Peculiarities of vascular tunic microstructure of the white rat eyeball under the effect of opioid

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Abstract
Objective: This article deals with determination of changes in the structural organization of vascular tunic of the eyeball under the effect of opioid. Materials and Methods: The study was carried out on 24 mature white male rats aged 3.0–4.5 months and 170–280 g weight. The research material included histological specimen and semi-thin sections of white rats’ eyeball vascular tunic. For the histological study, microscopic sections of the eyeball were stained with Hematoxylin and Eosin, Heidenhain’s Azan trichrome. Specimens were studied and photographed under the microscope magnification: ×600, ×1000. Results: The first signs of microstructure disorder in all parts of vascular tunic of the eyeball are noticeable after two weeks of nalbuphine injection to the white rats. During the next four weeks of the experiment, the pathological changes increase and are manifested by the swelling and polymorphonuclear infiltration of the iris, ciliary body, choroid and by deep destructive changes of eyeball hemomicrocirculatory bloodstream. Histological and ultramicroscopic studies of the white rats’ eyeball vascular tunic after six weeks of nalbuphine injections showed deep destructive changes in the structure of all parts of vascular tunic. Conclusions: Our study demonstrated a negative effect of the prolonged injection of opioid in the experiment on the state of microstructural organization of the eyeball vascular tunic. Development of angiopathy is the triggering for occurrence of destructive changes in the eyeball under the effect of opioid.

Keywords: eyeball, microstructure, nalbuphine, experiment, hemomicrocirculatory bloodstream.

Introduction
Due to the continuous growth of the number of drug addicts in the European countries, anti-drug strategy was elaborated there to overcome the problem of polyetiological pathology [1, 2]. Nevertheless, the majority of the materials deal with social, legal and psychological aspects without drawing attention to the fact that considerable metabolic and structural changes, early disability and death rate of patients with opioid dependence require a study of pathogenic mechanisms of development and advance of multiple organ comorbid conditions [3].

Chronic drug intoxication is proved to be causing deep inhibition of general body responsiveness and resistance to different infection processes and forms a chronic endogenous intoxication with the logical development of chronic polyorgan inefficiency [4]. The problem of the effect of opioid substances on the restructuring of organization of organs and systems is important and pressing for modern medicine. A number of problems pertaining to structural changes in tissue brought about by narcotic substances still remain unsolved. Data presented in professional literature are contradicting and require a more accurate clarification. As each function is based on the adequate organ structure and its disorder under the influence of pathogenic factors is a foundation for the development of a pathological process defining its character and peculiarities of clinical manifestations, there undoubtedly is a necessity for a study of morphological peculiarities of the organ [5].

Despite the progressive technologies applied in diagnostics and treatment, the number of invalids with vision disability caused by most of eye diseases has not dropped in recent decades. According to the World Health Organization (WHO), there are about 150 million patients with considerable deterioration of visual function, including 50 million of blind people. The number of blind people has increased by 12 million in the last 20 years. These data undoubtedly show the need to study pathology of the organ of vision. This is why the objective of the study is to determine changes in the structural organization of the eyeball vascular tunic under the conditions of administering nalbuphine.

Materials and Methods
The research was carried out on 48 mature white male rats aged 3.0–4.5 months and body weight 170–280 g. All animals were handled in accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (1986), International Guidelines (2011) and Guidelines on protection, care, and handling of laboratory animals prescribed by the Ukrainian legislation (2006, 2012). All experiments were approved by the University Animal Care and Use Bioethical Committee. The animals were housed under standard vivarium conditions [6].

The lab animals are divided into three groups: the first group (five rats) was intramuscularly injected with nalbuphine (Rusan Pharma Ltd., India) for two weeks (1\textsuperscript{st} week – 8 mg/kg per day, 2\textsuperscript{nd} week – 15 mg/kg per day); nalbuphine was intramuscularly injected to animals of the second group (five rats) for four weeks (1\textsuperscript{st} week– 8 mg/kg per day, 2\textsuperscript{nd} week – 15 mg/kg per day, 3\textsuperscript{rd} week...
– 20 mg/kg per day, IVth week – 25 mg/kg per day); the third group (five rats) was intramuscularly injected with nalbuphine for six weeks (Ist week – 8 mg/kg per day, IIth week – 15 mg/kg per day, IIIth week – 20 mg/kg per day, IVth week – 25 mg/kg per day, Vth week – 30 mg/kg per day, VIth week – 35 mg/kg per day). Sampling of the nalbuphine for six weeks (Ist week – 8 mg/kg per day, IIth week – 20 mg/kg per day, IVth week – 25 mg/kg per day); the polypropylene forms with the fresh mixture araldite for impregnated with resin, fragments were transferred to phthalate were added to 20 mL of this solution. Then, 0.4 mL of catalyst DY064 and 0.6 mL of dibutyl araldite M, hardener HY 964 1:1 (Sigma-Aldrich Chemie GmbH). 0.4 mL of catalyst DY064 and 0.6 mL of dibutyl phthalate were added to 20 mL of this solution. Then, impregnated with resin, fragments were transferred to polypyrrole forms with the fresh mixture araldite for 24 hours at 60°C for polymerization, locating them beforehand in the required plane. The formed blocks were cut to obtain a trapezoid form and, secured in the block holder, semi-thin sections were held using a glass knife on the ultramicrotome LKB-188 (Sweden), 1 μm thick. The glass knives were made using the device KnifeMaker LKB 7800. Sections were mounted on the microscope slide and heated using LKB-2208 MULTIPLE (Sweden) appliance. Sections attached to the microscope slide glass were stained with Methylene Blue–Basic Fuchsin (Sigma-Aldrich Chemie GmbH). The stained sections were placed into polystyrene solution in xylene (mounting-medium) and subsequently covered by cover glass. The obtained specimens were studied using light microscope Leica DM-2500 (Switzerland). Photofixation of the image was made using the digital camera Leica DFC450C and software Leica Application Suite ver. 4.4 [Build: 454] Leica Microsystems (Switzerland) Ltd.

To make histological specimens, the eyeball was fixed during 24 hours in Bouin’s solution; picric acid was washed out in 70% ethanol solution in distilled water in three portions until the light yellow color staining of ethanol. Then dehydration was made in a solution of isopropanol (Sigma) of the concentration increased from 50% to 100%. Then, the eyeball was embedded in paraffin blocks. Sections 7 μm thick were produced using sliding microtome MC-2. Sections were mounted on the microscope slide at the temperature of 45°C, dried in thermostat during 24 hours at the temperature of 40°C. The dried sections were deembedded during five minutes in xylene; washed in two portions of absolute ethanol during two minutes; two portions of 96% ethanol during two minutes; in 70% ethanol during two minutes; in distilled water during one minute; immersed in Mayer’s Hematoxylin for 5–10 minutes; nuclei were additionally stained with 0.15% Eosin during one minute. Dehydration was made in ethanol, xylene; covered by cover glass with an intermediate medium (Canada balsam).

To produce histological specimens stained with Heidenhain’s Azan trichrome, the sections were deembedded, passed through alcohols with decreasing concentrations, distilled water. Then, they were immersed in Azocarmine solution for 10–15 minutes being heated beforehand to the temperature of 56°C. Then, they were washed in distilled water; differentiated in aniline ethanol until the cells’ nuclei became stained. Immersed in acetic acid–ethanol for 30–60 seconds; fixed in 5% aqua phosphotungstic acid during 1–3 hours; washed in distilled water; differentiated in 96% ethanol. Passed through a series of ethanol increasing concentrations, xylene and covered.

To carry out morphometric analysis of angiography of the vascular tunic of the white rat eyeball, we measured diameters of the arterioles, capillaries and venules with the aid of the eyepiece ruler on specimens of the vascular tunic of the white rat eyeball. Actual diameter (D) of the vessels was measured with the aid of the eyepiece micrometer with microscope magnification: objective ×20, eyepiece ×8 and objective ×10, eyepiece ×8, bearing in mind division value (K) by the formula: D=d×K, where: D – actual diameter of the vessel; d – measured diameter of the vessel; K – coefficient of the eyepiece-measuring ruler. Division value of the eyepiece micrometer (K) was determined with the help of the standard net of Goryaev’s calculating camera. According to the camera’s certificate, the side of small quadrate is 50 μm. At magnification: objective ×10, eyepiece ×8 division value (K) attains 100 μm (0.1 mm), and at magnification: objective ×20, eyepiece ×8 division value (K) attains 50 μm (0.05 mm). Statistical data processing of the experiment results was made with the aid of a computer and set of applied programs for medical, biological and epidemiologic research “InStat”.

**Results**

**Changes in the vascular tunic of the eyeball after two weeks of nalbuphine injection**

The first signs of destructive changes in the vascular tunic of the eyeball were detected after two weeks of nalbuphine injection. Compared with the control group (Figure 1), blood vessels of the choroid are dilated, their walls are thinned, their lumens are largely filled with blood components (Figures 2 and 3). A slight swelling is seen around the vessels. The blood vessels of the ciliary body are also dilated, their contours uneven. Lumens of some vessels are without blood cells and aggregations of erythrocite were found in some others. Epithelium of ciliary processes is partly disorganized. The iris layers are clearly distinguishable, but some moderate changes have
been found in each of them. Anterior epithelium is somewhat disorganized and interrupted. Anterior border layer is thinned. The iris vessels are dilated, their contours tortuous. The iris relief is partly deformed. The said above is confirmed by the growth of arteriolovenular coefficient of the choroid of the eyeball up to 0.980±0.027 (control – 0.8036±0.070). Diameter of arterioles increase up 29.424±1.402 (control – 21.798±2.290), diameter of venules 29.984±0.845 (control – 27.092±1.438).

Changes in the vascular tunic of the eyeball after four weeks of nalbuphine injection

After four weeks of the experiment, there is observed an increase of destructive changes in all parts of the rats’ eyeball vascular tunic. Fibers of the connective tissue of the choroid are fluffy and contain numerous cell components, fragmentation of collagen fibers (Figure 4). Connective tissue of ciliary body was found to contain numerous fibroblasts, macrophages and distinct hyperemia. In most of vessel, lumens there is aggregation of erythrocytes, many capillaries of the ciliary process are destroyed (Figure 5). Edema and hemorrhage are seen around the vessels. The iris is thinned. The posterior border layer is delaminated and interrupted in some portion. The number of connective tissue fibroblasts and macrophages also increases among in between the iris fibers. The iris contours are uneven. Erythrocyte, platelet, leukocyte aggregates and aneurysmatic dilatation of arterioles have been found in most vessels of the iris (Figure 6). Swelling and hemorrhages were found around the vessels. Diameter of capillaries of papillary margin of the iris attains 6.2±0.1 μm (control – 5.6±0.3 μm). Diameter of the detected capillaries attains 7.2±0.2 μm (control – 6.8±0.4 μm). Diameter of the preserved capillaries of choroid attains 6.2±0.1 μm (control – 7.0±0.2 μm). Arteriolovenular coefficient of the choroid of the eyeball is 0.733±0.021.

Changes in the vascular tunic of the eyeball after six weeks of nalbuphine injection

After six weeks of injecting nalbuphine to the rats there are observed deep destructive changes in the vascular tunic of the eyeball. The choroid some places is thinned and some places thickened because of the bulky position of collagen and elastic fibers in its connective tissue. The number of cell elements is considerably lesser in between the fibers, there occurring occasional fibroblasts, macrophages. Thin-walled, extended venules prevail. Arterioles wall is thickened because of sclerosing. Choriocapillaris layer is destroyed. The number of capillaries decreases. Occasionally capillaries wall is damaged where outflow of blood from the vessels is observed (microhemorrhages). Numerous capillaries where found to contain no blood components, while in other capillaries there were aggregation of erythrocytes. Characteristic is the manifested paravasal edema (Figure 7). Deep changes have been detected also in the ciliary body. Fibers of connective tissue are bulky with occasional fibroblasts and macrophages lying between them. Venules are abruptly widened, their contours uneven. Arterioles are tortuous, their walls sclerotized, thickened, an aggregation of erythrocytes, adhesion is observed in most of them. Capillaries of ciliary process are mostly ruined, their walls interrupted. Evident perivascular edema, hemorrhages. Epithelium covering the ciliary process is disorganized, fragmented. Muscle layer of the ciliary body thinned. The iris layers are not distinguishable clearly. The iris venules are also dilated, their contours uneven, twisted. A considerable edema is observed around the vessels. Posterior border iris layer thickens, occasionally is laminated and in some places is thinned out and broken. Contours of the layer of anterior epithelium are uneven, tortuous, the layer is discontinuous. Anterior border layer is thinned. Fibers of connective tissue are bulky, edema is observed. Increase of the number and thickening of collagen fibers in the basic substance of the iris (Figure 8).

Diameter of capillaries of papillary margin of the iris increases up until 19.6±0.5 μm, arteriolovenular coefficient drops to 0.584±0.029, diameter of the venules in some places attains 48.744±2.585 μm.
Figure 3 – Choroid of white rats’ eyeball influenced by two weeks nalbuphine injection. Dilatation and overfilling with blood of the venule of choroid. Swelling of arteriole’s endothelium cytoplasm. Microphotograph stained with HE (>800).

Figure 4 – Choroid of white rats’ eyeball influenced by four weeks nalbuphine injection. Area of perivasal edema in choroid. Fragmentation of collagen fibers. Erythrocytes in base substance of connective tissue. Microphotograph stained Heidenhain’s Azan trichrome (>1000).

Figure 5 – Ciliary body of white rats’ eyeball influenced by four weeks nalbuphine injection. In the ciliary body conjunctive tissue are found a lot of fibroblasts, macrophages and explicit hyperemia. Microphotograph stained with HE (>600).

Figure 6 – Iris of white rats’ eyeball influenced by four weeks nalbuphine injection. Aneurysmatic dilatation of arteriole. Microphotograph stained with HE (>600).

Figure 7 – Choroid of the eyeball of the rat after six weeks of nalbuphine injections. Dilatation and overfilling with blood of the vessels of choroid. Area of the perivasal edema. Semi-thin section (1 μm) stained with Methylene Blue and Basic Fuchsin (>1000).

Figure 8 – Iris of white rats’ eyeball influenced by six weeks nalbuphine injection. Increase of the number and thickening of collagen fibers in the basic substance of the iris. Vacuolization of the iris epithelium cytoplasm. Microphotograph stained with Heidenhain’s Azan trichrome (>1000).
Discussion

The results of the present work are a fragment of the scientific research project “The structure of organs and their bloodstream in ontogenesis under the effect of laser irradiation and pharmaceutical agents in cases of blood supply disorders, reconstructive surgeries and diabetes mellitus” (State Registration Number 0110U001854), being conducted at the Department of General Anatomy of Danylo Halyszky National Medical University of Lviv in accordance with the State Plan and Program.

The studies we have carried out show that the first signs of damage of the microstructure of all parts of vascular tunic of the eyeball appear already after two weeks following injection of nalbuphine to the rats. First of all there are found intra- and extravascular changes in hemomicrocirculatory bloodstream of the eyeball. This is in line with the opinion of a number of authors that the vessels hemomicrocirculatory bloodstream are the first to react to pathogenic factors by the structural changes that serve as a foundation for the development of a pathological process and determine its character and peculiarities of its clinical manifestations [7].

The study of vessels’ structure, histological structure of their walls in the norm and under the effect of various factors allows studying importance of the vessel factor for morphofunctional inefficiency of internal organs. It is generally acknowledged that each function is based on the structure adequate thereto.

Edema of tissue of all parts of vascular tunic of the eyeball we detected after four and six weeks of nalbuphine injections in the experiment complies with the reports presented in professional literature on the effect of narcotic substances [8]. In particular, it was noted that signs edema and swelling of its tissue were found in the brain and in meningeal tunics under the effect of narcotic substances. This is explained by the combination of microcirculation disorder characteristic of acute drug intoxication. Aside from the drainage glia edema microscopically manifested by perivascular and pericytal edema, histological there have been found various disorder of microcirculation in subcortical regions and brainstem. It is generally acknowledged that each function is based on the structure adequate thereto.

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This project research work differs from other already known works of this kind by the fact that new data on the effect of opioid on the peculiarities of angioarchitecture of the vascular tunic of the white rat eyeball have been obtained based on a complex of micro-, macro- and electron microscopic studies. For the first time, we have investigated dynamics of structural changes of the vascular tunic of the rat’s eyeball under the effect of injection of opioid. Mathematical analysis allowed systematization of the obtained experimental data and present a comparative characteristic of angioarchitecture of the vascular tunic of the rat eyeball under the effect of opioid. The data obtained enable us to extend our notions and resolve the disputable problems of the effect of opioid on the eyeball vascular tunic structure, which will create the morphological basis for understanding pathogenesis of ophthalmologic diseases of drug users and patients who have to take opioids for an extended period of time, and for finding optimal methods of treatment. The obtained data are important for both, morphologists and clinicians.

Conclusions

The first signs of disorder of microstructure of all parts of vascular tunic of the eyeball are noticeable after two weeks of nalbuphine injection to white rats. During the next four weeks of the experiment pathological changes increase and are manifested in the form of edema and polymorphonuclear infiltration of iris, ciliary body and
vascular tunic proper as well as by deep destructive changes in parts of the eyeball hemomicrocirculatory bloodstream. Histological study of vascular tunic of the white rat’s eyeball after six week of nalbuphine injection showed deep destructive changes in the structure of all parts of vascular tunic of the eyeball.

Conflict of interests
The authors declare that they have no conflict of interests.

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