Effects of velnacrine maleate in the leukocyte-endothelial cell interactions in rat cremaster microcirculatory network

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Abstract. The expression of acetylcholinesterase in proinflammatory cells has supported the hypothesis that this protein plays a role in intercellular adhesion. Previous results of our group show that velnacrine, an acetylcholinesterase inhibitor, increases the number of adherent leukocytes in *post*-capillary venules of Wistar rats' mesentery muscle. This works intends to evaluate the local application of velnacrine and acetylcholine in the inflammatory response at the microcirculatory network by studying the leukocytes/endothelium interactions in *post*-capillary venules of Wistar rats' cremaster muscle. The number and the speed of the rolling leukocytes, the number of adherent leukocytes and hemodynamic parameters were determined and also the plasma levels of IL-1β. The results have shown that in the presence of velnacrine there is a significant increase of the rolling leukocytes (1.28 ± 0.39 *vs* 1.93 ± 0.20), as well as an increase of the adherent ones (0.86 ± 0.72 *vs* 1.02 ± 0.83). When acetylcholine is adding with velnacrine the number of rolling and adherent leukocytes decreases without changing the IL-1β plasma circulation induced by velnacrine. Our results suggest an anti-inflammatory role induced by ACh without full efficiency because the rolling leukocytes velocity was reduced without changes in the plasma level of IL-1β.

Keywords: Inflammation, leukocytes, endothelium, velnacrine, acetylcholine

1. Introduction

Acetylcholinesterase (AChE) is a protein with enzymatic function that is found anchored to the membranes of various cellular types, such as erythrocytes, platelets, leukocytes and endothelial cells, by different kinds of tails [8,11]. Structurally, it is presented as a globular protein with one ridge where the active site is found. The active site is composed by some functional places where the catalytic amino acid triad is located, as well as a secondary place, assigned as peripheral anionic center, for the acetylcholine (ACh) linking, the AChE's natural substrate [23]. The classical function of AChE is to regulate the duration of ACh-mediated neurotransmitter action on the postsynaptic receptors in cholinergic synapses, hydrolysing acetylcholine to acetate. Until now the presence of acetylcholinesterase in blood and in endothelial cells was identified without a description of is physiological function. Beyond the catalytic functions, other ones like intercellular adhesion were also described [22].

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The presence of AChE in erythrocytes is described since 1973 [40]. Although is function in the erythrocytes is already unknown. Tang et al. [36] demonstrated the presence of muscarinic type M1 receptors for ACh in erythrocytes. Other studies demonstrated that the presence of ACh in erythrocytes suspensions induces aggregation and decreases the hemoglobin affinity for oxygen [31]. It was also observed an increase of the erythrocyte deformability, nitric oxide and is metabolites concentrations [6,7,31]. The mechanisms leading to these effects are already unknown.

Other studies have demonstrated that both endothelial cells and T lymphocytes produce ACh [18,19] and [20]. At the microcirculation level, when red blood cells are oxygenated, NO previously linked to hemoglobin is released [16]. The red blood cells can behaviour either has NO scavenger or NO transporters, controlling the tissue oxygenation. In hypoxia conditions ACh is released from endothelial cells inducing NO synthesis and vasodilation in order to facilitate the blood flow to the tissues [5,12]. Studies of Borovikova [4] show the anti-inflammatory effect of ACh in the rats' systemic inflammatory response to endotoxin.

In the microcirculation level where the leukocyte/endothelial cell interaction is characteristic, recent studies have demonstrated that the velnacrine maleate, an inhibitor of AChE, previously used in the treatment of the Alzheimer illness, promotes an increase in the number of adherent leukocytes to the endothelium of post-capillary venules of the Wistar rats' mesentery in the presence of lipopolisaccharide (LPS) [34]. However, the systemic administration of velnacrine maleate lead to an increase of the number of leukocytes in rolling in the endothelium of *post*-capillary venules of Wistar rats cremaster muscle, without alteration of the adherent leukocytes [32].

The interaction of leukocytes with the endothelium facilitates its migration from the circulation to the tissues, thus allowing a bigger effectiveness of actuation in inflammatory states. There are few different stages of the migration of the leukocytes to the tissues: margination, rolling, adhesion, *diapedesis* and migration [9].

The many stages of the process of leukocytes/endothelial interaction are regulated by cytokines, as for example, the tumoral necrosis factor (TNFα) and interleukine-1β (IL-1β). The cytokines TNFα and IL-1β are synthesised and secreted by leukocytes (as T cells) and activated the endothelial cells, being the cyclic adenosine $3'$, $5'$-monophosphate (cAMP) one of the intracellular messengers involved in the synthesis and secretion of these cytokines [17,33].

The rolling state is a transitory state, unless the leukocytes are activated by molecules that provoke an adhesion to the endothelium. Thus, adhesion occur when there is a reduction of the amount of L-selectin and an increase of the molecules of the integrin family [33]. An important point is that many of adhesion molecules can mediate adhesion in the absence of flow, but are not able to mediate the attachment in the presence of flow. This suggests that there is a "no adhesion" state for cell attachment in flow [10]. During an inflammatory state, the leukocytes adhesion to endothelial cells is followed by *diapedesis* that consists in the migration of the adherent leukocytes to the junctions of the endothelial cells. During this migration there is a reduction of the adhesive interactions in the posterior part so that the leukocytes can pass to the surrounding tissues. This stage is called transmigration.

The aim of this work was to study of the influence of an acetylcholinesterase inhibitor, velnacrine maleate, in the inflammatory response at the microcirculatory network. For such, the leukocytes/endothelium interactions in post-capillary venules of Wistar rats cremaster muscle were studied, with the monotorization of the number of rolling leukocytes and the speed of rolling, the number of adherent leukocytes, the diameter of venule and the neighbouring arteriole. At the systemic level it was registered the cardiac frequency and the systolic and diastolic arterial pressures. Acetylcholine, the

natural substrate of acetylcholinesterase was also applied in order to verify if the possible inflammatory effect induced by the velnacrine, could be abolished. Knowing that some stages of the interaction process of the leukocytes with the endothelial cell are regulated by cytokines, the plasma concentration of IL-1β in blood samples of the studied Wistar rats was also determined.

2. Materials and methods

2.1. Animal preparation

The animals used in this study received human care in accordance with the Directive of the European Community n°86/609/CEE that mentions the protection of animals used for economic ends and other scientific ends.

In the experimental procedures 48 Wistar male rats (HsdBrlHan:Wist, Harlan Iberica), with a average weight of 279 ± 21.7 g, were kept in an animal facility with a 12 h light/dark cycle and housed in cages in a temperature controlled room. All animals were kept on a diet standard rat food and water *ad libitum* until 24 hours before the experiment. For the surgical procedures and microcirculatory measurements, the rats were anesthetized intraperitoneally with 1,5 g/Kg body weight urethane (Sigma-Aldrich) and intramuscular 50 mg/Kg body weight ketamine (Pfizer, Parke Davis) after 20 minutes. Body temperature was maintained between 35–37°C with auto-regulable heating platform. A tracheostomy was performed to maintain the animal in spontaneous breath during all the experiment. For drug administration, the right jugular vein was cannulated with polyethylene tubing; the left carotid artery was also cannulated for the measurement of mean arterial pressure and cardiac frequency. For the measurement of the arterial pressure the catheter was connected to a pressure transducer TRANSPAC® (Abbot, Sligo, Republic of Ireland); being the cardiac frequency and the systolic and diastolic pressures measured and registered through *hardware* and *software* system PowerLab/400 (AD Instruments, Castle Hill, Australia).

The preparation of cremaster for intravital microscopy is made in a Plexiglas support in accordance with [15]. Using an electrocauter a small incision in scrotum is made and the right testicule is brought outside. Then the conjunctive tissue that surrounds the cremaster is removed and once again with the aid of the electrocauter an incision in cremaster is made, fixing it to the support with silk sutures.

2.2. Intravital microscopy

The support with the animal is then placed in an inverted microscope Leitz® FLUOVERT FU (Leica, Heerbrugg, Whisker) adapted for intravital microscopy, equipped with a $40\times$ objective and a $10\times$ ocular. The images are caught through a video camera CCD-IRIS CXC-107AP (Sony), visualised in a colour monitor PVM 1440QM (Sony) and recorded in a video SVHS AG-MD830 (Panasonic) for later visualisation. After placing the support with the rat in the microscope, the venous catheter must be connected to a perfusion system with NaCl 0.9% pH 7.4, in a speed of 4 ml/h and the arterial catheter to the pressure transducer. The cremaster must be placed in perfusion of NaCl 0.9% pH 7.4 being the excess of liquid removed with a vacuum system. The preparations were allowed a 45 min *post-surgical* equilibration period.

After the equilibration period the cremaster observation is started. Post-capillary venules with 20–35 μm diameters were chosen for the quantification of the parameters previously defined. One notices that the register of venules must be of at least 1 min and the register of the arteriole of 30 s. At the same

time the cardiac frequency and the systolic and diastolic arterial pressures are also registered. From the recorded images the interactions leukocytes/endothelial cells are quantified by the parameters already established [28]. They count then the number of leukocytes in rolling and the rolling speed, the number of adherent leukocytes, measures the diameter of the venules and respective neighbouring arteriole is measured, and these are considered the basal values.

The leukocytes are considered in rolling on the endothelium if moving at a slower speed than erythrocytes in that vessel, and their number counted during 1 min. A leukocyte was considered adherent to the endothelium if it remained stationary for more than 30 s in a 100 μm length [39].

2.3. Experimental groups

Four experimental Wistar rats groups exist in order to observe the effect of diverse efectores in the leukocyte/endothelial interactions, thus we have Control Group ($n = 6$), Velnacrine Group ($n = 6$), Acetylcoline Group ($n = 6$) and Velnacrine + Acetylcoline Group ($n = 6$).

In the Control Group the cremaster remains in perfusion of NaCl 0.9% pH 7.4 during 60 minutes. The images of the venule and the arteriole should be registered at the end of 15, 30, 50 and 60 minutes. In the Velnacrine Group the cremaster is kept in perfusion of velnacrine (Hoechst-Roussel Pharmaceuthicals, Inc.) in a 10^{-3} M concentration and the images registered at the end of 15 and 30 minutes. After 30 minutes of perfusion with velnacrine we return to the perfusion with NaCl 0.9% pH 7.4, and data is registered at the end of 50 and 60 minutes. The same protocol is made using acethylcoline (Sigma-Aldrich) in a 10^{-3} M concentration for perfusion and also using velnacrine in set with acethylcoline. During this work the period corresponding to perfusion with NaCl 0.9% pH 7.4 (between 30 and 60 minutes) will be given the name of washout phase.

2.4. Determinations of the plasma IL-1β concentration present in the blood samples of the Wistar rats

For determination of the plasma concentration of IL-1β blood samples were collected from the 4 different experimental groups: Control Group, Velnacrine Group, Acetylcoline Group and Velnacrine + Acetylcoline Group. In the same way as experimental groups used for the microcirculation studies, cremaster is initially kept in perfusion of NaCl 0.9% pH 7.4 during 45 minutes for a postsurgical equilibrium period. After this equilibrium period the perfusion with each one of the different effectors in study is made. It is then collected about 1 ml of blood through the arterial catheter, using heparin for anticoagulation, after 30 minutes of perfusion with each one of the effectors and after 15 minutes of perfusion with NaCl 0.9% pH 7.4.

The collected blood is centrifuged for 1 minute at 9600 g in an eppendorf centrifugal Biofuge 15 (Heraus, Sepatech) and the plasma removed. The determination of the IL-1β concentration is made with ELISA kits of the Amershan Pharmacia Biotech, and the plates washed in a Micron Reader III Hyperion® (Hyperion Inc., Miami, USA).

2.5. Statistical analysis

The results gotten in the quantification of the leukocytes/endothelium interactions (adherent and rolling leukocytes, speed of rolling and venules and arterioles diameters) are normalized, relatively to the basal value in order to minimise the existing differences due to different basal values. The corresponding values of cardiac frequency and systolic and diastolic pressures, as well as the plasmatic concentration of IL-1β have been analysed in absolute value.
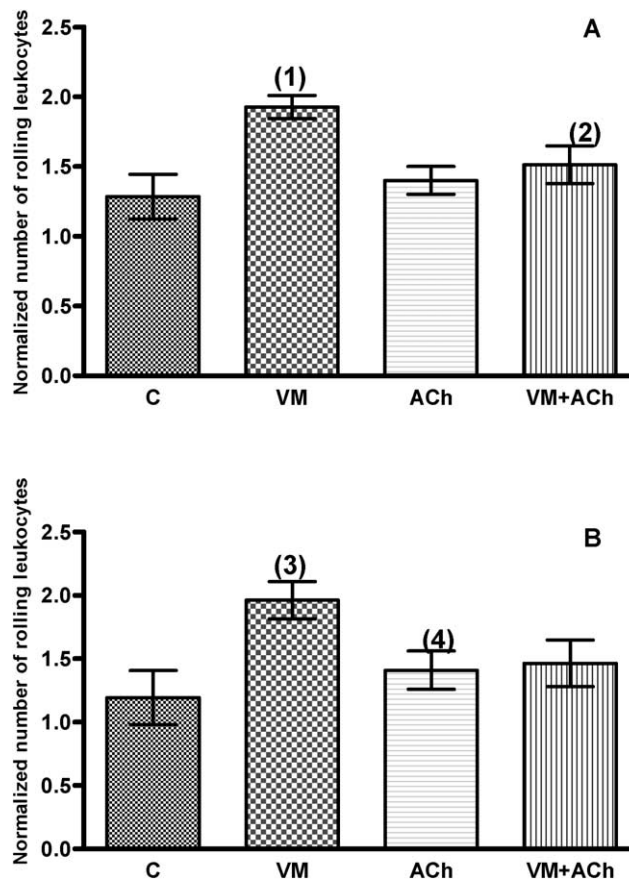
Fig. 1. Number of rolling leukocytes in the endothelium of post-capillary venules of Wistar rats cremaster muscle after 15 (A) and 30 minutes (B) of NaCl 0.9% C – Control and effector perfusion: V – Velnacrine 10^{-3} M; A – Acetylcholine 10^{-3} M; A+V – Acetylcholine 10^{-3} M + Velnacrine 10^{-3} M.
In relation with the Control group: (1) $p < 0.005$; (3) $p < 0.02$.
In relation with the Velnacrine group: (2) $p < 0.03$; (4) $p < 0.02$.

The results are presented as means $\pm$ standard deviation. Analysis was performed using the *t-test* for independent samples, in order to analyse the differences between the different experimental groups. The significance was considered for $p < 0.05$. The data analysis and the statistical treatment of the values were carried out in *GraphPad Prism software*.

3. Results

3.1. Rolling and Adhesion

In each of the four experimental groups of Wistar rats studied we verified that the normalised numbers of rolling and adherent leukocytes were constant during the 30 minutes of the experiment (Fig. 1, Table 1). The number of rolling leukocytes increased significantly in presence of velnacrine after 15 ($p < 0.005$) and 30 minutes ($p < 0.02$) of perfusion when compared with the control group. When only

Table 1

Normalized numbers of adherent (ADR) leukocytes in the endothelium of post-capillary venules of Wistar rats' cremaster muscle after 15 and 30 min of the effectors administration

Groups		15 min	30 min
Control	ADR	0.86 ± 0.72	0.88 ± 1.14
VM	ADR	1.02 ± 0.83	0.82 ± 0.88
ACh	ADR	0.75 ± 0.42	1.00 ± 0.63
VM + ACh	ADR	0.76 ± 0.60	1.26 ± 0.93

acetylcholine or both acetylcholine with velnacrine were present, although not statistically significant in relation to the control group, the number of rolling leukocytes increase. Comparing the normalised number of leukocytes rolling obtained in presence of acetylcholine with velnacrine perfusion with those obtained with velnacrine; we have a statistic significant decrease after 15 min ($p < 0.02$). After 30 min in presence of acetylcholine perfusion the number of rolling leukocytes was lower than that obtained with velnacrine ($p < 0.03$) (Fig. 1).

The number of adherent leukocytes increased in *post*-capillary venules in the presence of velnacrine in relation to the Control group. With the remaining effectors we observed an increase of the adherent leukocytes in the presence of velnacrine with acetylcholine, while no effect is produced with the acetylcholine perfusion. It is important to point out that though well visible these increases did not have statistic significance.

3.2. Rolling speed

The results obtained for speed values of the rolling leukocytes (Table 2) in the presence of each one of the effectors shows that: in the control group no changes are observed between 15 and 30 min of experience and the same happens with velnacrine and acetylcholine. In the presence of both effectors there is a decrease in the rolling speed of the leukocytes, although not statistically significant, in relation with the Control group.

3.3. Hemodynamic parameters

In Table 3 and 4 we can analyse the venules and arterioles diameters determined at cremaster microcirculatory network of all groups of Wistar rats studied. The velnacrine perfusion seems to decrease the venules diameters and acetylcholine seems to increase its, which corroborates with the vasodilator properties of acetylcholine. When the perfusion is made with both the effectors the gotten profile is very similar to the one obtained with velnacrine. For the arteriolar diameters, some variations are obtained: acetylcholine perfusion, as expected, has a vasodilator effect leading to an increase in the arteriolar diameter, with acetylcholine and velnacrine, like it happened with acetylcholine, an increase in the arteriolar diameter is observed. Velnacrine does not produce any changes in the arteriolar diameters.

Analysing the values obtained for systolic and diastolic blood pressures (Table 5) we observed that there were not, during the experiment, any significant changes in the those parameters. The basal values represented allow us to demonstrate the biological variability that exists between the different experimental groups used.

Table 2

Normalized leukocytes rolling velocity (mean ± standard deviation) in *post-capillary* venules of Wistar rats' cremaster muscle in all the studied groups after 15 and 30 min of the effectors administration

Groups	Rolling velocity (μm/s)	
	15 min	30 min
Control	0.97 ± 0.49	1.02 ± 0.46
VM	1.03 ± 0.64	1.11 ± 0.78
ACh	1.03 ± 0.74	1.36 ± 0.94
VM + ACh	0.65 ± 0.50	0.66 ± 0.53

Table 3

Normalized diameters (mean ± standard deviation) of *post-capillary* venules of Wistar rat's cremaster muscle in all the studied groups after 15 and 30 min of the effectors administration

Groups	Diameters (μm)	
	15 min	30 min
Control	1.01 ± 0.02	0.99 ± 0.06
VM	0.93 ± 0.10	0.93 ± 0.11
ACh	1.00 ± 0.12	1.06 ± 0.14
VM + ACh	0.96 ± 0.06	0.96 ± 0.18

Table 4

Normalized diameters (mean ± standard deviation) of arteries of Wistar rats' cremaster muscle. The values were obtained after 15 and 30 minutes of perfusion with each one of the effectors: velnacrine, acetylcoline and velnacrine with acetylcholine

Groups	Diameters (μm)	
	15 min	30 min
Control	1.02 ± 0.07	0.99 ± 0.10
VM	0.99 ± 0.10	1.05 ± 0.18
ACh	1.03 ± 0.12	1.10 ± 0.23
VM + ACh	1.18 ± 0.30	1.09 ± 0.20

3.4. Plasma IL-1β determinations

In order to verify if the perfusion of velnacrine maleate promote any changes in the plasma levels of IL-1β, after 30 minutes of perfusion the IL-1β concentrations were determined (Fig. 2). Analysing those results, we can observe an increase in the levels of IL-1β in the velnacrine group after 30 minutes of perfusion, as well as in the presence of velnacrine with acetylcholine. Acetylcholine perfusion did not produce any effect on the IL-1β concentrations after 30 min of perfusion at the cremaster microcirculatory network.

Table 5
Heart rate values (CF), systolic blood pressure (SBP) and diastolic blood pressure (DBP), represented as average $\pm$ standard deviation, for the several Wistar rats during each experiment

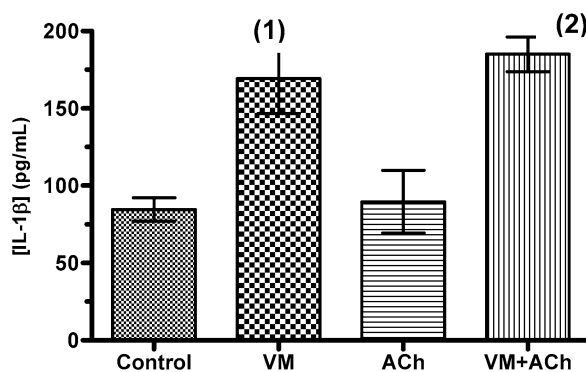| Groups		Basal	15 min	30 min
Control	CF	374 ± 40.28	362 ± 51.90	358 ± 48.51
	SBP	126 ± 15.35	122 ± 10.75	124 ± 19.68
	DBP	94 ± 13.78	88 ± 9.47	89 ± 18.10
VM	CF	400 ± 27.84	397 ± 23.87	399 ± 21.19
	SBP	124 ± 8.73	123 ± 7.88	122 ± 7.24
	DBP	85 ± 5.44	87 ± 5.72	85 ± 5.36
ACh	CF	387 ± 34.84	375 ± 19.48	371 ± 20.42
	SBP	121 ± 9.28	115 ± 4.92	113 ± 4.68
	DBP	84 ± 11.19	74 ± 7.29	72 ± 10.66
VM + ACh	CF	353 ± 41.48	341 ± 46.89	336 ± 38.18
	SBP	120 ± 12.22	119 ± 12.82	115 ± 10.68
	DBP	77 ± 7.08	72 ± 5.11	68 ± 9.15

Fig. 2. Values of IL-1β plasma concentrations in Wistar rats' blood samples obtained with NaCl 0.9% perfusion (C – Control) and effector perfusion V – Velnacrine 10^{-3} M; A – Acetylcholine 10^{-3} M; A+V – Acetylcholine 10^{-3} M + Velnacrine 10^{-3} M.
In relation with the Control group: (1), (2): $p < 0.05$.

4. Discussion

In this study the results showed that velnacrine maleate increased the number of rolling leukocytes in cremaster *post*-capillary venules at 15 min and 30 min after perfusion ($p < 0.005$, $p < 0.02$) *versus* the animal control group (Fig. 1). However, with acetylcholine at the same experimental conditions the number of rolling leukocytes at the cremaster *post*-capillary venules at 15 and 30 min after perfusion decreases ($p < 0.03$) in comparison with the velnacrine group (Fig. 1). Additionally, the presence of acetylcholine, either alone or with velnacrine, increases the number of rolling leukocyte in relation to the control group (Fig. 1).

Despite the non statistical significant differences obtained regarding the number of adherent leukocytes to the endothelium of *post*-capillary venules of Wistar rats' cremaster muscle (Table 2) an increase was verified in presence of velnacrine (1.02 ± 0.83 *versus* 0.86 ± 0.72) after 15 min of its perfusion, disappearing after 30 min (0.82 ± 0.88 *versus* 0.88 ± 1.14). However, when acetylcholine and velnacrine perfusion were done, the number of adherent leukocytes after 15 min decreased not significantly

(0.76 ± 0.60 *versus* 1.02 ± 0.83) and returned to the control level (0.76 ± 0.60 *versus* 0.86 ± 0.72). Higher normalized numbers were verified after 30 min of perfusion of both acetylcholine and velnacrine (1.26 ± 0.93 *versus* 0.88 ± 1.14). Concerning the control group, the number of adherent leukocytes increases after 30 min in presence of acetylcholine (1.00 ± 0.63 *versus* 0.88 ± 1.14) without statistical significant changes in the rolling velocity.

Both AChE substrate (ACh) and inhibitor (VM) perfusions in the cremaster microcirculatory network did not induce variations on the hemodynamic parameters' values namely arteries and *post*-capillary venules diameters (Table 3 and 4) as well as systolic and diastolic blood pressures and heart rate values in relation to the control group.

It is well known that acetylcholine produces blood vessels' relaxation in presence of an intact endothelial cell layer, as was verified for the first time by Furchgott and Zawadzki [12].

Nowadays, ACh infusion in the forearm blood flow is used to evaluate the functions of the endothelial cells' layer [21]. The vasoactive properties of ACh are regulated by acetylcholinesterase enzyme activity, hydrolysing it in to choline and acetate. Both pulmonary arteries and veins' endothelium cells have AChE activity [40] and so do lumen umbilical veins [6,7].

In our model, the perfusion of acetylcholine and velnacrine did not change the microcirculatory vessels' diameters. In addition, systemic inflammatory responses as an increase of the rolling leukocytes are observed. However, ACh did not modify the levels of plasma IL-1β and was not able to change the higher concentrations of this inflammatory interleukin, produced when the microcirculatory cremaster network is perfused with velnacrine (Fig. 2). An anti-inflammatory function was given to acetylcholine when TNF-α response was implicated by experimental activation of the cholinergic anti-inflammatory pathway by direct electrical stimulation of the efferent vagus nerves [38].

Regarding the Wistar rats' control group the results of this study suggest that the experimental procedure is by itself a promoter of IL-1β level increase because its concentration is not detected by the ELISA kit when in absence of any kind of animal manipulation. This was not removed by acetylcholine perfusion but an increase of the rolling leukocytes was obtained without any changes on IL-1β plasma levels. The presence of velnacrine worsens the acute inflammatory state, as verified by the increase in the IL-1β level, unimpaired by the acetylcholine addition. Despite the non-determinance of E-selectin concentrations in our study and its synthesis and expression occurrence after 4 h of IL-1β exposion as described by [29], it was also visualized by intravital microscopy, the rolling leukocyte inhibition when anti-selectin antibodies were used by [2,24].

Other studies have showed that the P-selectin influence the impairing of rolling leukocyte in mesentery, but more experimental exposition time was required to E-selectin began to play a role in the cremaster leukocytes rolling in ischemia/reperfusion situation [25].

However, other molecules such as α_4 integrins and vascular adhesion molecules have been reported as leukocyte rolling mediators [1,35] but its possible action was not object of the present study.

The transition of rolling to adhesion that occurs when a reduction in rolling velocity takes place was not verified in the presence of both acetylcholine and velnacrine. This could be explained by a change in the population of rolling cells over the course of the response time (e.g., from neutrophils to mononuclear cells as [24] pointed out in accordance with the limitation in identifying the type of rolling cells when intravital microscopy is used). The verified slowing down of rolling leukocytes in presence of ACh and VM can be mediated by pre-existent low-affinity integrins on circulatory leukocytes [3]. Several *in vitro* studies carried out with integrin antagonists indicated that leukocytes' arrest take place within fractions of seconds [30]. The high level of plasma IL-1β verified in both groups of Wistar rats with

VM or VM and ACh at cremaster microcirculatory network can probably activate the leukocytes integrins at remote locations from the endothelial post-capillary venules which do not modify its diameter (Table 3).

The results obtained in the present study can gives us a contribution for the acetylcholine anti-inflammatory already recognised action [38]. When ACh is added with VM at cremaster microcirculatory network, it decreases the number of rolling and adherent leukocytes without changing the IL-1β plasma circulation induced by VM. An inflammatory leukocyte endothelial interaction at the microcirculatory level was previously verified in the presence of velnacrine in mesenteric post-capillary venules [34]. Actually our results sustained that this tendency was also observed at another systemic domain and suggest an anti-inflammatory role induced by ACh without full efficiency.

Other studies have described similar protective effects of the endothelial cells, namely the role of reactive free radicals, such as oxygen free radicals and nitric oxide, in a protective process where the tolerance of a tissue to IR damage can be increased by a given stimulus [14] and the results obtained by [13] in chronic kidney diseases, with the alteration of the hemodynamic properties by vasodilators that restore renal function in those diseases.

The acute inflammatory state induced by the surgical procedure was not removed by ACh perfusion because the rolling leukocytes' velocity was reduced without changes in the plasma level of the pro-inflammatory cytokine IL-1β. These ACh role limitations could result either from its unattainable binding to AChE previously occupied by VM or from the ternary enzyme complex probably formed. In both hypothesis a cascade of signal transduction pathway have sustained plasma level of IL-1β as common response.

More studies are needed to understand the possible inflammatory function of the AChE and the molecular implications of velnacrine and acetylcholine on leukocytes subsets and selectins and integrins types. Regarding the alterations of IL-1β observed in this study it will be important to understand the signal transduction mechanism underlying the increase of IL-1β plasma concentrations in presence of velnacrine.

Acknowledgments

References

[1] R. Alon, P.D. Kassner, M.W. Carr, E.B. Fingerm, M.E. Hemler and T.A. Spronger, The integrin VLA-4 supports tethering and rolling in flow on VCAM-1, *J. Cell. Biol.* **128** (1995), 1243–1253.

[2] U.H. Andrian, J.D.Chambers, L.M. McEvoy, R.F. Bargatze, K. Arfors and E.C. Butcher, Two-step model of leukocyte-endothelial cell interaction in inflammation: Distinct roles for LECAM-1 and the leukocyte β_2 integrins *in vivo*, *Proc. Natl. Acad. Sci.* **88** (1991), 7538–7542.

[3] C. Berlin, R.F. Bargatze, J.J. Campbell, U.H. Andrian, M.C. Szabo, S.R. Hasslen, R.D. Nelson, E.L. Berg, S.L. Erlandsen and E.C. Butcher, α4 Integrins mediate lymphocyte attachment and rolling under physiologic flow, *Cell* **80** (1995), 413–422.

[4] L.V. Borovikova, S. Ivanova and M. Zhang, Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin, *Nature* **405** (2000), 458–462.

[5] C.M. Boulanger, K.J. Morrison and P.M. Vanhoutte, Mediation by M3-muscarinic receptors of bothe endothelium-dependent contraction and relaxation to acetylcholine in the aorta of the spontaneously hypertensive rat, *Br. J. Pharmacol.* **112** (1994), 519–524.

[6] F.A. Carvalho, R. Mesquita, J. Martins-Silva and C. Saldanha, Acetylcholine and choline effects on erythrocytes nitrites and nitrates levels, *J. Appl. Toxicol.* **24** (2004), 419–427.

[7] F.A. Carvalho, A. Calado, L.M. Graça, J. Martins-Silva and C. Saldanha, VIIIth International Meeting on Cholinesterases (2004), Perugia, Italy.

[8] G. Cicco, M. Vetrugno, M.T. Rotelli, G. Sborgia, M. Pennetta, P.P. Vico, V. Menneo, L. Nitti and C. Sborgia, Red blood cell (RBC) surface acetylcholinesterase showing a hemorheological pattern during glaucoma treatment, *Clin. Hemorheol. Microcirc.* **35** (2006), 149–154.

[9] D.B. Cines, Endothelial Cells in Physiology and in the Pathophysiology of Vascular Disorders, *Blood* **91** (1998), 3527–3561.

[10] K. Chang, D.F.J. Tees and D.A. Hammer, The state diagram for cell adhesion under flow: Leukocyte rolling and firm adhesion, *PNAS* **97** (2000), 11262–11267.

[11] X. Cousin, U. Strähle and A. Chatonnet, Are there non-catalytic functions of acetylcholinesteraes? Lessons from mutant animal models, *BioEssays* **27** (2005), 189–200.

[12] R.F. Furchgott and J.V. Zawadzki, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine, *Nature* **28**8 (1980), 373–376.

[13] N. Futrakul, P. Butthep, S. Patumraj, P. Siriviriyakul and P. Futrakul, Microvascular disease and endothelial dysfunction in chronic kidney diseases: Therapeutic implication. *Clin. Hemorheol. Microcirc.* **34** (2006), 265–271.

[14] T. Gori, L. Monica and S. Forconi, Ischemia and reperfusion: The endothelial perspective. A radical view, *Clin. Hemorheol. Microcirc.* **35** (2006), 31–34.

[15] M. Hill, B. Simpson and G. Meininger, Altered cremaster muscles hemodynamics due to disruption of the deferential feed vessels, *Microsvasc. Res.* **39** (1990), 349–363.

[16] L. Jia, C. Bonaventura, J. Bonaventura and J. Stamler, S. nitrosohaemoglobin: A dynamic activity of blood involved in vascular control, *Nature* **380** (1996), 221–226.

[17] R. Kacimi, J. Karliner, F. Koudssi and C. Long, Expression and regulation pf adhesion molecules 1 expression in mouse cremaster muscle vasculature, *Microcirculation* **4** (1998), 311–319.

[18] K. Kawashima, K. Kajiyama, T. Suzuki and K. Fujimoto, Presence of acetylcholine in blood and its localization in circulating mononuclear leukocytes of humans, *Biog. Amines* **9** (1993), 251–258.

[19] K. Kawashima, T. Fujii, Y. Watanabe and H. Misawa, Acetylcholine synthesis and muscarinic receptor subtype mRNA expression in T-lymphocytes, *Life Sci.* **62** (1998), 198–206.

[20] C. Kirkpatrick, F. Bitlinger, R. Unger, J. Kriesgsmann, H. Kilbinger and I. Wessler, The non-neuronal cholinergic system in the endothelium: evidence and possible pathobiological significance, *Jpn. J. Pharmacol.* **85** (2001), 24–28.

[21] T. Kishimoto, M. Jutila, E. Berg and E. Butcher, Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors, *Science* **245** (1989), 1238–1241.

[22] A. Klegeris, T.C. Budd and S.A. Greenfield, Acethylcholinesterase-induced respiratory burst in macrophage: evidence for the involvement of the macrophage mannose-fucose receptor, *Biochimica et Biophysica Acta* **1289** (1996), 159–168.

[23] J. Kua, Y. Zhang and J.A. MacCammon, Studying enzyme binding specificity in acetylcholinesterase using a combined molecular dynamics and multiple docking approach, *J. Am. Chem. Soc.* **124** (2002), 8260–8267.

[24] P. Kubes and S. Kanwar, Histamine induces leukocyte rolling in post-capillary venules. A P-selectin-mediated event, *J. Immuol.* **152** (1994), 3570–3577.

[25] P. Kubes, M. Jutila and D. Payne, Therapeutic potencial of inhibiting Leukocyte Rolling in ischemia/reperfusion, *J. Clin. Invest.* **95** (1995), 2510–2519.

[26] P. Kubes and S. Kerfoot, Leukocyte Recruitment in the Microcirculation: the Rolling Paradigm Revisited, *News Physiol. Sci.* **16** (2001), 76–80.

[27] C. Krieglstein and D. Granger, Adhesion molecules and their role in vascular disease, *Am. J. Hypert.* **14** (2001), 44S–54S.

[28] K. Ley, Histamine can induce leukocyte rolling in rat mesenteric venules, *Am. J. Physiol.* **267** (1994), H1017–H1023.

[29] S.K. Lo, B. Bevilacqua and A.B. Malik, E-selectin ligands mediate tumor necrosis factor-induced neutrophil sequestration and pulmonary edema in guinea pig lungs, *Circ. Res.* **75** (1994), 955–960.

[30] A.F.H. Lum, C.E. Green, G.R. Lee, D.E. Staunton and S.I. Simon, Dynamic regulation of LFA-1 activation and neutrophil arrest on intercellular adhesion molecule 1 (ICAM-1) in shear flow, *J. Biol. Chem.* **277** (2002), 20660–20670.

[31] R. Mesquita, I. Pires, C. Saldanha and J. Martins-Silva, Effects of acetylcholine and spermineNONOate on erythrocyte hemorheologic and oxygen carrying properties, *Clin. Hemorheol. Microcirc.* **25** (2001), 153–163.

[32] R. Mesquita, Comunicação colinérgica no sangue e endotélio vascular, *RFML*, Série III, **7** (2002), 83–88.

[33] W. Min and J. Pober, TNF initiates E-selectin transcription in human endothelial cells throught parallel TRAT-NF-kappa B and TRAF-RAC/CD42-JNK-c-Jun/ATF2 pathways, *J. Immunol.* **159** (1997), 3508–3518.

[34] H.S. Rosário, C. Saldanha and J. Martins e Silva, The effect of velnacrine in the lipopolysaccharide-induced leukocyte-endothelial interaction, in: *21st European Conference on Microcirculation* (B. Fragell, Ed.), Monduzzi Editore, Bologna, Italy, 2000, 79–82.

[35] M. Salmi, S. Tohka and S. Jalkanen, Human vascular adhesion protein-1 (VAP-1) plays a critical role in lymphocyte-endothelial cell adhesion cascade under shear, *Circ. Res.* **86** (2000), 1245–1251.

[36] L. Tang, Human erythrocyte as a model for investigating muscarinic agonists and antagonists, *Gen. Pharmacol.* **22** (1991), 485–490.

[37] K. Tracey, The inflammatory reflex, *Nature* **420** (2002), 853–859.

[38] S.C. Tromp, *In vivo modulation of leukocyte-endothelium interactions*, Universitaire Pers Maastricht, Masstricht, 1999.

[39] L. Walch, C. Taisne, J.P. Gascard, N. Nashashibi, C. Brink and X. Norel, Cholinesterase activity in human pulmonary arteries and veins, *Brist. J. Pharmacol.* **121** (1997), 986–990.

[40] D. Wright and D. Plummer, Multiple forms of acetylcholinesterase from human erythrocytes, *Biochem. J.* **133** (1973), 521–527.