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Evaluation of the efficacy of apoE gene transfection

Żanna Białoszycka St. Vincent de Paul Hospital, ul. Wójta Radtkego 1, 81-348 Gdynia, Poland
https://orcid.org/0000-0002-3136-7687
dr.zannabialoszycka@gmail.com

Monika Białoszycka School of Medicine, Collegium Medicum, University of Warmia and Mazury Aleja Warszawska 30, 11-082 Olsztyn, Poland
https://orcid.org/0009-0005-9371-0398
monika.margaretka@gmail.com

Alisa Pachevska Department of Pediatric Stomatology of National Pirogov Memorial University st. Pirogova 56, 21018 Vinnytsya, Ukraine
https://orcid.org/0000-0002-6041-3814
alisa.paczewska@gmail.com

Valerij Istoshyn Department of Biochemistry of National Pirogov Memorial University st. Pirogova 56, 21018 Vinnytsya, Ukraine
https://orcid.org/0000-0002-1857-3195
walery.istoszyn@onet.pl

Alina Biloshytska Department of Medical Biology of National Pirogov Memorial University st. Pirogova 56, 21018 Vinnytsya, Ukraine
https://orcid.org/0000-0003-2790-060X
alina.biloszycka@gmail.com

Irina Simonova Department of Medical Biology of National Pirogov Memorial University st. Pirogova 56, 21018 Vinnytsya, Ukraine
https://orcid.org/0000-0002-3501-1813
arinasimonova@gmail.com

Corresponding author:

Żanna Białoszycka St. Vincent de Paul Hospital, ul. Wójta Radtkego 1, 81-348 Gdynia, Poland
https://orcid.org/0000-0002-3136-7687
dr.zannabialoszycka@gmail.com

Abstract

Introduction: Pathology of organs of the male reproductive system remains one of the most urgent problems of modern medicine, which is associated not only with patients' impaired quality of life, but also with high rates of male infertility, which tends to increase.

The aim: To evaluate the efficacy of apoE gene transfection on the morphofunctional state of rat testes in experimental atherosclerosis.

Materials and methods: The study was conducted on 30 sexually mature male rats weighing 150-170 grams. Experimental animals were divided into 3 groups. Group 1 -

intact animals. Group 2 – animals with experimental atherosclerosis; Group 3-animals with experimental atherosclerosis, which were intramuscularly injected with the apolipoprotein E gene at a dose of 50 μg of DNA per animal on the first day of modeling atherosclerosis. To study the efficiency of apolipoprotein E gene transfection on morphofunctional changes in testes, the following research methods were used: 1) histological; 2) biochemical; 3) reverse transcriptase PCR diagnostic method.

Results and discussion. Experimental cholesterol loading leads to impaired lipid metabolism, decreased serum testosterone levels, degenerative and dystrophic changes in the testis of experimental rats, which is manifested by luminal occlusion of blood vessels, overgrowth of connective tissue in the organ interstitium, decreased number and qualitative change of Sertoli and Leydig cells, as well as spermatogenic epithelium thinning and inhibition of gamete maturation process in it.

Conclusions: intramuscular transfection of the apoE gene inhibits lipid metabolism disorders and has a positive effect on the morphofunctional state of the testes of experimental animals.

Keywords: atherosclerosis, apoE gene, transfection, testes, Sertoli, Leydig cells.

1.INTRODUCTION.

Pathology of organs of the male reproductive system remains one of the most urgent problems of modern medicine, which is associated not only with impaired quality of life of such patients, but also with high rates of male infertility, which has a growing tendency [1-3].

Researchers have established a relationship between the development of atherosclerosis, metabolic syndrome, age, and decreased spermatogenesis and testosterone levels in experimental animals [2].

It is known that atherosclerosis is a chronic disease of elastic and elastic-muscular arteries resulting from lipid metabolism disorders and accompanied by deposition of cholesterol and some lipoprotein fractions in the vessel intima. The deposits are formed as atheromatous plaques, and further growth of connective tissue (sclerosis) and calcification of the vascular wall leads to its deformation and narrowing of the lumen. [4-6].

The largest epidemiological studies (Framingham, MRFIT, 7 countries, 1947), have shown a clear correlation between blood cholesterol concentration and the mortality rate from coronary heart disease [7-9]. Currently, there are several theories of atherosclerosis development. But it should be noted immediately, that all modern theories of atherosclerosis occurrence do not contradict, but only mutually complement each other. Thus, all theories and hypotheses of the pathogenesis of atherosclerosis are put within the limits of two concepts. One of them assumes that the development of atherosclerosis is due to the disruption of lipoproteins and certain proteins (e.g., blood plasma fibrinogen) and that the onset of atherosclerosis is thus "introduced" into the arterial wall from the blood. Another concept combines theories and hypotheses in which changes in cellular, connective tissue and other factors are of primary importance as the root cause of the development of the atherosclerotic process [10-11]. At the same time, atherosclerosis refers to a multifactorial pathology, in the development of which, along with environmental influences, heredity plays a role. The results of a large number of population, clinical and experimental studies indicate that there is a relationship between the presence of mutations of individual genes and their variability on the one hand, and lipid metabolism disorders on the other. Thanks to advances in molecular biology, it has been proved that the cause of lipoprotein metabolism disorders are mutations of the low-density lipoprotein receptor gene apoE (accompanied by increased LDL levels), the high-density lipoprotein major protein (apoA) and mutations of the apoC gene, which contribute to a significant increase in the level of triglycerides at normal cholesterol levels [12-13].

In recent years, new methods of treating atherosclerosis have been developed, and gene therapy is of great interest. This method considers the introduction into the body (with the help of viral, cellular or other vectors) of certain genes expressing proteins that affect lipid metabolism. Most often, these are the genes of apolipoproteins and β-LP-R, as they have all already been mapped and cloned [14]. The following types of gene therapy are considered for practical use: gene replacement therapy - used to treat genetic defects caused by the loss of gene function, in this case, copies of a normal "therapeutic" gene are introduced into cells and conditions for its expression are created to replace the function of the defective gene; inhibitor therapy - treatment of genetic diseases caused by excessive gene activation – the strategy is aimed at inserting a gene, the product of which will block the expression of the pathological gene and thereby inhibit the development of the disease; the elimination of certain cells – involves the destruction of a specific population of cells, in particular transformed ones [15]. An extremely important problem of gene therapy is the choice of delivery method of the therapeutic gene to the target tissue. An ideal "therapeutic" gene delivery system should provide: high efficiency of target absorption of the "therapeutic" gene by target cells; its minimal intracellular destruction during transport to the nucleus; high level of expression providing therapeutic effect; no rearrangements or mutations; no immunogenicity of the expression product. Takis Athanasopoulus [16] has developed the direct introduction of the DNA construct into target cells by injection which is the simplest method of delivering a transgene (transferred gene) to cells in vivo, in which DNA is introduced directly into the tissue by injection. The use of this method has so far been limited to such tissues as skin, thymus, striated muscles, some solid (those growing in a dense nodule) tumors. Long enough (up to a year) transgene expression is observed mainly in muscle tissue [17]. A promising method to directly introduce DNA construct into target cells is to deliver the genetic construct in liposomes [18-19]. In particular, in cationic liposomes with a positive charge, a negatively charged DNA molecule forms a DNA-lipid complex - lipocomplex. The advantages of using such complexes, compared to viral vectors, are the ability to carry a larger volume of information, the impossibility of recombination and the emergence of infectious properties. The constructs are less likely to trigger an immune or inflammatory response, and are simpler and cheaper to manufacture [20].

2.AIM

To evaluate the efficacy of apoE gene transfection on the morphofunctional state of the rat testes in experimental atherosclerosis.

3. MATERIALS AND METHODS

The study was conducted on 30 sexually mature male rats weighing 150-170 grams, which were kept on a standard diet in conditions of the scientific research facility of National Medical University. Animal housing and manipulations were performed in accordance with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1985), "General Ethical Principles of Animal Experiments", adopted by the First National Congress on Bioethics (Kyiv, 2001), as well as the bioethics committee of National Medical University (Minutes № 11, June 7, 2012). All procedures with animals were performed under light ether anesthesia [21].

The model of experimental atherosclerosis was created according to the classic Anichkov method by feeding cholesterol to animals with sunflower oil. Rats were intragastrically injected with cholesterol in a dose of 0.5 g/kg and additionally 4(6)-methyl-2-thiouracil in a dose of 12 mg/kg to suppress the thyroid gland function with the help of a probe with oil for 30 days. Experimental animals were divided into 3 groups.

Group 1 – intact animals kept under usual conditions of the experimental clinic; Group 2 – animals with experimental atherosclerosis; Group 3– animals with experimental atherosclerosis, which were injected intramuscularly with the apolipoprotein E gene at a dose of 50 μg of DNA per animal on the first day of modeling atherosclerosis. The cationic liposomes/DNA preparation was produced using the DOTAP Methosulfate reagent manufactured by SIGMA-ALDRICH (USA) in accordance with the manufacturer's instructions. 5 μg of DOTAP Methosulfate per 1 μg of DNA was used. A mixture of cationic liposomes and DNA was prepared immediately before administration according to the manufacturer's instructions (by shaking the DNA with cationic liposomes for 10 minutes) [Sigma/Molecular Biologia]. The fact that the apoE gene is active was checked by the traditional reverse transcriptase polymerase chain reaction method.

The following research methods were used to study the effectiveness of apolipoprotein E gene transfection on morphofunctional changes in testes: 1) histological; 2) biochemical; 3) reverse transcriptase PCR diagnostic method.

For the morphological study, the right testis was taken and immersed in a 10% solution of neutral formalin for fixation. The fixed organ was washed under running water for one day to remove the fixative, then dehydrated in alcohols of increasing concentration and embedded in paraffin. Blocks were cut using a MS-2sledge microtome. The obtained sections were stained with hematoxylin-eosin and Van Gieson. Hematoxylin-eosin staining provides data on the nature of the structure of the testis, parenchyma, stroma, and blood vessels. Van Gieson staining was performed with hematoxylin- picrofuxin, which allows identifying collagen fibers of connective tissue in the studied organ. Micropreparations were evaluated under a MIKMED-1 microscope at different magnifications (ocular lens x10; objective x8, x20, x40, x90).

For a biochemical study, after decapitation, rats' blood was taken to obtain serum, in which testosterone levels were determined. Testosterone levels were determined in a standard analytical grade [Immunoanalysator (CENTOWER); Siemens Medical Solutions, Erlangen, Germany].

To determine the transfection efficiency, a section of the muscle where the liposome+plasmid complex was injected was cut with a scalpel and placed in liquid nitrogen for subsequent RT-PCR study.

4. RESULTS AND DISCUSSION

The efficiency of transfection was evaluated using the method of reverse transcriptase polymerase chain reaction - RT-PCR (Fig. 1).

1 2 3 4 5 6 7 8 M 9 10 11 12

Fig. 1. Electrophoregram of apoE gene RNA amplification products (230 n. s.):
№ 1 – "-" –control; №№2-9 – muscle tissue samples of rats injected with a plasmid carrying the human apoE gene; № 10 – "+" – control; №№ 11-12 – muscle tissue samples of control rats (without introduction of plasmid DNA).

Biochemical examination of rats serum showed that cholesterol loading leads to a decrease in testosterone levels in the group with experimental atherosclerosis, an increase in total cholesterol and β-lipoprotein cholesterol levels, and a recovery of these indicators in the group where the apoE gene was transfected (Table 1).

Table 1 Biochemical examination of rats serum

Group of animals index	Intact (n=10)	Atherosclerosis (n=10)	Atherosclerosis and gene transfection apoE (n=10)
Testosterone, nM/l	28,5 7,1	7.3 0,01 nM/l *	19,8 5,3 nM/l *#
Total cholesterol, mmol/l	1,435±0,025	4,5628±0,2028 *	1,383±0,085 *#
β-lipoprotein cholesterol, mmol/l	0,309±0,03	3,8662±0,205 *	1,035±0,0047 *#

Notes: * – the difference is significant in comparison with the group of intact animals ($p \leq 0.05$), # – the difference is significant in comparison with the group of animals with experimental pathology ($p \leq 0.05$).

Macromorphological examination of the testes of intact animals showed that tunica albuginea of the testes was formed by dense fibrous tissue which was uniformly thick and had a smooth surface. Seminiferous tubules with 3-4 rows of cells of the spermatogenic epithelium are visualized on histological sections. The wall of convoluted tubules has a typical three-layer structure, including a basement membrane, a continuous layer of myoid cells, and a fibrous sheath. The spermatogenic epithelium, tightly adjacent to the basement membrane, is represented by rows of germ cells filling its entire lumen. Cells at different stages of spermatogenesis, ranging from spermatogonia to spermatozoa, which are freely located in the lumen of convoluted tubules can be identified. Secondary spermatocytes and spermatids at different stages of spermiogenesis are predominant among the cells of the spermatogenic epithelium. The presence of a large number of spermatogonia mitoses and meiotic divisions of spermatocytes is noteworthy. The tails of mature spermatozoa of the inner row of cells are directed into the lumen of the tubules and in most cases almost

completely overlap it, their nuclei are large in size and contain mainly heterochromatin (Fig. 1). Sertoli cells closely adhere to the basement membrane of tubules. In their population, cells containing euchromic nuclei can be detected, indicating active transcriptional processes in them, which are certainly related to their important involvement in the regulation of spermatogenesis. There are also cells with an optically compacted nucleus, indicating their metabolic inertness (Fig. 2). Numerous Leydig cells are observed in the interstitium of the testes, which are located in small variable-sized clusters in the connective tissue layers between the convoluted seminiferous tubules of the testicle. Leydig cells are round or polygonal in shape. They are characterized by the presence of large round nuclei centrally located in the cell, light cytoplasm with a significant content of vacuole-like structures, indicating high hormone-producing activity of these cells (Fig. 3).

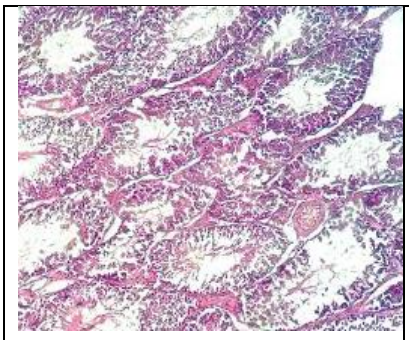

Figure 1. Microstructure of the testis of an intact rat. Stained with hematoxylin and eosin. ×100.

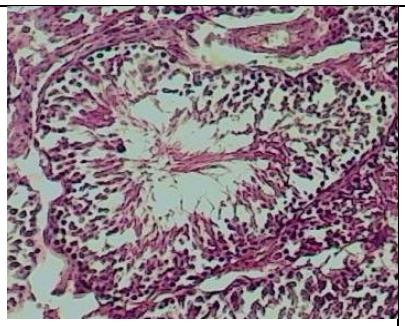

Figure 2. Microstructure of the testis of an intact rat. Sertoli cells Stained with hematoxylin and eosin. ×200.

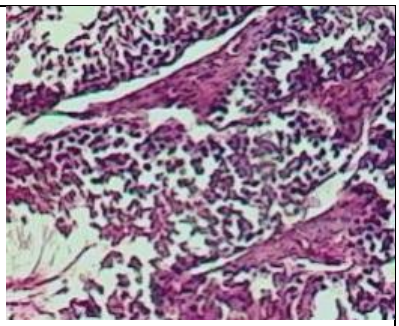

Figure 3. Microstructure of the testis of an intact rat. Leydig cells Stained with hematoxylin and eosin. ×200.

The fibrous membranes of the tubule walls are in close contact with each other. At the points of contact of the walls of 3-4 tubules, cracks are formed, in which blood vessels mostly of small caliber are passed, moderately filled with blood corpuscles. (Fig. 4)

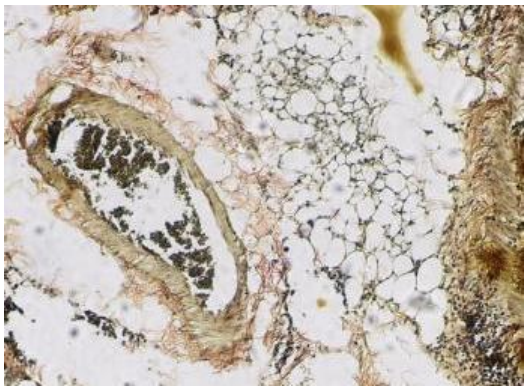

Fig. 4. Microstructure of the testis of an intact rat. Vessel. Van Gieson's stain. × 400.

Macromorphological examination of animal testes with experimental atherosclerosis notes significant swelling of fibrous stroma. Histological examination reveals thinning of the spermatogenic epithelium, an increase in the space between cells, and their cleaning from the basement membrane (Fig. 5).

In contrast to intact animals, the secondary spermatocytes and spermatides predominate. The number of maturation cells and the sperm is actually decreased in the lumen of convoluted tubules. Formed spermatozoa make up molds and eosinophilic units in the lumen of the convoluted tubules. A significant number of gametes contain large nuclei, there are multinucleated spermatides. Considerable structural changes are detected in the spermatozoid head, which are manifested by its vacuolation and amorphia, indicating the absence or lysis of the acrosome. The contours of the sperm head are irregular, it is divided into fragments and amorphia in the structure. There is significant deformation of the major and intermediate part of the flagella sperm and their heterogeneity in the structure along the entire length with a pronounced cytoplasmic excess. The number of Sertoli cells decreases. Most of them have optically dense cytoplasm and pycnomorphic nuclei. The wall of convoluted tubules retains its structural integrity. But there is swelling of the interstitial space, more pronounced in the subcapsular region, the growth of fibrous tissue in the intratubular spaces (Fig. 6).

The number of Leydig cells is significantly reduced, indicating inhibition of testosterone-producing function in this group of animals (Fig. 7), which is observed in the serum biochemical examination.

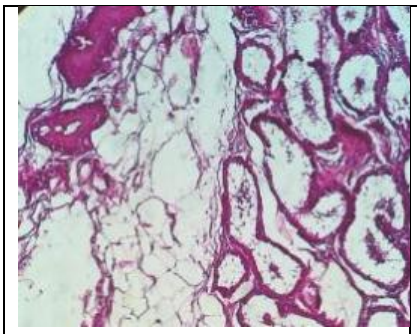	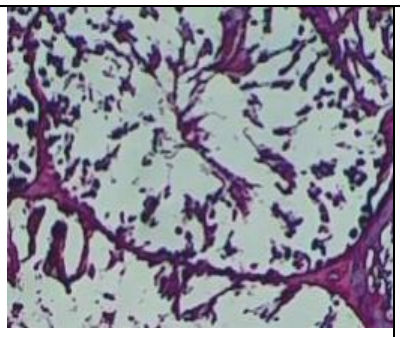	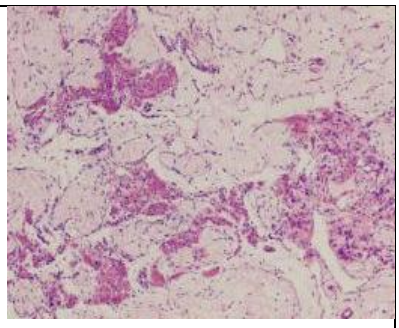
Fig. 5. Microstructure of the rat testis with experimental atherosclerosis. Hematoxylin and eosin stain. × 100.	Fig. 6. Microstructure of the rat testis with experimental atherosclerosis. Hematoxylin and eosin stain. Sertoli cells. ×200.	Fig. 7. Microstructure of the rat testis with experimental atherosclerosis. Hematoxylin and eosin stain. Leydig cells. ×200.

Concurrently, signs of connective tissue degeneration in the form of pronounced overgrowth of fibrous tissue in intratubular spaces are revealed. Single vessels of the hemomicrocirculatory bed are characterized by narrowing of the lumen, up to its complete obliteration and thickening of the vessel wall, making it difficult for the trophic of both spermatogenic epithelium and Leydig cells, which are located next to the vessels (Fig. 8).

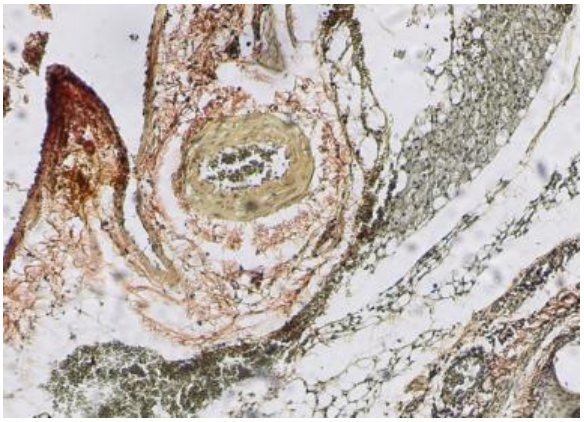

Fig. 8. Microstructure of the rat testis of Group 2. Van Gieson's stain. × 100.
A muscular type artery with a narrowed lumen in the gate of the testis with a thickened wall due to atherosclerotic lesions.

Histological examination of testis preparations of the third group of animals injected with the apoE gene on the 1st day of the experiment, revealed areas with slight thinning of the

spermatogenic epithelium, some increase in the space between cells, and their partial detachment from the basement membrane.

The number of maturing cells and actually spermatozoa in the lumen of convoluted tubules increases significantly (Fig. 9). At the same time, the number of areas with tubules preserving signs of active spermatogenesis increases compared to the second group. The thickness and qualitative composition of the spermatogenic epithelium in such tubules differ little from that of sexually mature rats. More of these functionally active convoluted tubules are found in the central part of the organ (Fig. 10). The number of Leydig cells is significantly increased, which explains the restoration of their testosterone-producing function in this group of animals (Fig. 11).

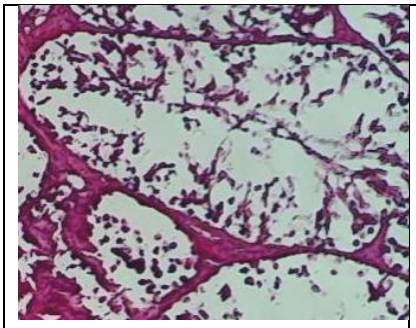	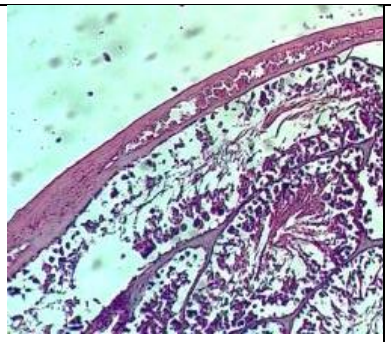	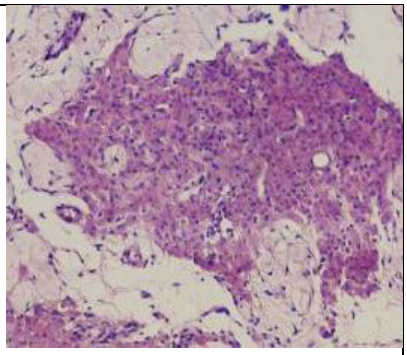
Fig. 9. Microstructure of the rat testis with experimental atherosclerosis and apoE gene transfection. Hematoxylin and eosin stain. ×200.	Fig. 10. Microstructure of the rat testis with experimental atherosclerosis and apoE gene transfection. Sertoli cells. Hematoxylin and eosin stain. ×200.	Fig. 11. Microstructure of the rat testis with experimental atherosclerosis and apoE gene transfection. Leydig cells. Hematoxylin and eosin stain. ×200.

Morphological manifestations of pathological reactions of the arterial walls in the rat testis become less noticeable. The larger caliber arteries appear more stretched due to their filling with blood (Fig. 12).

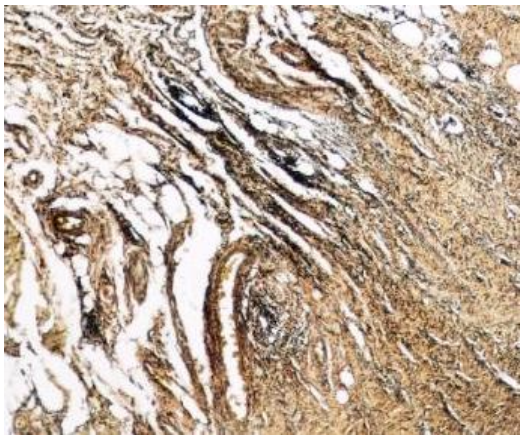

Fig. 12. Microstructure of the rat testis of Group 3. Artery of muscular type Van Gieson's stain. × 100.

5. CONCLUSIONS

(1) Experimental cholesterol loading leads to impaired lipid metabolism in research animals.

(2) Experimental cholesterol loading leads to decreased serum testosterone level, degenerative and dystrophic changes in the testis of experimental rats.

(3) The apoE gene transfection prevents the development of dyslipidemia and morphofunctional disorders in the testes of experimental animals.

Author's contribution

Conceptualization Żanna Białoszycka, Monika Białoszycka, methodology Alina Biloshytska, Alisa Pachevska, Valerij Istoshyn, software Monika Białoszycka, Alisa Pachevska, Irina Simonova, check Żanna Białoszycka, Alina Biloshytska, Valerij Istoshyn, formal analysis Żanna Białoszycka, Alisa Pachevska, Irina Simonova investigation Monika Białoszycka, Irina Simonova, resources Żanna Białoszycka, Monika Białoszycka, Alina Biloshytska, Valerij Istoshyn, data curation Monika Białoszycka, Alina Biloshytska, Irina Simonova, writing-rough preparation Alisa Pachevska, Valerij Istoshyn, Irina Simonova, writing review and editing Alisa Pachevska, Valerij Istoshyn, visualization Żanna Białoszycka, Monika Białoszycka, Irina Simonova, supervision Alina Biloshytska, Valerij Istoshyn, project administration Żanna Białoszycka, Alisa Pachevska.

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Data Availability Statement

Not applicable.

Acknowledgments

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Ethical approval

The minutes for this study was approved by the institutional ethics committee.

Conflict Of Interest:

The authors declare no conflict of interest.

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