapillaries in different fields of view showed the presence of different diameters, and the average diameter was equal to 6.06±0.16 μm. The diameter of the sinusoidal hemocapillaries of the subcapsular zone of intact rats was slightly larger than the average and was up to 13 μm, while the diameter of the sinusoidal capillaries in the depth of the parenchyma was smaller and approached the average values,

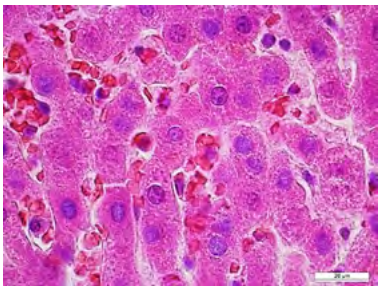

Fig. 4. Sinusoidal hemocapillaries of an intact rat liver filled with erythrocytes, Kupffer stellate cells present in the lumen of the sinusoids (own research, control group). Staining with hematoxylin and eosin. x1000

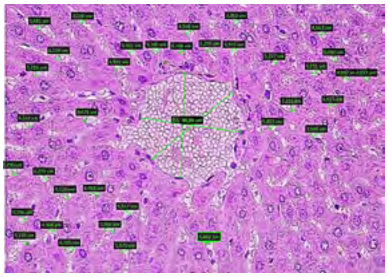

Fig. 5. Morphometric study of the diameter of sinusoidal capillaries in an intact rat (own research, control group). Staining with hematoxylin and eosin. x400

although in some fields of view the measurements were from a minimum value of 2.04 μm to a maximum – 8.48 μm. Closer to the central vein, the diameter was larger (Fig. 5).

An important role in the vascular system of the liver is assigned to the central vein *(Mak, 2020)*. The central vein is named so because of its central position in the classical lobe of the liver *(Ross, 2016)*. The central vein is also called the terminal hepatic vein *(Lieber, 1982)* because it is the terminal branch of the hepatic veins. The term centrilobular vein was sometimes used *[Porto, 1989; Chevallier, 1994)*.

The central vein is a thin-walled vessel with numerous sinusoidal entrances that drain sinusoidal blood (Fig. 6).

According to Lamers et al. *(Lamers, 1999)* the diameter of the central vein is not uniform; the diameter increases as the central vein drains into the sublobular vein. When leaving the lobule, the diameter is 150 μm or less, and when entering the sublobular vein, the diameter increases by more than 3 times and is more than 500 μm *(Lamers, 1999)*.

The results of our morphometric studies of the diameter of the central vein showed indicators: from 87.79 μm to 90.89 μm (Fig. 7).

The authors demonstrated in their experimental studies that sinusoids flow not only into central veins, but also into sublobular veins, which in turn converge and form collecting veins. Collecting veins are classified as right, middle and left hepatic veins *(Elias, 1955; Bhunchet, 1998; Lamers, 1999)*. There are also interesting data on the nature of the branching of the central veins. According to Lamers et al. found that from two to four central veins flow into the sublobular vein at the same time, and not one central vein, as previously believed *(Lamers, 1999)*.

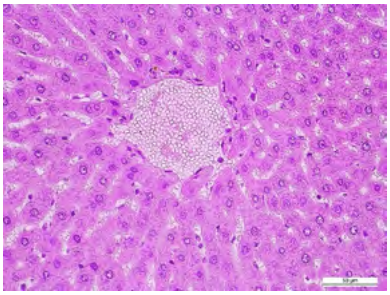

Fig. 6. The central vein is round, filled with erythrocyte masses, the endothelium is preserved, the endothelial cells are flattened and elongated. Expanded sinusoidal hemocapillaries are clearly visualized. Kupffer cells of a typical structure are visualized in sinusoidal hemocapillaries (own research, control group). Hematoxylin and eosin staining. x400

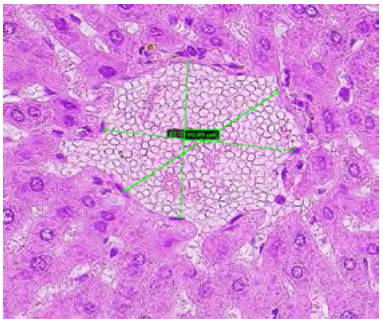

Fig. 7. Morphometric study of the diameter of the central vein in an intact rat (own research, control group). Staining with hematoxylin and eosin. x400

3.2.Microscopic architecture of the liver after 2 weeks cannabidiol oil (CBD) applications in experimental rats

With the daily (once a day) addition of 3 mg of 10% oil (5 drops) of cannabidiol to the basic feed after 2 weeks of the experiment, histological and morphometric studies of the liver were performed.

The analysis of the histological picture of the liver showed that the structure of the liver lobe was not changed. The hepatic artery of the portal tract of the lobule is oval, slightly narrowed, erythrocyte aggregates are present in the lumen (Fig. 8). Hepatocytes in beams are in two rows. Hepatocyte nuclei are clearly contoured, mostly with one nucleolus. The cytoplasm of individual hepatocytes is oxyphilic and contains small vacuoles. Dark hepatocytes are diagnosed in small groups near the hepatic artery.

The wall of the hepatic artery is slightly thickened, its inner lining consists of preserved endothelium. The nuclei of endothelial cells are flattened and elongated. The endothelium that lines the wall of the hepatic artery is associated with a small amount of loose connective tissue.

Sinusoidal hemocapillaries are narrowed, but there are isolated dilated ones, sometimes significantly.

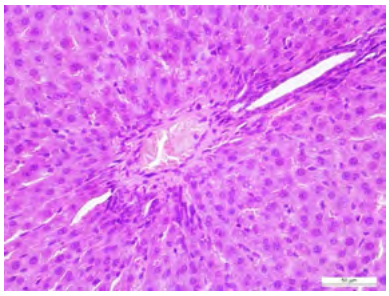

Fig. 8. Histological structure of the liver 2 weeks after exposure to CBD. The portal tract of the lobule. Hepatic artery with erythrocyte aggregates in the lumen, the smooth muscle layer is normal, with clear endothelium, the lumen is empty. Staining with hematoxylin and eosin. x400.

We measured the internal diameter of the hepatic artery and the thickness of its wall. The transverse, longitudinal and diagonal dimensions of the lumen were determined, as well as wall thickness measurements along the perimeter in different places. The number and diameter of arteries in the triad can vary widely.

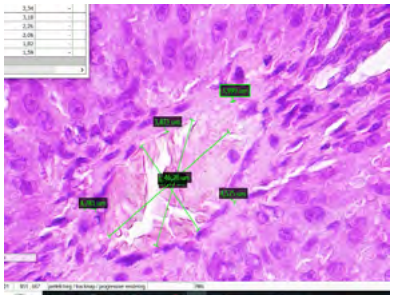

Fig. 9. Histological structure of the liver 2 weeks after exposure to CBD. The portal tract of the lobule. The diameter of the hepatic artery and the thickness of its wall are within normal limits. Staining with hematoxylin and eosin. x400

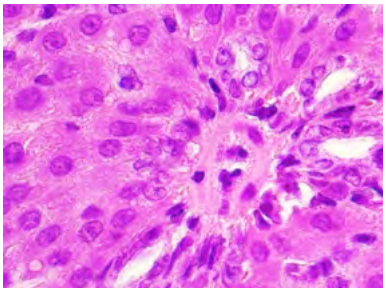

Fig. 10. Histological structure of the liver 2 weeks after exposure to CBD. The portal tract of the lobule. Slight swelling of the endothelium of the hepatic artery. The bile ducts are of normal structure, several sinusoids are dilated and filled with blood, the vein is of normal structure. Staining with hematoxylin and eosin. x1000

The longitudinal measurement of the diameter of the hepatic artery was the largest and amounted to 61.88 μm, diagonal – 46.20 μm, transverse – 37.63 μm. The thickness of the wall around the perimeter was uneven and varied from 3.87 μm to 4.78 μm in different places (Fig. 9).

In other cases, the lumen of the hepatic artery in the portal tracts is narrowed due to swelling of the endothelium and leukostasis. Bile ducts (3 ducts) are of normal structure, several sinusoids (2 sinusoids) are dilated and filled with blood, hepatic vein is of normal structure, microvesicular dystrophy of individual hepatocytes is diagnosed (Fig. 10).

Sinusoidal hemocapillaries were narrowed in many lobes, although some were slightly dilated, Kupffer cells (stellate reticulo-endothelial phagocytic) were clearly visualized (Fig. 11).

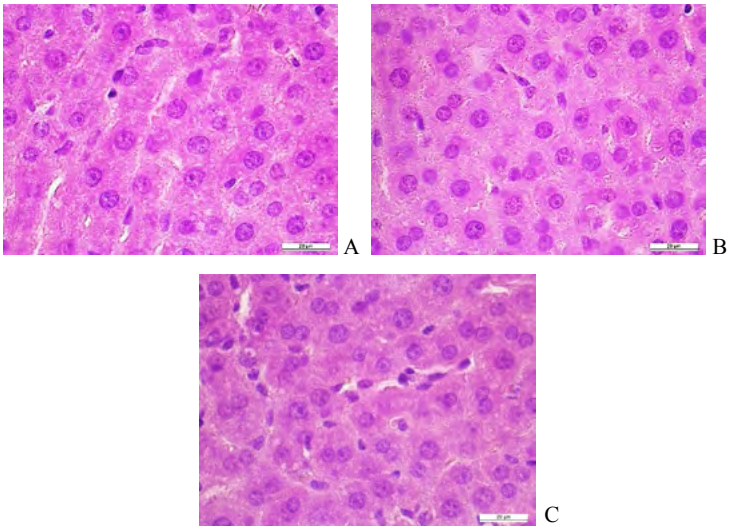

Fig. 11. Histological structure of the liver 2 weeks after exposure to CBD. Sinusoidal hemocapillaries in some places narrowed (A, B). Some slightly enlarged, clearly visualized Kupffer cells (C). Staining with hematoxylin and eosin. x1000

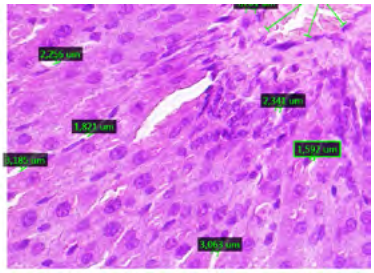

Fig. 12. Histological structure of the liver 2 weeks after exposure to CBD. A separate sinusoid is significantly dilated near the hepatic artery of the portal tract. Staining with hematoxylin and eosin. X1000

The morphometric indicators of the diameter of sinusoids were from 1.59 μm to 3.18 μm. In separate fields of view, dilated sinusoids were visualized, which resembled the hepatic vein near the portal tracts. The lumen of dilated sinusoidal capillaries was 7.18±0.13 μm. In addition, dark hepatocytes in dense rows were next to the fibrous plate of the triad (Fig. 12).

4. Conclusions

The literature presents the results of experimental studies in which the authors express concern about the development of hepatotoxicity caused by cannabidiol *(Marx T.K., 2018)*. In recent clinical trials, elevated liver enzymes were observed in 5–20% of patients treated with cannabidiol, and several patients were excluded due to the threat of fulminant liver failure *(Devinsky O., 2017, 2018; Thiele E.A., 2018)*.

We conducted an experimental study of the effect of CBD on the liver 2 weeks after its use as a dietary supplement. The main group consisted of 18 rats to which 5 drops (3 mg 10%) of cannabidiol oil were added to the main feed once a day for 2 weeks. The control group consisted of 6 sexually mature white male rats, which were provided with water and food without any restrictions. Our research results showed that the use of CBD oil as a food supplement did not have a toxic effect on the liver, did not cause any damage.

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