ORIGINAL PAPER

THE PHARMACOLOGICAL ACTION OF CHOLINERGIC SUBSTANCES ON NEW CORNEAL BLOOD VESSELS IN RATS

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Abstract. *This study aimed to evaluate the existence of muscarinic receptors in the new corneal blood vessels, experimentally induced with ketamine in rat pups. The experimental model of neovascularization was performed on 15-day-old Wistar rats, in which 5 intraperitoneal injections of ketamine were administered at a dose of 150 mg/kg body weight at 5-day intervals. Examination of the reactivity of new corneal blood vessels was performed on day 45 of life. Each eye that developed neovascularization was evaluated with a Nikon stereomicroscope, coupled to a Mshot video camera, and the total magnification of the system was 400X. The vascular diameter was measured at a chosen point, the same point /points for each recording made to that eye, and the acquisition of images was performed at set time intervals, every 60 seconds for each eye to be examined, throughout the recording period. The parameters under investigation were the variations of the vascular diameter, and the processing of the obtained data was performed with the help of Microsoft Office Excel. Our results suggest that muscarinic receptors are present in the new corneal blood vessels because the administration of conjunctival instillations of acetylcholine caused statistically significant vasodilation, while atropine, a blocker of muscarinic receptors, antagonized this effect.*

Keywords: *acetylcholine; atropine; new blood vessels; cornea; vasodilator.*

1. INTRODUCTION

Acetylcholine is a neurotransmitter synthesized from acetyl-coenzyme A and choline in the cytoplasm of cholinergic synapses, under the influence of choline acetyltransferase, and is concentrated in the synaptic vesicles where it is stored until release [1]. Acetylcholine is released from the cholinergic nerve endings at rest, as well by stimulation. Acetylcholine is predominantly enzymatically inactivated in the synaptic cleft by specific acetylcholinesterase

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(AchE) and outside the synaptic space by pseudocholinesterase, capable of cleaving other choline esters [2]. Acetylcholine catabolism results in acetic acid and choline.

Two classes of acetylcholine receptors, muscarinic and nicotinic receptors, have been described. Five subtypes of muscarinic receptors are described, denoted from M1 to M5 [3]. Probably, all organs have several types of muscarinic receptors, but in general, at least in some organs, a certain type predominates. M3 receptors predominate in the eye and vascular endothelial cells, although other types of muscarinic receptors are also found [4-6]. M1 and M3 receptors are responsible for vasodilation in the lingual artery in dogs, which is conditioned by the existence of an intact endothelium [7]. Stimulation of M1 and M3 receptors in human pulmonary arteries causes vasodilation by endothelial mechanisms [8]. By binding acetylcholine to muscarinic receptors in the vascular endothelium, there is an increase in the production of nitric oxide and prostaglandins, with the relaxation of vascular smooth muscle and vasodilation [9-10].

Atropine is a natural alkaloid obtained from Atropa belladonna, being a non-selective muscarinic antagonist with parasympatholytic action. Atropine is a blocker of muscarinic receptors, competing with acetylcholine (the main neurotransmitter of the parasympathetic nervous system), which relaxes the smooth gastrointestinal, genitourinary, or bronchial muscles, decreases the secretion of the salivary glands and sweat glands, causing dry mouth and dry skin [11]. At the level of the heart, atropine decreases the intensity of vagal reflexes, leading to an increase in heart rate [12-13]. Atropine is a racemic mixture of two isomeric forms, *dextro*- and *levo*-hyoscyamine, its activity being almost entirely due to the *levo*-isomer [14]. Atropine is well absorbed from the conjunctival mucosa during topical ocular administration [15].

The cornea is an avascular optical structure under normal conditions. Corneal neovascularization, as well as neovascularization in the ocular structures, is a pathological condition that frequently causes problems of management and curative treatment because it can lead to impairment of visual function. Currently, the treatment of ocular neoformation vessels includes laser treatment, treatment with VEGF inhibitors, and, last but not least, corneal transplantation in the case of corneal vascularization [16-18]. The study of the various pharmacological receptors in the new blood vessels and how they can be medically influenced may represent a future method of treatment to regress / or inhibit them.

This study aimed to evaluate corneal neoformation vessels to investigate their possible reactivity to acetylcholine. In this sense, acetylcholine was administered in conjunctival instillations, and the evolution of the vascular diameter under the effect of this substance was followed. Obtaining dynamic changes in the vascular diameter was considered proof of the existence at this level of the corresponding pharmacological receptors. By administering acetylcholine after atropine, a muscarinic receptor blocker, it was possible to analyze what type of pharmacological receptors exist in these new blood vessels. At a later stage, the question arose whether at the level of these vessels there is a phase control or a tonic control exerted by the active substances in the cholinergic field. For this, the effect of atropine on the motility of new blood vessels was studied. It was considered that if atropine changes the vascular diameter, there is a tonic control of acetylcholine, and if atropine does not change the vascular diameter, the control exerted by acetylcholine is a phase control.

2. MATERIALS AND METHODS

The animals used were Wistar rats in which, a possible experimental model of corneal vascularization was previously obtained by repeated injections with ketamine, administered intraperitoneally, at a dose of 150 mg/kg body weight. We started the experiment with a number of 75 rat pups in which, starting from the 15th day of life, by 5 repeated injections of ketamine, at an interval of five days between administrations, we obtained optimal corneal neovascularization for analysis of the reactivity of new corneal blood vessels. On the occasion of the fifth administration of ketamine, rats with corneal blood vessels were selected and divided into groups of 6 eyes with neovascularization/experiment, a total of 19 groups on which substances from the adrenergic, cholinergic, and histaminergic fields were tested. Corneal blood vessel reactivity testing was performed on 45-day-old rats, using 3 batches for the evaluation of cholinergic substances, 2 batches consisting of 4 rats/6 eyes with neovascularization/experiment, and a group of 3 rats/6 eyes with neovascularization/ experiment, a total of 11 rats. The experimental animals were provided by the Biobase of the University of Medicine and Pharmacy "Carol Davila" Bucharest. The animals were brought to the working laboratory, where they were accommodated and fed according to the animal welfare conditions used in the experiments. 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 ketamine, 10% solution (CP-Ketamine 10%, CP-Pharma, Germany, veterinary medicine), distilled water (Zentiva SA, Romania), acetylcholine (Acetylthiocholine iodide, Merck, Germany), and atropine sulfate Takeda1mg /mL solution for injection (TAKEDA GmbH – Germany). Ketamine was administered intraperitoneally, and distilled water, acetylcholine, and atropine were administered in conjunctival instillations.

We administered equimolar solutions for each batch of experiments. Distilled water was used as a solvent for the preparation of the administered substances. We used molar concentration to express the concentration of substances. Atropine sulfate has a molecular weight of 676.8 g/ mol and a molar concentration of approximately 1.5 mM/mL. We prepared, by successive dilutions, a solution of atropine with a concentration of 150 μM/ mL. The molecular weight of acetylcholine iodide is 289.18 g/ mol, and to prepare a solution with a molar concentration of 1.5 mM, we used 0.43 mg of the pure substance to 1 mL of distilled water, and then we used successive dilutions to reaches a molar concentration of acetylcholine solution of 150 μM.

The substances to be investigated, acetylcholine and atropine, were administered by conjunctival instillation at times T1 = 30 seconds and T6 = 330 seconds. The moments chosen for measuring vascular diameters were: 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 moments when the substances to be investigated, respectively the moments T1 and T6, were not analyzed.

Three experiments were performed. In the first experiment, distilled water was administered at time T1 and acetylcholine at time T6, in the second experiment, atropine was administered at time T1 and acetylcholine at time T6, and in the third experiment, distilled water was administered at time T1 and atropine at time T6.

Each eye was evaluated with a Nikon stereomicroscope, model SMZ 1270, coupled to a Mshot video camera, model MSX2-C, connected to a computer, the total magnification of

the system being 400X, which allowed the measurement of neovascular diameter in micrometers. The system was manually calibrated using the "Mshot Imaging Analysis System" software and the Nikon micrometric calibration blade, type B (1 Div = 0.1 mm = 100 μm), J28004 series.

The anesthetized rats were placed in a restraint device, lying on the side, to have optimal access to the eyeball, and the eyelid slit was kept open by manual traction. The examination was done for each eye with neovascularization, 6 eyes per experiment. Image recording was performed at set intervals of 60 to 60 seconds for each eye to be examined throughout the recording period. To have the same magnification factor, the recordings were made from the same working distance for each eye, and then the data was processed. For each eye, a video recording of 630 seconds was made, saved in MP4 format, as well as 12 images saved as jpg files. The images were processed in the Mshot Imaging Analysis System program. For each eye, respectively for each image, of the chosen moment T0-T11, 3 diameter measurements were performed at the same points, for which the average was calculated.

The parameters under investigation were the variations of the vascular diameter (vasodilation/vasoconstriction), and the measurements were expressed in micrometers. For each moment of each determination, the average of the percentage change in the outer diameter of the vessel reported at time 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. Positive values of Drel represent increases in diameter or vasodilation, while negative values represent a decrease in vascular diameter or vasoconstriction. The data obtained were analyzed using Microsoft Office Excel.

For each group and each moment of the determinations, the mean and the standard error were calculated. Using the T-Student test, the variant for paired samples (2-tailed, 1 paired), the statistical significance of the difference between each moment and the T0 moment was calculated, comparing Drel with the value from the T0 moment. The results were considered statistically significant if $p < 0.05$.

3. RESULTS AND DISCUSSIONS

Experiment 1. THE EFFECT OF ACETYLCHOLINE ON THE NEW CORNEAL BLOOD VESSELS

The administration of distilled water did not cause statistically significant changes. The administration of acetylcholine 150μM at time T6 led to vasodilation from time T7 to time T11, the average percentage change in vascular diameter reported at time T0 being 13.63% ± 3.31 at time T7, 11.72% ± 2, 88 at T8, 13.08% ± 2.8 at T9, 15.65 % ± 2.6 at T10 and 17.86% ± 2.19 at T11. The changes are statistically significant, with p between 0.001 and 0.0004. The data are presented in Table 1 and Fig. 1.

Table 1. Evolution over time of the average percentage change of vascular diameter after administration of distilled water at time T1, subsequently after administration of acetylcholine 150 µM at time T6.

Image capture time (seconds)	Average percentage change in vascular diameter %	Standard error	p-value (t-test)
T0 - 0 s	0.00	0.00	0.000
T1 - 30 s- administration of distilled water			
T2 - 90s	5.95	1.96	0.770
T3 - 150s	1.57	2.05	0.470
T4 - 210s	2.52	1.36	0.120
T5 - 270s	1.99	1.62	0.270
T6 - 330s- administration of acetylcholine			
T7 - 390s	13.63	3.31	0.009
T8 - 450s	11.72	2.88	0.009
T9 - 510s	13.08	2.8	0.005
T10 -570s	15.65	2.6	0.001
T11 -630s	17.86	2.19	0.0004

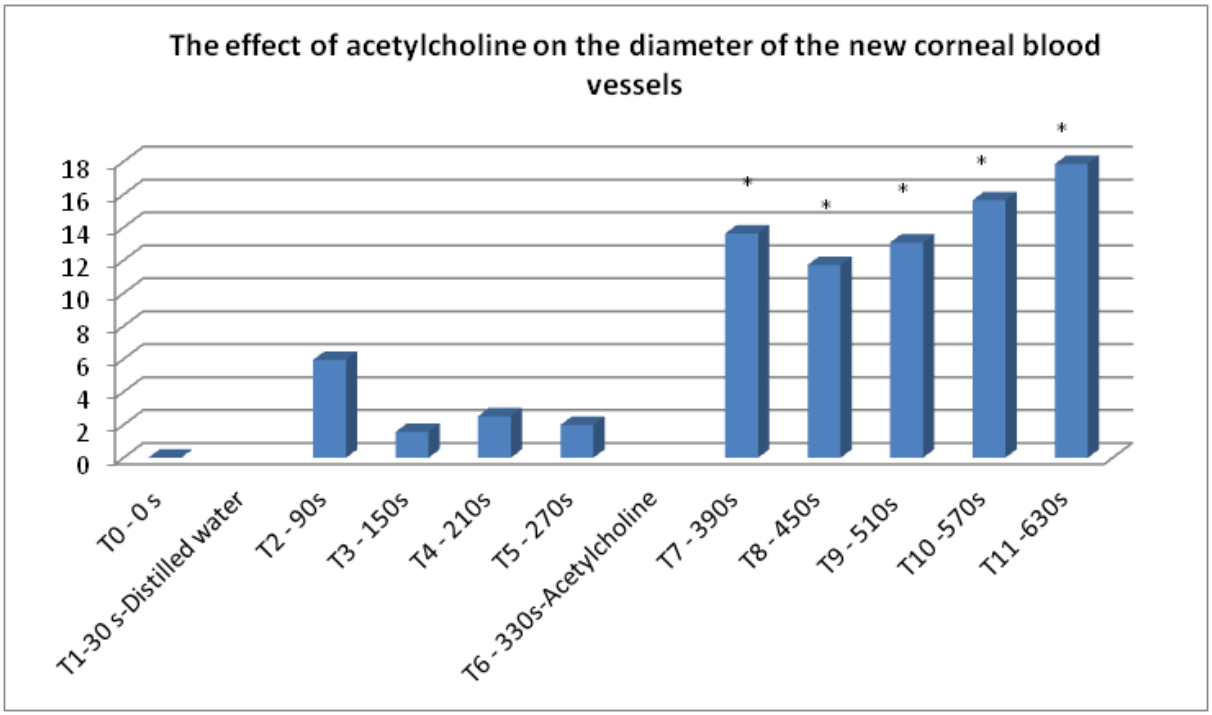

Figure 1. The evolution over time of the average percentage change of the vascular diameter after the administration of distilled water at time T1, respectively the administration of acetylcholine 150 µM at time T6, compared to time T0 of the recording. The horizontal axis represents the moments when the measurements were performed, the vertical axis or the height of the columns represents the percentage of the change of the vascular diameter related to the T0 moment of the recording. Statistically significant changes were in the moments T7-T11(* p <0.05).

Experiment 2. THE EFFECT OF ACETYLCHOLINE AFTER ATROPINE ON THE NEW CORNEAL BLOOD VESSELS

The percentage change in corneal vascular diameter in Experiment 2 is shown in Table 2 and Fig. 2. Neither the administration of atropine at time T1 nor the administration of acetylcholine at time T6 caused statistically significant changes at any of the times examined.

Table 2. Evolution over time of the average percentage change of vascular diameter after administration of atropine 150µM at time T1, subsequently after administration of acetylcholine 150µM at time T6.

Image capture time (seconds)	Average percentage change in vascular diameter %	Standard error	p-value (t-test)
T0 - 0 s	0.00	0.00	0.00
T1 - 30 s- administration of atropine			
T2 - 90s	3.21	2.27	0.21
T3 - 150s	3.00	3.12	0.38
T4 - 210s	4.52	2.99	0.19
T5 - 270s	4.77	2.81	0.15
T6 - 330s- administration of acetylcholine			
T7 - 390s	6.19	3.84	0.16
T8 - 450s	3.84	3.87	0.36
T9 - 510s	2.44	4.06	0.57
T10- 570s	2.55	5.27	0.64
T11- 630s	4.43	4.51	0.37

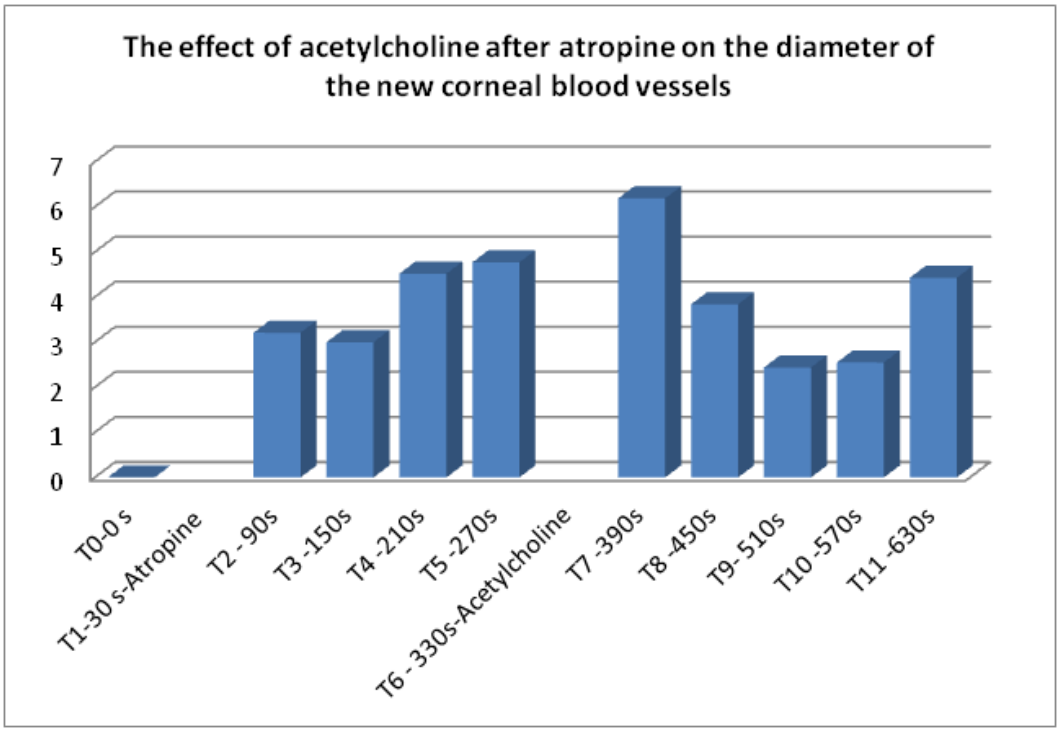

Figure 2. The evolution over time of the average percentage change of the vascular diameter after the administration of atropine 150 µM at time T1, respectively the administration of acetylcholine 150 µM at time T6, compared to time T0 of the recording. The horizontal axis represents the moments when the measurements were performed, the vertical axis or the height of the columns represents the percentage of the change of the vascular diameter related to the T0 moment of the recording.

Experiment 3. THE EFFECT OF ATROPINE ON THE NEW CORNEAL BLOOD VESSELS

In our experimental conditions, neither distilled water nor atropine caused statistically significant changes in vascular diameter. The above data are presented in Table 3 and Fig. 3.

Table 3. Evolution over time of the average percentage change of neovascular diameter after administration of distilled water at time T1, then after administration of atropine 150µM at time T6.

Image capture time (seconds)	Average percentage change in vascular diameter %	Standard error	p-value (t-test)
T0 - 0 s	0.00	0.00	0.00
T1 - 30 s - administration of distilled water			
T2 - 90s	3.20	1.50	0.08
T3 - 150s	2.95	1.67	0.13
T4 - 210s	3.02	2.19	0.22
T5 - 270s	1.70	2.91	0.58
T6 - 330s- administration of atropine			
T7 - 390s	4.43	2.35	0.11
T8 - 450s	5.83	2.51	0.06
T9 - 510s	6.43	2.65	0.06
T10 -570s	4.10	2.47	0.15
T11 -630s	4.26	2.99	0.21

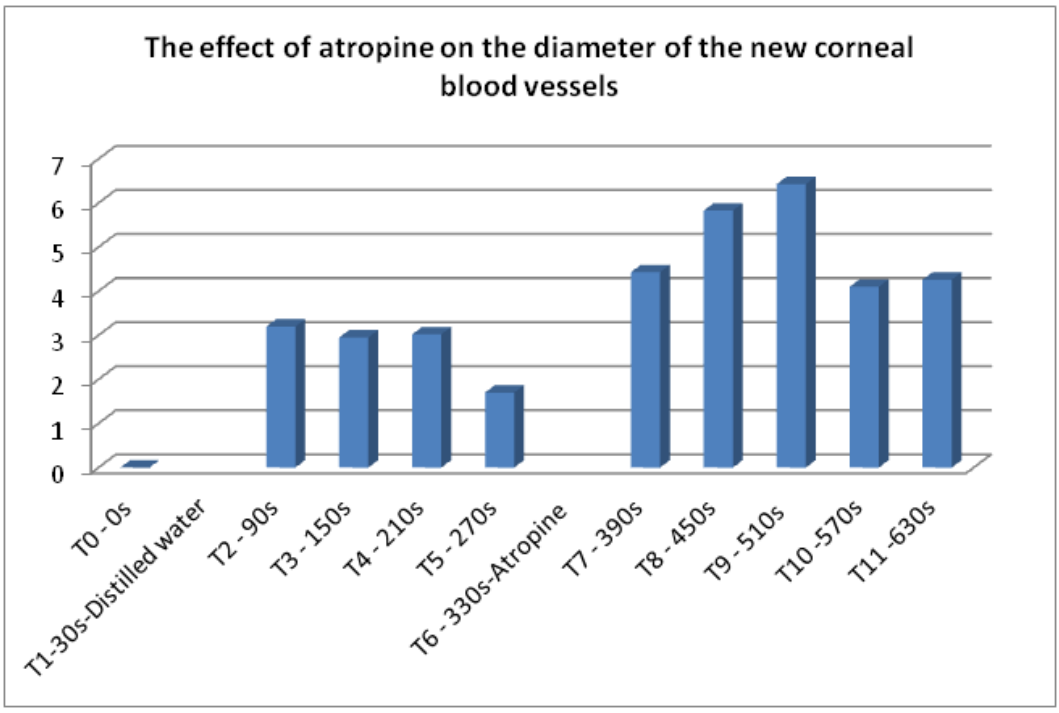

Figure 3. The evolution over time of the average percentage change of the vascular diameter after the administration of distilled water at time T1, respectively the administration of atropine 150 µM at time T6, compared to time T0 of the recording. The horizontal axis represents the moments when the measurements were performed, the vertical axis or the height of the columns represents the percentage of the change of the vascular diameter related to the T0 moment of the recording.

The neovascularization model was developed during experimental research on the occurrence of cataracts in rat pups. Induction of lens opacity was performed in 15-day-old rat pups by subcutaneous injection of sodium selenite, and ketamine was used as a general anesthetic at a dose of 150 mg/kg body weight to examine lens changes. At the second microscopic examination, so after two successive administrations of ketamine, administered intraperitoneally, for anesthetic purposes, the examination of cataracts was no longer possible due to changes in corneal transparency. Subsequent experiments concluded that corneal transparency changes and neovascularization, at this level, were given by ketamine. Our statements agree with the data described in the literature [19-22].

In the first experiment of this study, the administration of acetylcholine in conjunctival instillations produced statistically significant vasodilation in the corneal blood vessels. This allows us to state that these blood vessels contain cholinergic receptors, probably of the muscarinic type, as the entire systemic vascular territory. There are numerous studies on the effects of acetylcholine in various vascular areas, both in humans and animals.

Acetylcholine produces vasodilation in dogs, in the lingual arteries, with intact vascular endothelium, vasodilation probably achieved through muscarinic M1 and M3 receptors [7]. In human pulmonary arteries with an intact endothelium, M1 and M3 receptors mediate acetylcholine-induced vasodilation, while M3 receptors mediate acetylcholine-induced vasoconstriction when the vascular endothelium is denuded [8]. Acetylcholine produces, in murine, vasodilation in the ophthalmic arteries with intact endothelium and vasoconstriction in the denuded ophthalmic arteries [23]. Acetylcholine produces, in rats, dose-dependent vasodilation in the mesenteric arteries with an intact endothelium, while on denuded arteries, only high doses of acetylcholine cause vasodilation, arterial relaxation being achieved through muscarinic M1 and /or M3 receptors [6]. Acetylcholine-induced endothelial-dependent relaxation has also been reported in perforating branches of the human internal mammary artery, predominantly via M1-type receptors [24]. Studies in canines have shown that acetylcholine has led, through M1 and M3 type muscarinic receptors, to the relaxation of intranasal veins and the contraction of extranasal veins [25]. In newborn pigs, acetylcholine produces cerebral vasoconstriction through M1 receptors [26], while in humans and bovines, it led to vasodilation in the brain microvascularization through muscarinic M5 receptors [27-28]. The effects of acetylcholine on vascular motility may differ depending on the investigated vascular territory, but also depending on the species or the quality of the vascular endothelium (Table 4).

Table 4. Effects of acetylcholine on vascular motility in different territories and species

VD	VC	Territory, species, type of receptors
+		isolated canine lingual arteries; M1, M3[7]
+		murine retinal arterioles; M3 [29]
+		ophthalmic arteries in mice; M3 [30]
+	+	murine ophthalmic arteries; M3: endothelium-dependent vasodilation and endothelium-independent vasoconstriction [23]
+		the choroid of pigeons; M3 [31]
+		rat mesenteric arteries; M1, M3 [6]
+	+	In canines, intranasal venodilation, extranasal veins contraction; M1, M3 [25]
+		human pulmonary arteries; M1, M3 [8], [32]
	+	human pulmonary arteries with endothelial denudation; M3[8]

Table 4. (*continued*)

VD	VC	Territory, species, type of receptors
+		human pulmonary veins; M1 [33]
+		rat and rabbit pulmonary artery; M3 [32]
	+	pulmonary vasoconstrictions in rabbit- M1, in humans-M3, and the dog-M1, M2 [32]
+		perforating branch of the human internal mammary artery; M1 [24]
+		coronary arteries in mice; M3 [34]
+		bovine and human intracortical arterioles; M5[27]
+		in mice, in the cutaneous, skeletal muscle, and renal interlobar arteries; M3 [35]
	+	cerebrovascular constriction in the newborn pig; M1[26]
+		human brain microvascular endothelial cells; M5 [28]
+		bovine and/or human intracortical arterioles; M5 [27]
+		femoral and aortic artery in the mouse; M3 [36]
+		cat femoral artery; M3 [37]

VD=vasodilation; VC=vasoconstriction

In response to acetylcholine-induced vascular relaxation, vascular smooth muscle cells may release nitric oxide only if the vascular endothelium is intact, while vascular endothelial alteration is accompanied by vasoconstrictor effects due to lack/ alteration of endothelium-derived relaxation factor [38]. Acetylcholine binds to muscarinic receptors present in the vascular endothelium, which leads to an increase in nitric oxide production, which causes vascular smooth muscle relaxation [9]. In mammals, nitric oxide causes vasodilation through increased synthesis of cGMP [39]. Nitric oxide inhibits voltage-gated Ca^{2+} channels and decreases cytosolic Ca^{2+} concentration, causing vasodilation [40]. In most studies, acetylcholine causes vasodilation dependent on an intact endothelium.

In the second experiment of our study, the administration of acetylcholine after atropine did not produce the relaxation of the corneal blood vessels. The administration of atropine produced the relaxation of the new corneal blood vessels, but the vasodilation remained without statistical significance, and the administration of acetylcholine after the administration of atropine also remained without statistical significance. The above statement that muscarinic receptors are present in new corneal blood vessels has been confirmed by the fact that atropine, a non-selective muscarinic receptor antagonist previously administered to acetylcholine, has blocked its vasodilating effect.

Similarly, atropine has blocked the vasodilating effect of acetylcholine in human pulmonary veins [33], in retinal arterioles in wild-type mice [29], in ophthalmic arteries in mice [30], or in cutaneous, muscular, and renal interlobar arteries in mice [35]. Atropine has antagonized the vasodilating effects of acetylcholine in the perforating branches of the mammary artery (in humans) [24], or the femoral artery in cats [36]. Also, in the coronary arteries and aorta in wild-type mice, atropine reduced the relaxation given by acetylcholine [34] or blocked the cerebrovascular constriction given by acetylcholine in the newborn pig [26]. All these studies demonstrate the existence of muscarinic receptors in different vascular territories, atropine antagonizing the vascular effects of acetylcholine.

In the third experiment, the administration of atropine did not produce statistically significant changes in the diameter of the new corneal blood vessels, so we can say that at their level, there is no tonic cholinergic control, as there is no other vascular territory, but a phase control by acetylcholine possibly existing in the bloodstream is possible.

Also, atropine administration remained without vascular effects in the human retinal arteries (in basal conditions) [41], while in the rat mesenteric arteries, atropine produces endothelium-dependent vasodilation, but also vasodilation dependent on vascular smooth muscle cells (mediated by Ca^{2+} ion-dependent processes) [42]. Both in the vascular endothelium and the vascular smooth muscle, atropine produces vasodilation partially mediated by voltage-gated K^+ channels [43]. According to the data presented, atropine exerts vascular effects through multifactorial mechanisms, probably not fully elucidated.

4. CONCLUSIONS

In conclusion, the novelty of our study would be the testing of the reactivity of corneal blood vessels on a model of corneal vascularization obtained with ketamine. Also, to date, numerous studies are showing the vascular effects of active cholinergic substances in different vascular territories, in many species, including humans, but we have not found studies related to research on these substances in new corneal blood vessels.

In our experimental conditions, at the level of ketamine-induced corneal neoformation vessels, in rat pups, there are muscarinic receptors, as it results from the pharmacodynamic reactivity highlighted in our experiment. At the level of the new corneal blood vessels, there is no tonic type cholinergic control, but possibly a phase-type cholinergic control, because atropine does not change the diameter of these blood vessels when given alone. Future research on ocular neoformation vessels could lead to drug influence of these vessels, with beneficial effects on visual function.

Compliance with ethics requirements: *The animal experiments complied with the ethical standards according to Directive 2010/63/EU, as well as the Romanian legislation in force regarding the protection of animals used for scientific purposes.*

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