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Medicine

EFFECTS OF TIMOLOL AND ADRENALINE IN THE CONTROL OF CORNEAL NEOVASCULAR MOTILITY IN RATS

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ABSTRACT:
THIS STUDY AIMED TO EVALUATE THE EXPRESSION OF ALPHA OR BETA-TYPE ADRENERGIC RECEPTORS IN CORNEAL NEW BLOOD VESSELS.
THE EXPERIMENTS WERE PERFORMED ON WISTAR RATS, IN WHICH A MODEL OF CORNEAL NEOVASCULARIZATION WAS OBTAINED BY REPEATED INJECTIONS OF KETAMINE (150MG/KG BODY WEIGHT), ADMINISTERED INTRAPERITONEALLY. THE VARIATION OF THE EXTERNAL DIAMETER OF THE CORNEAL NEOFORMATION VESSELS WAS FOLLOWED, AT A TOTAL MAGNIFICATION OF APPROXIMATELY 400X, OVER 630 SECONDS.
ADRENALINE PRODUCED STATISTICALLY SIGNIFICANT VASOCONSTRICTION IN THE CORNEAL NEOVESSELS VIA ALPHA-ADRENERGIC RECEPTORS. TIMOLOL PRODUCED STATISTICALLY SIGNIFICANT VASODILATION, REGARDLESS OF THE DOSE ADMINISTERED, BY A MECHANISM INDEPENDENT OF BETA-ADRENERGIC RECEPTORS. TIMOLOL CAUSED VASODILATION IN A DOSE-DEPENDENT MANNER, THE VASODILATION BEING INVERSELY PROPORTIONAL TO THE DOSE. FOLLOWING THESE RESULTS, WE CAN SAY THAT AT THIS LEVEL THERE ARE ALPHA-1 ADRENERGIC RECEPTORS, BUT WE CAN NOT EXCLUDE THE EXISTENCE OF BETA-ADRENERGIC RECEPTORS WHOSE STIMULATION PRODUCES VASODILATION.

KEYWORDS: ADRENALINE, TIMOLOL, NEW CORNEAL BLOOD VESSELS, CORNEA, VASOCONSTRICTION, VASODILATOR

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INTRODUCTION

Corneal neovascularization, as well as ocular neovascularization, is one of the causes of blindness worldwide. About 1.4 million people develop corneal blood vessels annually, of which about 12% go blind[6]. Avascular optical structure, the cornea plays a fundamental role in refraction, so that blood vessels developed at this level is always a pathological condition, with a negative impact on visual function and quality of life, which often poses problems of curative treatment.

Adrenergic receptors, first described by Ahlquist in 1948, are divided into two categories: alpha-adrenergic receptors (α receptors) and beta-adrenergic receptors (β receptors)[7]. For alpha-adrenergic receptors, the α1 subtypes were highlighted with postsynaptic and α2 localization, located predominantly presynaptic, but also postsynaptic and extrasynaptic[8,9,10]. Alpha1 adrenergic receptors are found in the vascular smooth muscle, and their stimulation causes vasoconstriction[11]. Stimulation of β2 adrenergic receptors causes vasodilation by relaxing the smooth arteriolar and venous muscles[12]. Although most studies in the literature show that β2 adrenergic receptors are expressed at the vascular level, there are also studies that state the presence of β1 adrenergic receptors[13,14].

Adrenaline or epinephrine is a catecholamine that has agonist effects on all types of adrenergic receptors, the final response depending on the types of receptors to which they bind and their density at the cell surface. Where there is a higher density of α1 adrenergic receptors, adrenaline leads to vasoconstriction[15], while in territories with an increased density of beta2 adrenergic receptors adrenaline causes vasodilation[16]. Adrenaline causes vasodilation and lowering of blood pressure in small doses, while high doses cause vasoconstriction and increased blood pressure[17,18].

Timolol is a non-selective beta-blocker of β-adrenergic receptors, with no notable intrinsic sympathomimetic action. Blood flow from the root of the iris, ciliary body, and choroid is significantly reduced by the topical conjunctival application of timolol 0.25%. The decrease in the rate of aqueous humor is not due to the inhibition of ATPases, carbonic anhydrase, β-adrenergic receptors, or prostaglandin receptors, but is related to decreased

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blood flow to the root of the iris, probably correlated with the concentration of dopamine at this level[19]. Timolol may have local anesthetic effects, and systemically it is a beta-blocker without cardioselectivity[20].

In 1985, Nielsen C.B. and Nielsen P.J. stated that timolol administration inhibits the secretory mechanism of the endothelial pump by blocking endothelial β-adrenergic receptors, resulting in an increase in corneal thickness[21]. Blockade of β-adrenergic receptors of corneal epithelial and endothelial cells by timolol results in decreased intracellular cyclic adenosine monophosphate (cAMP) and inhibition of protein kinase A activity, leading to an increase in corneal thickness[22].

Timolol (non-selective β-blocker) and betaxolol (selective β1-blocker) caused dose-dependent vasodilation in the ciliary arteries in rabbits, previously treated with Krebs solution with a high concentration of K + ions, which determined their vasoconstriction[23].

There are studies that say that timolol significantly reduces the area of corneal neovascularization in rabbits[24] and that it alters the normal angiogenic response in rat cornea[25].

The aim of the present study was to examine the adrenergic receptors expressed in the new corneal blood vessels, by investigating their reactivity to the administration of adrenaline and timolol.

Future research on ocular neoformation vessels could lead to drug influence of these vessels, with beneficial effects on visual function.

MATERIALS AND METHODS

We started the experiment with a number of 75 Wistar rat pups, aged 15 days, in which we developed a possible model of corneal neovascularization by successive administration of ketamine. The rat pups were injected with a dose of 150mg/kg body weight ketamine, administered intraperitoneally, to obtain corneal neovascularization on days 15, 20, 25, 30, and 35 of life. At the last administration of ketamine to obtain neovascularization, on day 35 of life, the rats that showed neovascularization were selected (figure 1-1) and were divided into batches of 6 eyes with neovascularization/experiment, a total of 19 batches on which substances from the adrenergic, cholinergic and histaminergic fields were tested.

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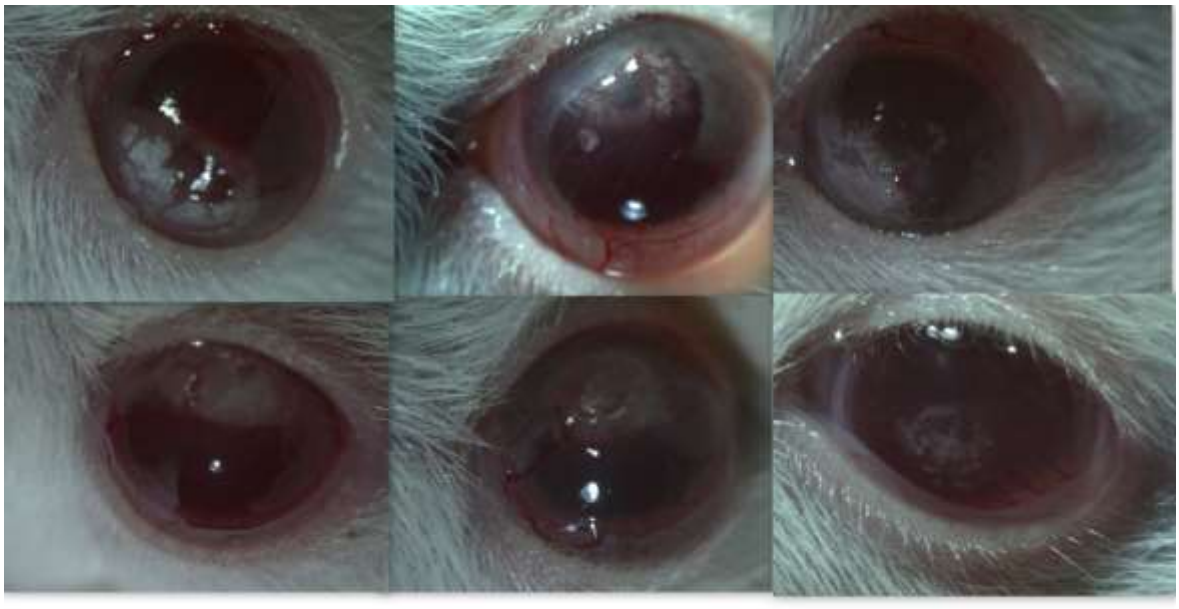

Figure 1-1. Examples of images with new corneal blood vessels

Testing the reactivity of corneal vessels to adrenaline and timolol was performed on rats aged 45 days. A total of 19 rats were divided into 5 groups of animals. We used 2 batches of 3 rats/ 6 eyes with neovascularization /experiment, a batch 5 rats/ 6 eyes with neovascularization /experiment, and 2 batches of 4 rats/ 6 eyes with neovascularization /experiment. The animals were provided by the Biobase of the "Carol Davila" University of Medicine and Pharmacy, Bucharest. Batches of animals were brought to the working laboratory and kept in standard environmental conditions. The animals had ad libitum access to food and water. *The experiments were carried out with the approval of the Ethics Commission of the University of Medicine and Pharmacy "Carol Davila" Bucharest, as well as by the provisions of Directive 2010/63/EU on the protection of animals used for scientific purposes, and their transposition into internal law, by Law No. 43/2014.*

The substances used were: adrenaline Therapy 1 mg/ ml solution for injection adrenaline (SA Therapy, Romania), timolol 0.5% (Timolol, solution 5 mg/ml, eye drops, Eipico Med SRL, Romania), ketamine 10% solution (CP-Ketamine 10%, CP-Pharma, Germany, veterinary medicine), distilled water (Zentiva SA, Romania).

Ketamine was injected intraperitoneally, while distilled water, timolol, and adrenaline were administered topically in conjunctival instillations. For the preparation of the solutions, we used distilled water as a solvent. Adrenaline, 1mg /ml solution, with a molecular weight of 183.204 g /mol, has CM = 5.45mM, approximately 5.5mM /ml solution. The molecular weight of timolol is 316.421 g /mol. We made the appropriate dilution to obtain timolol at 5.5mM /ml, but for testing, we also calculated lower dilutions of timolol, of 2.75mM /ml, respectively 0.55mM /ml.

On day 45 of life (10 days after the last ketamine injection), rats were anesthetized with ketamine at a dose of 150 mg /kg body weight for examination. Distilled water, adrenaline, and timolol were administered topically, at the conjunctival level, at moments T1(30 seconds) and T6 (330 seconds). The measurement of vascular diameters was at the following times: T0(0 seconds), T1(30seconds), T2(90 seconds), T3(150 seconds), T4(210 seconds), T5(270 seconds), T6(330 seconds), T7(390 seconds), T8(450 seconds), T9(510 seconds), T10(570

seconds) and T11(630 seconds). The application moments of the substances to be investigated, T1 and T6, were not analyzed.

Three experiments were performed. In the first experiment, we examined the effect of adrenaline on new corneal blood vessels. In the second experiment, we aimed to influence the effect of adrenaline by administering timolol. In the last experiment, we followed the reactivity of corneal neoformation vessels by administering different concentrations of timolol (5.5mM, 2.75mM, 0.55mM). The calibration of the system was done using Nikon micrometric blade, type B (1Div = 0.1mm = 100µm), J28004 series, and the "Mshot Imaging Analysis System" software.

The anesthetized rats were placed in lateral decubitus, and the eye was kept open by manual traction of the eyelids. The examination was performed for each eye that presented neovascularization, 6 eyes per experiment. The recordings were made from the same distance, for each eye, in order to have the same magnification factor. The shooting was performed every 60 seconds for a period of 630 seconds. For each eye, there are 12 images, saved as jpg files and a video recording. The images were processed in the Mshot Imaging Analysis System program.

The diameter of the neoformation vessels was measured at a chosen point, the same point (s) for each recording made to that eye throughout the recording period. For each image of each measured moment (T0-T11), 3 measurements of the external diameter were performed at the same points, for which the average was calculated.

Variations in vascular caliber were followed, and the measurements were expressed in micrometers. For each moment of each determination the percentage variation of the vascular diameter relative to the moment T0 was calculated according to the following formula:

$$Drel = \left(\frac{Dx - D0}{D0}\right) x100$$

where Drel represents the percentage variation of the vascular diameter from the moment T0, Dx represents the diameter in µm of the vessel at the measured moment, D0 represents the diameter in µm from the moment T0. The increase in diameter/vasodilation is given by the positive values of Drel, while the decrease in diameter/vasoconstriction is represented by the negative values. With the help of Microsoft Office Excel, the data obtained were analyzed

For each moment of the determinations and each group, the mean and the standard error were calculated, after which the T-Student test was applied, the variant for paired samples, comparing Drel with the value from the T0 moment. The results were considered statistically significant if $p < 0.05$.

RESULTS

Experiment 1. The effect of adrenaline on corneal neoformation vessels.

By administering distilled water at time T1, no statistically significant changes in vascular diameter were observed at any of T2-T5 times.

After administration of adrenaline, the mean percentage change in vascular diameter ± standard error was -6.28% ± 2.55 (p=0.057) at time T7, -19.92% ± 4.79 (p=0.008) at time T8, -35.22% ± 8.56 (p = 0.009) at time T9, -46.94% ± 4.34 (p = 0.0001) at time T10 and -46.29% ± 5.78 (p = 0.001) at time T11., for the last 3 values the differences being statistically significant compared to time T0. In conclusion, adrenaline produces statistically significant

vasoconstriction for the last four measurements. The results are presented in: (table 1); (figure 1).

Table 1. Evolution over time of the percentage change of the diameter of the neoformation vessels after the administration of distilled water at the time of T1, subsequently after the administration of adrenaline at the time of T6.

Image capture time (seconds)	The mean percentage change in vascular diameter (%)	Standard error	p-value (t-test)
T0 - 0s (control)	0	0	0
T1 - 30s	Administration of distilled water		
T2 - 90s	1.18	0.84	0.22
T3 - 150s	0.82	0.81	0.35
T4 - 210s	0.4	0.83	0.52
T5 - 270s	-0,01	0.84	0.57
T6 - 330s	Adrenaline 5.5mM administration		
T7 - 390s	-6.28	2.55	0.057
T8 - 450s	-19.92	4.79	0.008
T9 - 510s	-35.22	8.56	0.009
T10 -570s	-46.94	4.34	0.0001
T11 -630s	-46.29	5.78	0.001

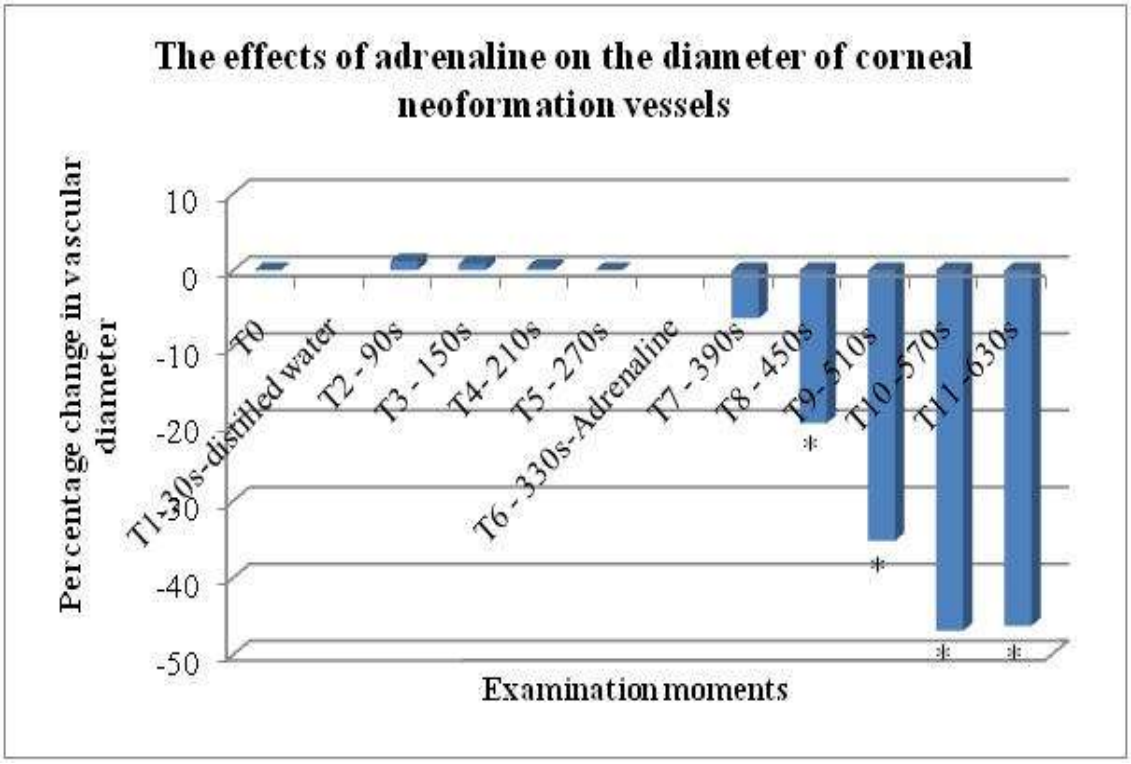

Figure 1. The evolution over time of the mean percentage change of the diameter of the neoformation vessels after the administration of distilled water at time T1, respectively after the administration of 5.5mM adrenaline at time T6. There were statistically significant changes for moments T8, T9, T10, and T11 (* $p < 0.05$).

Experiment 2. The effect of adrenaline after timolol on corneal neoformation vessels

In this experiment, the effect of adrenaline was followed after the previous administration of timolol, a non-selective beta blocker. Administration of 5.5 mM timolol at time T1 resulted in vasodilation from time T2 to time T5, as follows: mean percentage change in vascular diameter ± standard error was 14.82% ± 7.28 (p = 0, 09) at time T2, 20.37% ± 7.59 (p = 0.04) at time T3, 28.63% ± 7.73 (p = 0.01) at time T4 and 40.01 % ± 7.51 (p = 0.003) at time T5. Administration of 5.5 mM adrenaline at time T6 continued vasodilation at time T7, when the mean values of the percentage change in vascular diameter ± standard error were 24.09% ± 8.69 (p = 0.03). However, from T8 to T11, adrenaline caused vasoconstriction, and the mean percentage change in vascular diameter ± standard error was -9.07% ± 5.73 (p = 0.15) at T8, -13.29% ± 6.03 (p = 0.07) at T9, -16.11% ± 5.44 (p = 0.03) at T10 and -17.74% ± 6, 06 (p = 0.03) at time T11. These results are presented in: (table 2); (figure 2). Administration of 5.5mM timolol produces statistically significant vasodilation from time T3 to time T5. The administration of adrenaline produces statistically significant vasoconstriction for the T10 and T11 moments of the examination (p=0.03).

Table 2. Evolution over time of the percentage change of the diameter of the neoformation vessels after the administration of timolol at the time of T1, subsequently after the administration of adrenaline at the time of T6.

Image capture time (seconds)	The mean percentage change in vascular diameter %	Standard error	p-value (t-test)
T0 – 0s (control)	0	0	0
T1 -30s	Timolol 5.5mM administration		
T2 - 90s	14,82	7,28	0,09
T3 - 150s	20,37	7,59	0,04
T4 - 210s	28,63	7,73	0,01
T5 - 270s	40,01	7,51	0,003
T6 - 330s	Adrenaline 5.5mM administration		
T7 - 390s	24,09	8,69	0,03
T8 - 450s	-9,07	5,73	0,15
T9 - 510s	-13,29	6,03	0,07
T10 - 570	-16,11	5,44	0,03
T11 - 630s	-17,43	6,06	0,03

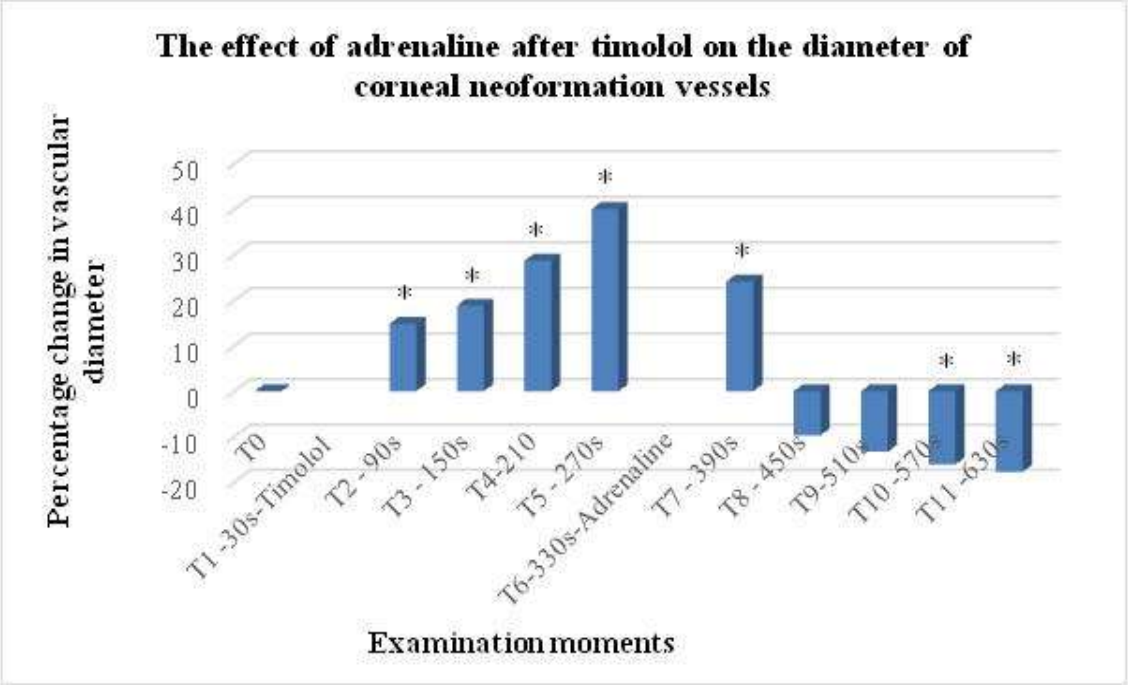

Figure 2. Evolution over time of the mean percentage change of the diameter of the neoformation vessels after the administration of 5.5 mM timolol at the time of T1, respectively after the administration of 5.5 mM adrenaline at the time of T6. There were statistically significant changes for moments T3, T4, T5, T7, T10 and T11 (* p <0.05).

Experiment 3. The effect of timolol on corneal neoformation vessels

In the third experiment, we tested the reactivity of corneal neoformation vessels to the administration of timolol in conjunctival instillations. Due to the results obtained in the previous experiment, in which the administration of timolol had a vasodilating effect, we tested different concentrations of timolol in order to determine if there is a dose-effect relationship. The results are shown in (table 3); (figure 3).

In batch number 1 (timolol 5.5mM) the administration of distilled water at time T1 did not produce statistically significant changes in vascular caliber, so the mean percentage change in the diameter of the neoformation vessels ± standard error was 0.56% ± 1.11 p = 0.63) at time T2, 0.85% ± 1.15 (p = 0.49) at time T3, 2.31% ± 1.37 (p = 0.15) at time T4, and of 2.07% ± 0.93 (p = 0.07) at time T5. At time T6 5.5 mM timolol was administered, when the mean percentage change in the diameter of the neoformation vessels ± standard error was 26.27% ± 3.15 (p = 0.0004) at T7, 34.82% ± 5.6 (p = 0.001) at time T8, 40.1% ± 6.19 (p = 0.001) at time T9, 44.73% ± 7.76 (p = 0.002) at time T10 and 42.67% ± 7.77 (p = 0.002) at time T11. The changes were statistically significant compared to time T0 for all times after timolol 5.5mM administration, with $p < 0.05$. The results are presented in: (table 3); (figure 3).

In batch number 2 (timolol 2.75mM) by administration of distilled water at time T1 there were no statistically significant changes in the caliber of corneal neoformation vessels, and the mean percentage change in vascular diameter ± standard error was -0.69% ± 1.5 (p = 0.66) at time T2, 0.4% ± 0.75 (p = 0.61) at time T3, 0.57% ± 1.01 (p = 0.59) at time T4 and 0.71% ± 1.96 (p = 0.73) at time T5. Timolol administration of 2.75 mM resulted in a mean percentage change in the diameter of the neoformation vessels ± standard error of 24.91% ± 3.86 at T7, 31.61% ± 6.15 at T8, 33, 73% ± 5.81 at T9, 47.48% ± 13.29 at T10 and 43.6% ±

11.84 at T11. The changes given by the administration of timolol 2.75mM were statistically significant compared to the initial time, with $p<0.05$ for all moments from T7 to T11 as follows: $p = 0.001$ at time T7, $p = 0.003$ at time T8, $p = 0.002$ at time T9, $p = 0.01$ at time T10 and $p = 0.01$ at time T11. The results are presented in: (table 3); (figure 3).

In batch number 3 (timolol 0.55mM) the administration of distilled water at the time of T1 did not cause statistically significant changes in vascular caliber, and the mean percentage change in the diameter of the neoformation vessels ± standard error was 1.48% ± 1.08 ($p = 0.22$) at time T2, 0.52% ± 1.4 ($p = 0.72$) at time T3, -0.46% ± 1.57 ($p = 0.77$) at time T4 and of -0.12% ± 1.43 ($p = 0.93$) at time T5. At time T6, 0.55mM timolol was administered, and the mean percentage change in the diameter of the neoformation vessels ± standard error was 27.74% ± 8.41 ($p = 0.02$) at T7, 43.42% ± 9.17 ($p = 0.005$) at time T8, 53.91% ± 12.43 ($p = 0.007$) at time T9, 56.79% ± 11.3 ($p = 0.004$) at time T10 and 57 .82% ± 12.49 ($p = 0.005$) at time T11. The changes were statistically significant compared to the initial time (T0) for all moments after timolol administration of 0.55 mM, with $p < 0.05$. The results are presented in: (table 3); (figure 3).

The changes were statistically significant from baseline (T0) for all moments after administration of 0.55mM timolol, with $p < 0.05$.

Table 3. Evolution over time of the mean percentage change in the diameter of the neoformation vessels after the administration of distilled water at time T1, respectively after the administration of timolol at time T6, compared to the three batches used

Capture time	Timolol 5.5mM			Timolol 2.75mM			Timolol 0.55mM		
	Mean %	Std.Er	p-value	Mean %	Std.Er	p-value	Mean %	Std.Er	p-value
T0 – 0s	0	0	0	0	0	0	0	0	0
T1 -30s	Administration of distilled water								
T2 - 90s	0,56	1,11	0,63	-0,69	1,5	0,66	1,48	1,08	0,22
T3 - 150s	0,85	1,15	0,49	0,4	0,75	0,61	0,52	1,4	0,72
T4 - 210s	2,31	1,37	0,15	0,57	1,01	0,59	-0,46	1,57	0,77
T5-270s	2,07	0,93	0,07	0,71	1,96	0,73	-0,12	1,43	0,93
T6 - 330s	Timolol administration								
T7-390s	26,27	3,15	0,0004	24,91	3,86	0,001	27,74	8,41	0,02
T8 -450s	34,82	5,6	0,001	31,61	6,15	0,003	43,42	9,17	0,005
T9 - 510s	40,1	6,19	0,001	33,73	5,81	0,002	53,91	12,43	0,007
T10-570s	44,73	7,76	0,002	47,48	13,29	0,01	56,79	11,3	0,004
T11-630s	42,67	7,77	0,002	43,6	11,84	0,01	57,82	12,49	0,005

Std.Er.=Standard error

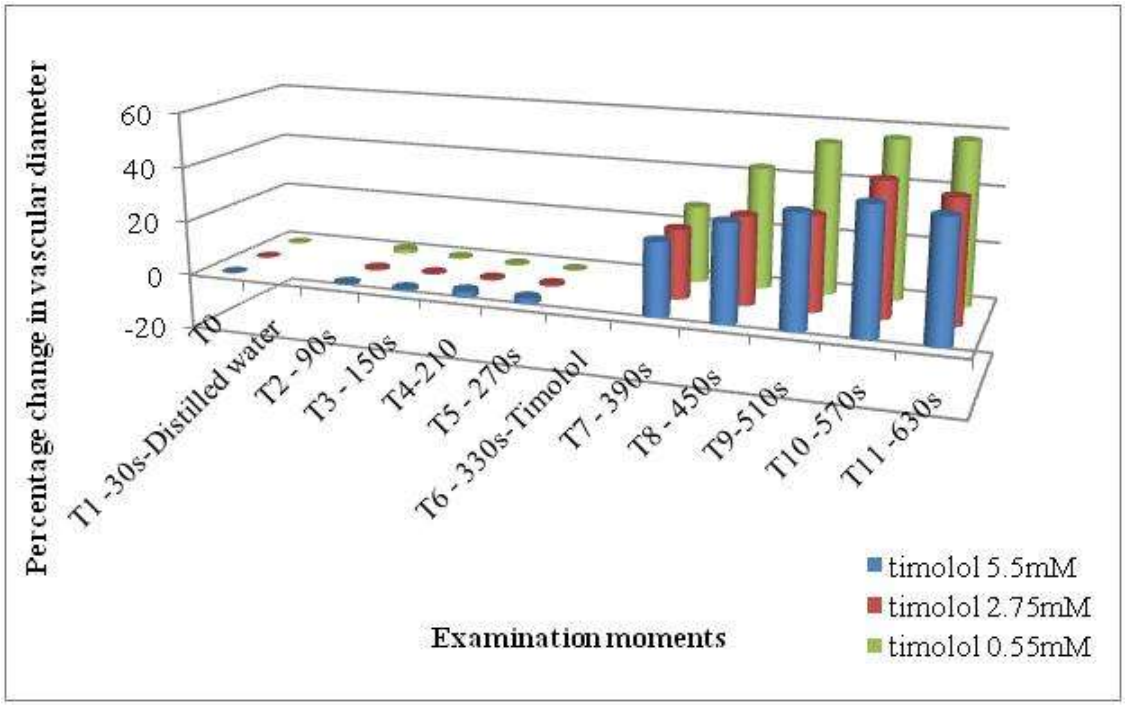

Figure 3. Evolution over time of the mean percentage variation of the diameter of the neoformation vessels after the administration of distilled water at time T1, respectively after the administration of timolol at time T6, compared to the three batches used. Horizontally, the moments at which the determinations were performed are presented, vertically being represented the percentage variation of the vascular diameter.

DISCUSSIONS

The neovascularization model started from the initial study of experimentally induced cataracts with sodium selenite in rat pups. Subsequently, we identified ketamine, used as a general anesthetic, as an inducer of corneal opacities and neoformation vessels, which no longer allowed the evaluation of lens opacities, which agrees with the existing data in the literature[26,27,28,29].

In the first experiment, the administration of adrenaline produced a statistically significant decrease in the diameter of the neoformation vessels, which allows us to state that, at the level of the corneal neovascularization, there are alpha1 adrenergic receptors, whose stimulation produces vasoconstriction.

In the second experiment, timolol administration produced vasodilation in the corneal neoformation vessels, statistically significant vasodilation for T3-T5 moments of the examination. From a pharmacological point of view, it is known that blockade of vascular β-adrenergic receptors causes vasoconstriction, but in this case, timolol produced vasodilation, either by a vasodilating mechanism mediated by nitric oxide or by a mechanism similar to blockers of calcium channels or through a combination of these mechanisms. There are data

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in the literature that state that timolol has a vasodilating action. In 2006, Dong and colleagues studied the vasodilation caused by timolol and betaxolol and showed that they act similarly to calcium channel blockers by lowering the concentration of Ca2 + in vascular smooth muscle, vasodilation that was not inhibited by pre-treatment with an inhibitor of nitric oxide synthase nor by denuding the vascular endothelium[30]. Some studies state that timolol did not cause significant vasodilation in human retinal arterioles, but did cause a small but significant dilation in fresh and cryopreserved porcine retinal arterioles[31]. Administration of 0.5% timolol to rhesus monkeys resulted in iridial vasodilation[32].

The administration of adrenaline at time T6, continues vasodilation at time T7, with statistical significance ($p = 0.03$). From the T8 moment of the measurements, the adrenaline determines the vasoconstriction, with statistical significance for the T10 and T11 moments, produced through the α1 adrenergic receptors.

Due to the unexpected effect of timolol, we could not conclude the distribution of adrenergic receptors, in terms of alpha/beta ratio.

In the third experiment, the administration of distilled water did not produce statistically significant changes in any of the batches presented above and at any of the times examined, while timolol administration resulted in statistically significant changes for all 3 concentrations used and for all times examined T7-T11. Comparing the results obtained above (table 3; figure 3), we can say that the vasodilating effect of timolol was the more intense, the lower the concentration of timolol.

Among the advantages of researching corneal neoformation vessels, we can list their easy and in vivo examination, the cornea is located in the anterior part of the eyeball, respectively the ease of administration, in conjunctival instillations, of the substances to be researched. Also, topical conjunctival administration of medicinal substances has a low risk of systemic adverse reactions, smaller amounts of the active substance are required than in systemic administration, and costs are relatively low. As for disadvantages, we mention the relatively small dimensions of the eyeball in rat pups, as well as the need for general anesthesia for examination.

The limits of the present study would be represented by too small groups, 6 eyes with neovascularization /experiment, requiring the extension of research on larger groups, as well as the extension of testing on a wider range of agonists-antagonists.

CONCLUSIONS

1. Adrenaline causes vasoconstriction achieved through alpha1 adrenergic receptors.
2. Timolol produces vasodilation in the corneal neoformation vessels through a mechanism independent of beta-adrenergic receptors.
3. Timolol caused vasodilation in the corneal neoformation vessels, regardless of the dose administered.
4. The greater the vasodilation produced by timolol, the lower the concentration of timolol administered.

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COMPLIANCE WITH ETHICS REQUIREMENTS

None of the authors had any financial interest in this article. The animal experiments complied with the ethical standards according to the European Directive 86/609 / EEC, as well as the Romanian legislation in force regarding the protection of animals used for scientific purposes.

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