Quarta-feira, *8 de Setembro, 2004*

14h00 - 14h15
Opening Address
J. Martins e Silva, F. Ramoa Ribeiro, C. Saldanha, Braz Nogueira, V. Oliveira - Portugal A.R. Pries - Germany, A. Shore - UK

14h15 - 15h00
Plenary Lecture
Perspectives of Microcirculation
Giuseppe Ambrosio - Italy

15h00 - 16h45
Plenary Symposium
Illusion of reperfusion: Microvascular integrity as the key factor in successful postischemic reperfusion
Giuseppe Ambrosio-Italy, Michael Menger — Germany, Isabella Tritto-Italy, G. Schmid-Schönbein-USA, Eric Brochet-France, B. Gerber-Belgium

17h00 - 18h30
Plenary Symposium
Microcirculatory mechanisms of hypertension
Eric Vicaut- France, Giuseppe Ambrosio-Italy, H. Struijker Boudier-The Netherlands, Akos Koller-Hungary, D. Rizzoni-Italy

18h30 - 19h00
Recital "Quarteto de Cordas"

19h00 - 20h30
Welcome Reception
Porto offered by the Lisbon City Hall

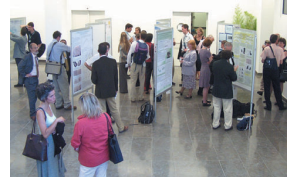
Quinta-feira *9 de Setembro, 2004*

08h30 - 09h15
Plenary Lecture
Microvascular Mechanisms for Inflammation and organ injury in hypertension
G. Schmid-Schönbein-USA

09h15 - 10h45
Plenary Symposoium
Clinical Microcirculation
Can Ince, Jerome Lindeboom, Christian Boerma, Keshen Mathura- The Netherlands

11h00 - 12h30
Plenary Symposium
Critical experimental studies: a more effective way to translate basic research into therapeutic options?
H. Struijker Boudier-The Netherlands, D. Seiffge-Germany, J.M.Harlan-USA, A.R. Pries-Germany

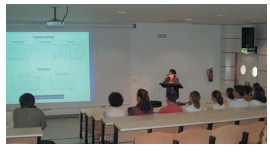
13h30 – 14h15
Guided Poster Tour



14h45 – 16h00
Free Oral Communications
Assessment Techniques and Others
J. Carvalho de Sousa-Portugal

16h15 - 17h45
Parallel Symposium
Microvascular Assessment: advanced techniques for intravital microscopy
D.Slaaff, M.Zandwoort-the Netherlands, A.R.Pries, M.Menger, W. Kübler-Germany, S. Charpak-France

16h15 – 17h45
Parallel Symposium
Retinal Microcirculation
E.Vicaut-France, A. Harris-USA, M.Pâques, A.Gaudric-France, L.Metzner Serra, A.Castanheira Diniz-Portugal



16h15 - 17h45
Parallel Symposium
Red Blood Cell Interaction with Blood Vessel Wall Endothelium in Circulatory Disorders
S. Yedgar-Israel, A.Popel, D.Kaul, T.Wick-USA, D.Anstee-UK

16h15 - 17h45
Parallel Symposium
Physiological versus Pathological Angiogesenis
A. Cidadão, D. Henrique, S. Dias-Portugal

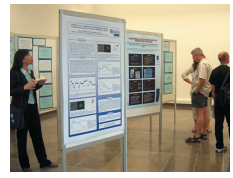
Sexta-feira
10 Setembro 2004

08h30 – 09h15
Malpighi Award Lecture
Brian Duling-USA

09h15 – 10h45
Plenary Symposium
Gap junctions and vascular cell communication
U.Pohl, C. de Wit, I.Fleming-Germany, J.Pearson, T. Grifith-UK

11h00 – 12h30
Plenary Symposium
Programming of vascular dysfunction
C. Torrens, G.F. Clough, J. Coleman-UK, E.Villamor-The Netherlands, M.Pinho Franco-Brazil, M. Norman-Sewden

13h30 – 14h15
Guided Poster Tour



14h45 – 16h00
Free Oral Communications
Ischemia and Reperfusion
D.Gama, V. Oliveira-Portugal

16h15 – 17h45
Parallel Symposium
Leukocyte trafficking in inflammation and disease
B. Walzog, M. Sperandio, J. Schimeisky-Germany, K.Ley-USA, B.Engelhrdt-Switzerland, K.Norman-UK

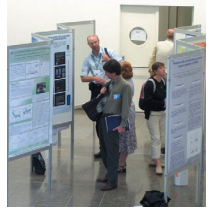
16h15 – 17h45
Parallel Symposium
Cerebral vascular disease
J. Ferro, V. Oliveira, J.Costa-Portugal, C. Navas, F.P.Goméz-Spain

16h15 – 17h45
Parallel Symposium
Pressure measurements in human microvessels: fancy or future?
J. Tooke, C.C.Michel, A.C.Shore-UK, D. Ubbink, D. Slaaff, J.C.Graaf-The Netherlands, B.Aman-Vesti-Switzerland

Sábado
11 de Setembro 2004

09h00 – 10h15
Free Oral Communications
Angiogenesis, Pharmacology, Gene therapy and Hypertension
J. Fernandes e Fernandes-Portugal

10h15-11h00
Guided Poster Tour



11h15–12h45
Parallel Symposium
Molecular mechanisms of arteriosclerosis
M. Carrageta, J. Gonçalves, M. E. Macedo, P.M. Silva, J.M. Silva, A.S. Coelho-Portugal

11h15–12h45
Parallel Symposium
Frontiers in angiogenesis: mechanisms and applications
J. Molema, F. Noble-The Nethgerlands, M. Weiss, C. Heeshen, C. Kupatt-Germany

11h15–12h45
Parallel Symposium
From endothelial dysfunction to atherothrombosis
J. Fernandes e Fernandes, L.M. Pedro, C. Saldanha-Portugal, W. Paaske-Dannmark, A.Koller-Hungary

11h15–12h45
Parallel Symposium
Molecular imaging of apoptosis in the vessel wall
L. Hofstra, A.Houben, C. Reutelingsperger –The Netherllands, J. Narula-USA

13h00–13h15
Final acknowledgments

13h00–13h15
Invitation to next ESM meeting in Amsterdam

POSTERS

PHYTOSTEROLS IN MILK EFFECTS ON PLASMA CHOLESTEROL CONCENTRATIONS: EXPERIMENTAL EVIDENCE IN PORTUGUESE HEALTHY SUBJECTS

S. Gonçalves, A. S. Silva, C. Branco, C. Saldanha, J. Martins e Silva*

Instituto de Biopatologia Química, Unidade de Biopatologia Vascular, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, 1649-028 Lisboa Portugal.
*sabreu@fm.ul.pt

INTRODUCTION

Cholesterol is important for the body's functions. It contributes to cell membranes configuration and properties and in synthesis of certain hormones. Absorbable cholesterol comes from three sources: the diet, the liver and from intestinal cell turnover. Artery-clogging plaques may be formed from excess of plasma cholesterol levels, increasing vascular event. Plant phytosterols are known to suppress intestinal cholesterol absorption even through the mechanisms has not been established [1,2]. ***The aim of this study is to show how phytosterols-milk intake influenciates plasma cholesterols levels in healthy subjects***.

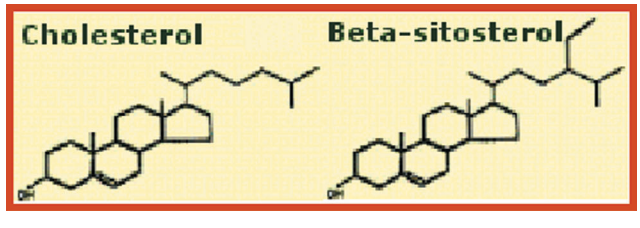


Figure 1. Structures of Cholesterol and Sitosterol molecules.

MATERIALS AND METHODS

Subjects Twenty (aged 27-50 y) healthy subjects were selected.

Protocol The subjects were fed a milk-sterol containing (2mg/day) for thirty days. The subjects were instructed to maintain the same dietary intake during the study.

Analyses Blood samples were collected from subjects before breakfast on days 0, 15 and 30 of the trial. Plasmatic concentrations of Total cholesterol, HDL- and LDL-cholesterol were determinate.

Statistical methods The data were evaluated by using t-Student test for paired samples (level of significance $P<0.05$).

RESULTS

Parameter	*Mean 0 Days*	*Mean 15 Days*	*Mean 30 Days*
Total Cholesterol (mg/dL)	**200 ± 40**	**182 ± 40**	**182 ± 29**
HDL (mg/dL)	**59 ± 15**	**56 ± 17**	**55 ± 11**
LDL (mg/dL)	**122 ± 38**	**108 ± 35**	**111 ± 26**

Table N.º1. Total cholesterol, HDL- LDL cholesterol values obtained during the study.

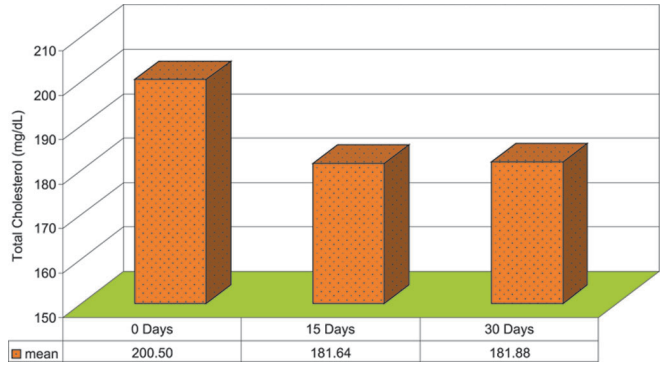


Figure 1. Effect of the phytosterol-enriched milk on Total cholesterol concentrations of healthy subjects.

CONCLUSIONS

- After fifteen days of treatment Total cholesterol and LDL-cholesterol concentrations decreased significantly by 9%, $P < 0.05$ and 11.48%, $P < 0.05$ respectively.
- HDL cholesterol levels did not change significantly.
- After thirty days, the values did not change significantly in relation with the results obtained at fifteen days.
- These results show a positive effect in cholesterol plasma levels in healthy subjects.

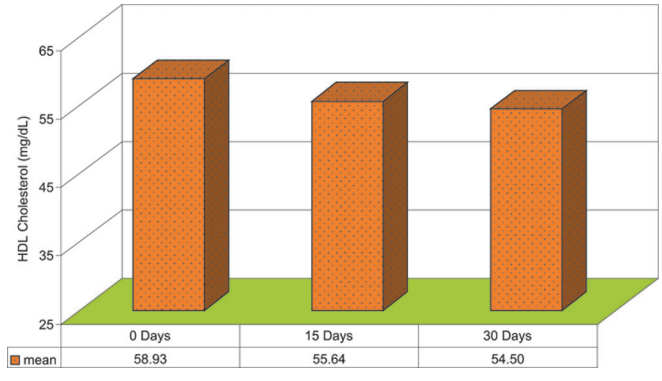


Figure 2. Effect of the phytosterol-enriched milk on HDL cholesterol concentrations of healthy subjects.

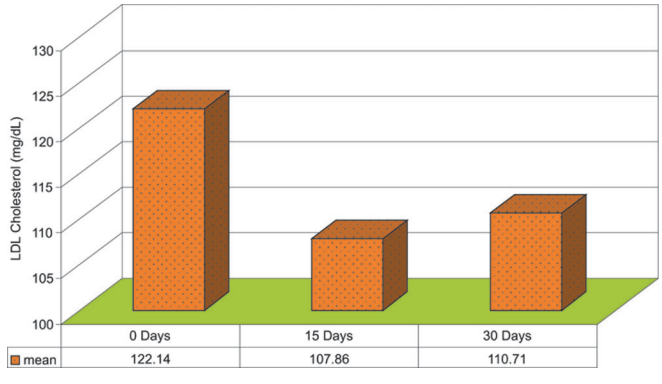


Figure 3. Effect of the phytosterol-enriched milk on LDL cholesterol concentrations of healthy subjects.

REFERENCES

[1] K.B. Hicks and R.A. Moreau. Food Technology. 55:1 (2001)

[2] M.H. Moghadasian and J.J. Frohlich. Am. J.Med. 107: 588-594 (1999)

APOIO

Mimosa

CNAM - Centro de Nutrição e Alimentação Mimosa

PHYTOSTEROLS IN MILK AS A DEPRESSOR OF PLASMA CHOLESTEROL LEVELS: EXPERIMENTAL EVIDENCE IN HYPERCHOLESTEROLEMIC SUBJECTS

S. Gonçalves1, A. S. Silva1, C. Saldanha1, J. Martins e Silva1, A. V. Maria*[2]

[1] Instituto de Biopatologia Química, Unidade de Biopatologia Vascular, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, 1649-028 Lisboa Portugal.
[2] Instituto de Medicina Preventiva, Faculdade de Medicina, 1649-028 Lisboa Portugal
*sabreu@fm.ul.pt

INTRODUCTION

Cholesterol is a fatty substance that forms deposits in the blood vessel. Artery-clogging plaques may formed when among others factors, cholesterol levels become too high, so blood vessels become blocked and are unable to supply blood to the heart or brain, triggering a heart attack or stroke. Plant sterols have been reported to decreased plasma concentrations of cholesterol without any side effects. ***The aim of this work is to show the effect of phytosterol milk containing in the treatment of the subjects with hypercholesterolemia.***

MATERIAL AND METHODS

Subjects Thirty seven (aged 30-45 y) hypercholesterolemic patients (LDL cholesterol > 130mg/dL) were selected.

Protocol. Nineteen subjects were treated with enriched phytosterols milk (2mg/day), the other eighteen were used as control group (milk without phytosterols). Both groups drunk the milk during thirty days.

Analyses Blood samples were collected from subjects before breakfast on days 0, 15 and 30 of the trial. Plasmatic concentrations of Total cholesterol, HDL- and LDL-cholesterol were determinate.

Statistical methods The data were evaluated by using t-student method (two tails paired with a level of significance P<0.05).

RESULTS

Parameter	*Phyto Milk*	*Normal Milk*
Total Cholesterol (mg/dL)	***i*** **245 ± 37*** ***f*** **223 ± 33**	**253 ± 37**** **247 ± 34**
HDL (mg/dL)	**63 ± 16**** **58 ± 13**	**52 ± 7**** **52 ± 7**
LDL (mg/dL)	**156 ± 37*** **137 ± 36**	**166 ± 31**** **156 ± 28**

Table No.1 Effect of phytosterol enriched and none enriched milk on mean of Total cholesterol, HDL- LDL cholesterol concentrations of hypercholesterolemic subjects after fifteen days of milk intake.
i inicial value, f final value; * P < 0.05, ** P > 0.05

Parameter	*Phyto Milk*	*Normal Milk*
Total Cholesterol (mg/dL)	**248 ± 38 *** **229 ± 29**	**255 ± 38*** **239 ± 45**
HDL (mg/dL)	**62 ± 15 **** **61 ± 14**	**52 ± 8**** **51 ± 9**
LDL (mg/dL)	**157 ± 38 *** **143 ± 26**	**166 ± 33*** **146 ± 40**

Table No.2 Effect of phytosterol enriched and non enriched milk on mean of total cholesterol, HDL- LDL cholesterol concentrations of hypercholesterolemic subjects after thirty days of milk intake
P < 0.05, ** P > 0.05

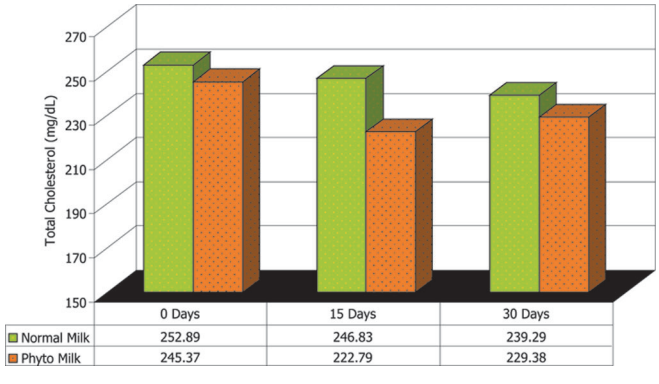


Figure 1. Effect of phytosterol enriched and non enriched milk on mean of Total cholesterol concentrations of hypercholesterolemic subjects.

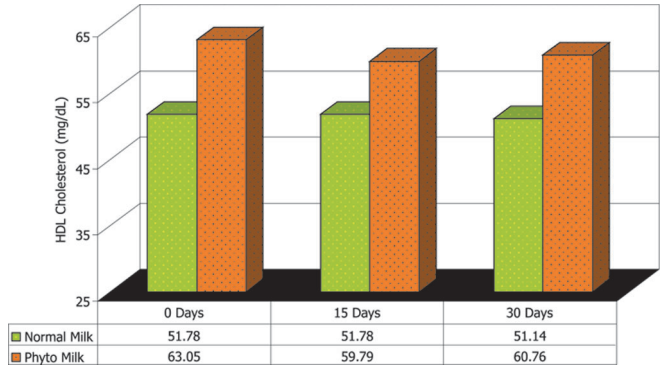


Figure 2. Effect of phytosterol enriched and none enriched milk on mean of HDL cholesterol concentrations of hypercholesterolemic subjects.

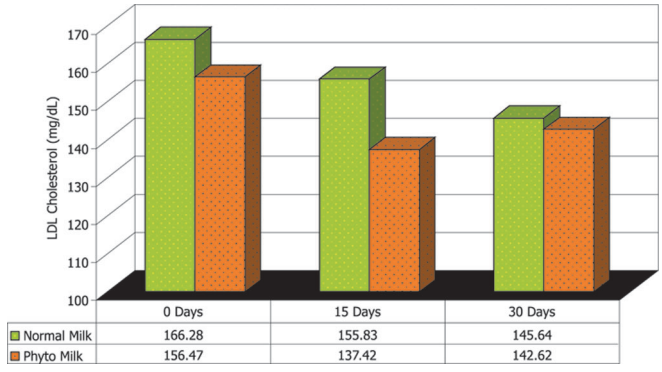


Figure 3. Effect of phytosterol enriched and none enriched milk on mean of LDL cholesterol concentrations of hypercholesterolemic subjects.

CONCLUSIONS

- After fifteen days of treatment Total cholesterol and LDL cholesterol concentrations decreased significantly by 10.08%; $P < 0.05$ and 12.74%; $P < 0.05$, respectively.
- After thirty days, the values did not change significantly in relation with the results obtained at fifteen days.
- The control group do not show significative diference after fifteen and trirty days of normal milk.
- These results show a positive effect with the milk as a good food vehicle as lowering plasma cholesterol in the treatment of hypercholesterolemia.

APOIO

Mimosa

CNAM - Centro de Nutrição e Alimentação Mimosa

CONFORMATIONAL CHANGES DURING FIBRINOGEN-LIGAND BINDING FOLLOWED BY FLUORESCENCE SPECTROSCOPY

S. Gonçalves, N. C. Santos, J. Martins e Silva, C. Saldanha*

Instituto de Biopatologia Química, Unidade de Biopatologia Vascular, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, 1649-028 Lisboa Portugal.
* sabreu@fm.ul.pt

INTRODUCTION

An unknown binding mechanism can be relate increased erythrocyte aggregation with plasma fibrinogen concentration. Several studies have shown that an increase in fibrinogen levels is associated with the risk of cardiovascular disease and cerebrovascular disorders, infections, inflammations, trauma and to others factors such as hypertension and obesity. A previous *in vitro* study has shown an impaired erythrocyte aggregation in the presence of b Estradiol[1]. The study of ligand binding to fibrinogen could provide information about the functionality of this protein during erythrocyte aggregation. ***The aim of this work is*** **to study fibrinogen-binding properties in the presence of different ligand concentrations by using fluorescence spectroscopy.**

METHODS

Measurements Absorbance measurements were carried out in a Genesys 10 UV ThermoSpectronic at 280nm. Fluorescence measurements were carried out in a Varian Cary Eclipse Fluorescence Spectrophotometer. For Fibrinogen-ligand studies emission spectrum was recording by setting the excitation wavelength to 280 nm and acquired the emission spectrum between 300-400nm. For quenching experiences the excitation wavelength was 295 nm and the emission wavelength was 344nm.

Analysis: The results were fitted by using the Lehrer[2] equation and the fraction of exposed quenchers were obtained:

$$\frac{I0}{I} = \frac{1 + K_{sv}[Q]}{(1 + K_{sv}[Q])(1 - f_B) + f_B}; f_B = \frac{I_{0,B}}{I_0}$$

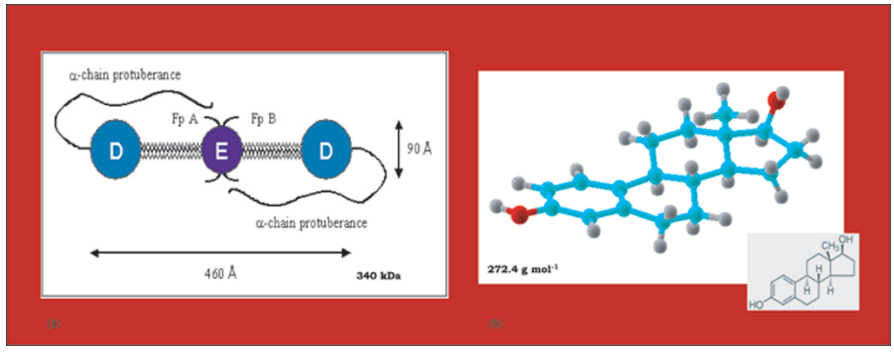


Figure 1. Structures of Fibrinogen (a) and β Estradiol (b) molecules

RESULTS

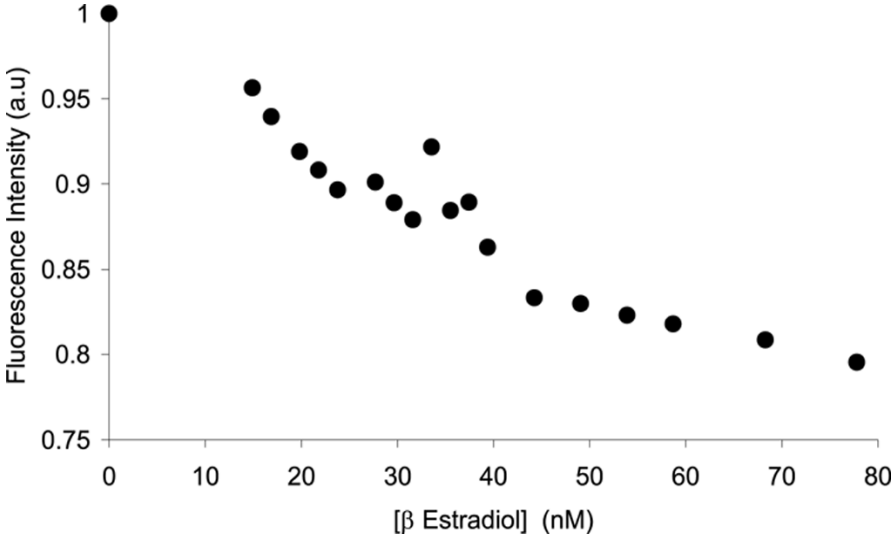


Figure 2. Fluorescence intensities for Fibrinogen-β Estradiol binding.

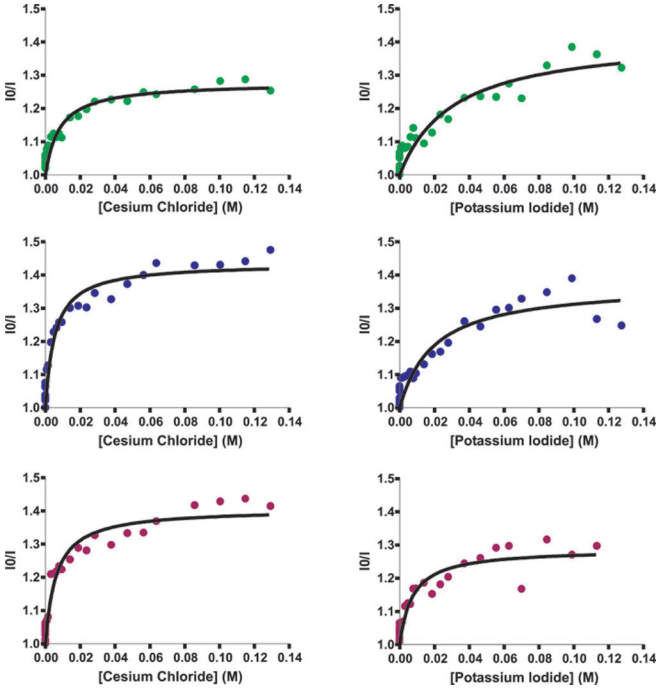


Figure 3. Fitting results obtained for the different Fibrinogen-ligand concentration.

Quencher	β- Estradiol concentration		
	0 nM	22 nM	65 nM
CsCl	*Ksv* = **158 ± 75** *fB*= **0.22 ±0.02**	*Ksv* = **268 ± 47** *fB* = **0.3 ± 0.01**	*Ksv* = **230 ± 41** *fB* = **0**.29 ± 0.01
KI/Na2S2O3	*Ksv* = **46 ± 12** *fB* = **0.29 ± 0.02**	*Ksv* = **70 ± 17** *fB* = **0.27 ± 0.01**	*Ksv* = **176 ± 46** *fB* = **0.22 ± 0.01**

Table 1 *Ksv** and *fB* ** values obtained after fitting the experimental results
* Stern Volmer constant, **fraction of exposed residues.

CONCLUSIONS

- In the range of the concentrations studied for Fibrinogen- b-Estradiol interaction no shifts in the fluorescence maxima were observed.
- The quenching parameters obtained at different b-Estradiol concentrations (22 or 65 nM)

show alterations that can be related with either possible conformational changes or a discrete reorganization of tryptophan residues during fibrinogen-ligand binding.

- The Fibrinogen- b-Estradiol binding induces an enhanced exposure of tryptophan residues.

REFERENCES

[1] Gonçalves, I., Saldanha, C. and Martins e Silva, J. 2001, Clinical Hemorheology and Microcirculation, 25,127

[2] Lehrer,S.S. 1971, Biochemistry, 10, 3254.

APOIO FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA E DO ENSINO SUPERIOR Portugal

EFFECTS OF VELNACRINE MALEATE IN THE LEUKOCYTE-ENDOTHELIAL CELL INTERACTIONS IN RAT CREMASTER MICROCIRCULATORY NETWORK

A.S. Silva, C. Saldanha, J. Martins e Silva*

Instituto de Biopatologia Química,Unidade de Biopatologia - Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Portugal
* anarmsilva@fm.ul.pt

ABSTRACT

The expression of acetylcholinesterase (AChE) in proinflammatory cells has supported the hypothesis that this protein plays a role in intercellular adhesion, beyond its acetylcholine hydrolysis ability. Previous results of our group show that velnacrine (VM), an acetylcholinesterase inhibitor, increases the number of rolling leukocytes in post-capillary venules of Wistar rats' cremaster muscle. In order to evaluate the influence of the local application of velnacrine and acethylcoline (Ach), on the microcirculation in post-capillary venules of Wistar rats' cremaster muscle, the numver of rolling leukocytes was monitorized. The number of adherent leukocytes, the diameter of the respective venule and the neighboring arteriole was also determined. The results have shown that in the presence of velnacrine there is

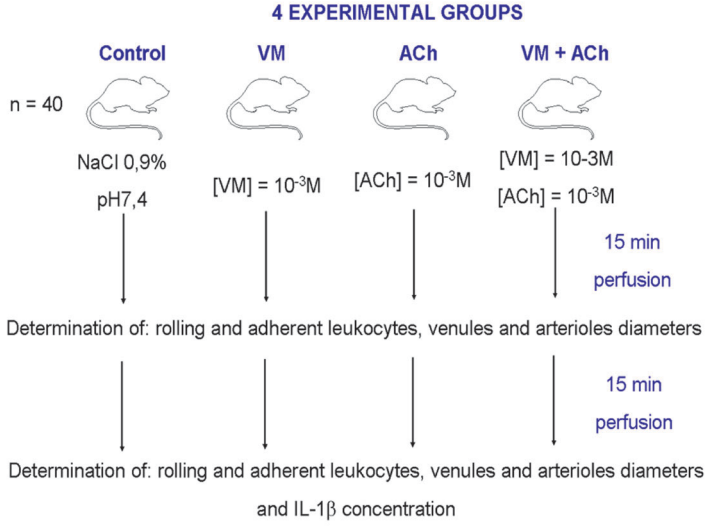
significant decrease of the rolling leukocytes (1.36 ± 1.05 vs 1.96 ± 1.15), as well as an increase of the adherent ones (1.04 ± 1.03 vs 1.05 vs 1.20). When ACh was also added the results obtained suggest that the effect of the velnacrine counters the one of acethylcoline, leading to the hypothesis that there is a specific interaction between velnacrine and acethylcolinesterase. The measurement of the venules and arterioles diameters allows us to conclude that acethylcoline induced vasodilatation either alone or in association with velnacrine. In opposition, in the venules, both the effectors and velnacrine alone lead to vasoconstriction. We raise the hypothesis that the different vasoactive effects of VM and Ach on the venular and arteriolar sectors could be mediated by different signal transduction mechanism of AChE.

AIMS

The presence of acetylcholinesterase in the sanguineous cells and in the endothelial cells has come to base the hypothesis of this protein to play, beyond the catalytic functions, functions of intercellular adhesion. It is known that the systemic administration of velnacrine maleate (VM), an inhibitor of AChE, takes to an increase of the number of leukocytes in rolling in the endotelium of post-capillary venules of Wistar rats cremaster, without alteration of the adherent ones. This work tends to evaluate the influence of the local application of VM and acetylcholine on the microcirculation.

METHODS

- Anaesthesia – i.p with 1,5g/Kg body weight and i.m. 50mg/Kg body weight with ketamine
- Tracheotomy – to maintain the animal in spontaneous breath



- Catheterization – of right jugular for drug administration and left carotid artery for arterial pressure and cardiac frequency measure
- Cremaster preparation for intravital microscopy in an inverted microscope.
- 45 minutes of postsurgical equilibration period in NaCl 0,9% pH 7,4 perfusion

RESULTS

- in the presence of velnacrine there is a significant increase of the rolling leukocytes
- with velnacrine the number of adherent leukocytes increases
- the rolling speed increases with velnacrine and acetylcholine and decreases with both the effectors at the same time.
- the venular diameter decreases in presence of velnacrine and increases with acethylcoline, a profile similar with velnacrine is

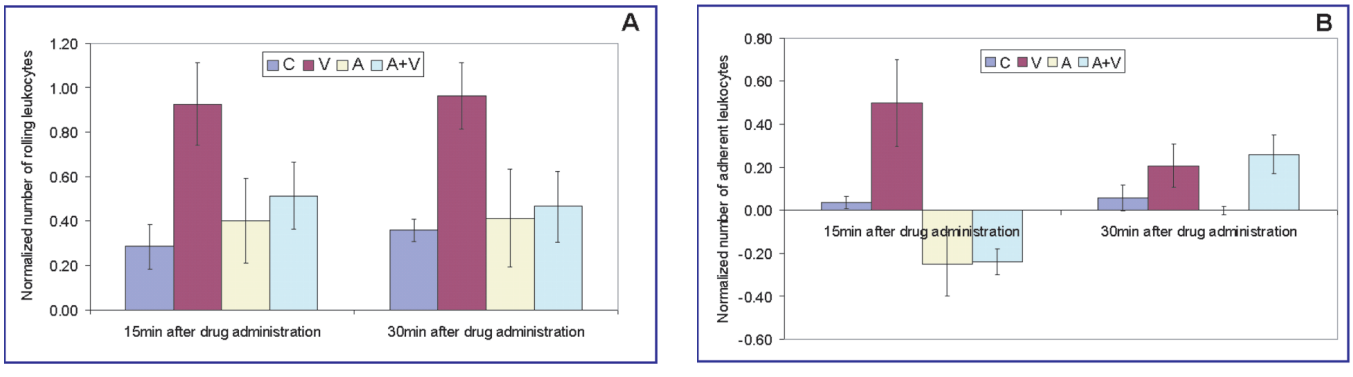


Figure 2. Number of rolling **(A)** and adherent leukocytes **(B)** in the endothelium of post-capillary venules of Wistar rats cremaster muscle.
C - Control; V - Velnacrine; A - Acetylcholine; A+V – Acetylcholine + Velnacrine

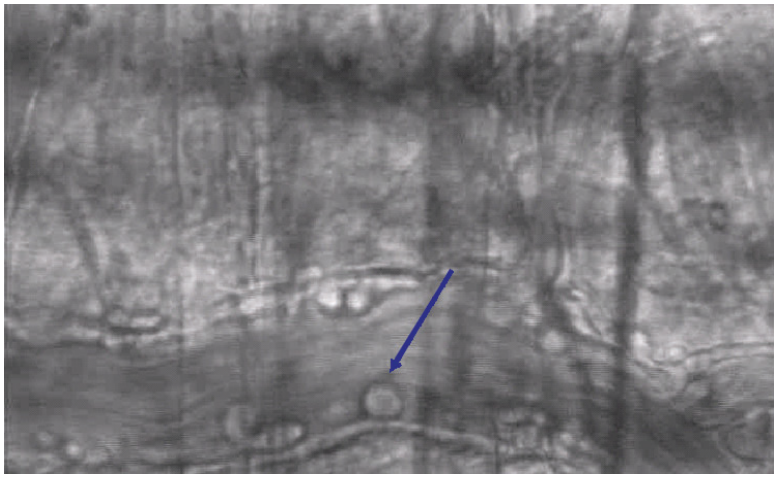


Figure 2. Image of a post-capillary venule, observed on the optical microscope (400x) where we can observe the rolling leukocytes (arrow). Image obtained through the *AquaCosmos* software *Version 2.0.*

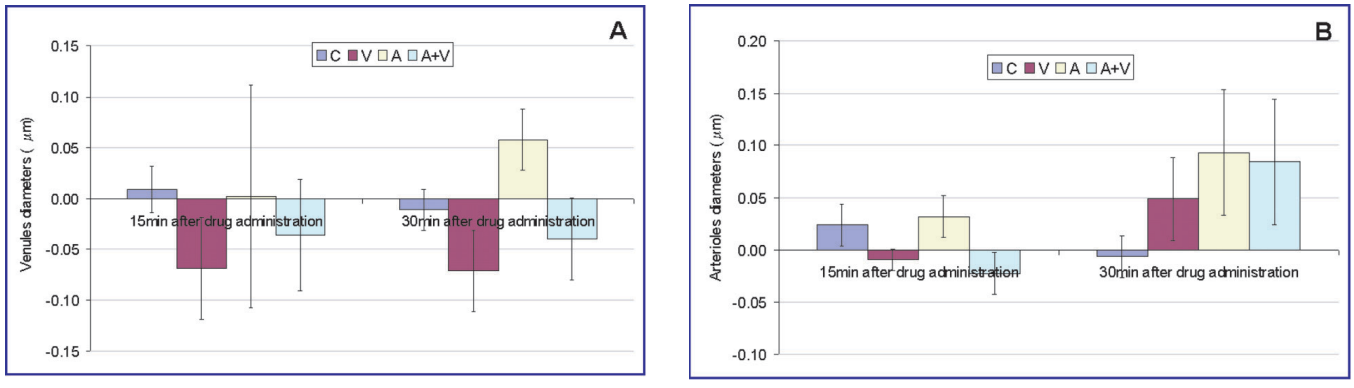


Figure 3. Variation of the venules **(A)** and arterioles **(B)** diameters.
C- Control; V- Velnacrine; A- Acetylcholine; A+V – Acetylcholine + Velnacrine

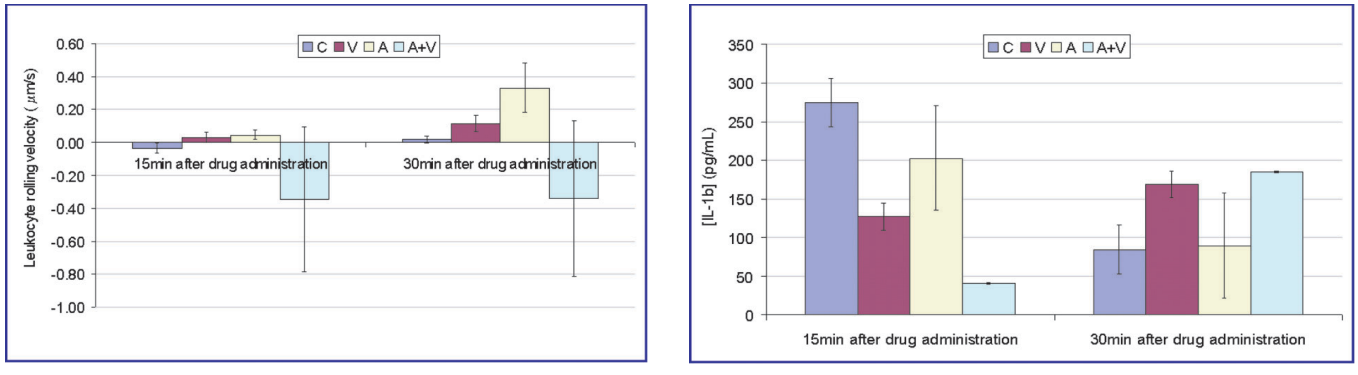


Figure 4. Leukocytes rolling velocity after the administration of velnacrine, acetylcholine and velnacrine + acetylcholine

Figure 5. IL-1b plasmatic concentrations in Wistar rats' blood samples

obtained with both the effectors used.

- in the arteriolar sector, the acetylcholine has a vasodilator effect

CONCLUSIONS

The rolling state of the leukocytes is a transitional state in the inflammation process. At the endothelial cell level, the effects caused by the velnacrine and by ACh are different. The vasoactive effects of VM and ACh on the venular and arteriolar are also different, this raises the hypothesis that two forms of AChE are present. One active form, when ACh is used as an effector, characterized by a decrease in the adherent leukocytes, an increase in the rolling velocity and vasodilatation properties. The AChE/Velnacrine complex describes an inactive form of the enzyme leading to an increase of the rolling and adherent leukocytes and a decrease of the rolling velocity. The two described

forms of the enzyme, active and inactive, appear to be related, respectively, with an anti-inflammatory and a proinflammatory state.

APOIO FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA E DO ENSINO SUPERIOR Portugal

EFFECTS OF PHYTOSTEROLS SUPPLIED IN HALF-FAT AND LOW-FAT MILK, IN THE SERUM CHOLESTEROL CONCENTRATIONS IN RATS

A.S. Silva , C. Branco, J. Martins e Silva, C. Saldanha*

Instituto de Biopatologia Química,Unidade de Biopatologia - Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Portugal
* anarmsilva@fm.ul.pt

23rd Lisbon, September 8-11, 2004
European Conference on Microcirculation
Microcirculation and Vascular Biology a Basis for Research and a Reason for Life

ABSTRACT

Clinical and experimental studies have shown that the use of phytosterol esters as a food ingredient reduces the plasmatic concentrations of cholesterol and LDL cholesterol, not affecting the HDL cholesterol levels. Resulting from the use of phytosterols as a food component, a number of studies have demonstrated the efficacy of these components in a variety of foods, such as margarines. Knowing that there is a positive correlation between artherosclerosis and the LDL cholesterol levels and negative between the HDL cholesterol, the use of phytosterols as a food ingredient, due to their cholesterol lowering properties can have a beneficial effect on health by reducing the risk of developing artherosclerosis and coronary heart disease. In this basis we have conducted a 30-day feeding study in a 40 rats group, using half-fat and low-fat milk containing phytosterols, in order to

cholesterol lowering properties can have a beneficial effect on health by reducing the risk of developing artherosclerosis and coronary heart disease. In this basis we have conducted a 30-day feeding study in a 40 rats group, using half-fat and low-fat milk containing phytosterols, in order to evaluate the serum cholesterol concentrations after a 30-day feeding period. Thus we have used milk containing the following concentrations in phytosterols: 0 (as the Control group), 0.2, 0.3, and 0.4 g of phytosterols per 100mL of milk. Throughout the study, clinical observations, body weight and food and milk consumption were measured. The fourth concentrations studied were tolerated as evidenced by the absence of clinical changes or major abnormalities in growth, food and milk consumption, the minor changes in hematological parameters, were considered to be with no biological or toxicology significance. For the plasmatic cholesterol concentration there were no significant differences in the cholesterol and HDL cholesterol levels, either with low-fat or half-fat milk, but there was a decrease of about 70% in the LDL cholesterol level with low-fat milk and of about 50% with half-fat milk.

AIMS

Phytosterols (PE) effects in lowering the plasma cholesterol concentrations are already known, thus they can have a beneficial effect on health maintenance by reducing the risk of developing artherosclerosis and coronary heart disease. Other studies show the efficacy of PE as a food ingredient. Thus, the aim of this study is to analyse the milk as a vehicle for phytosterol induce lowering of plasma cholesterol levels.

METHODS

Schematical representation

RESULTS

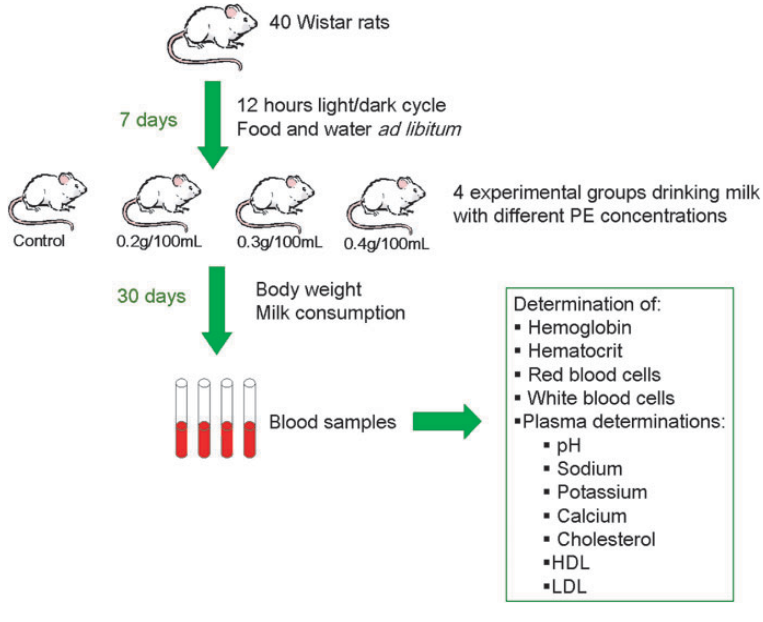


Figure 1. The rats were kept for 1 week in an animal facility with a 12h light/dark cycle, on a diet standard rat food and water *ad libitum*. After this adaptation period the animals started drinking low and half-fat milk containing phytosterols in four different concentrations for 30 consecutive days. After the 30-day feeding period, blood samples were collected for further analysis.

	Milk PE concentration (g/100mL milk)						
	Control	0.2		0.3		0.4	
		LF	HF	LF	HF	LF	HF
Weight (g)	306 ± 58	310 ± 17	316 ± 25	295 ± 36	306 ± 31	295 ± 36	307 ± 11
Mean consumption (ml/rat/day)	30 ± 2	39 ± 19	30 ± 8	34 ± 19	35 ± 6	34 ± 19	34 ± 6
Haemoglobin (g/dL)	16.0± 1.1	16.5 ± 1.0	16,3 ± 0,9	15.9 ± 1.0	16,5 ± 0,9	15.9 ± 1.0	16,7 ± 0,6
Haematocrit (%)	52 ± 12	49.3 ± 2.7	52 ± 2	46 ± 2.0	52 ± 3	46 ± 2.0	54 ± 2
Mean cell haemoglobin concentration (g/dL)	0.31 ± 0.03	0.33 ± 0.01	0,31 ± 0,01	0.34 ± 0.02	0,32 ± 0,01	0.34 ± 0.02	0,309 ± 0,003
Red blood cell count ($\times 10^{12}$/L)	8.00 ± 0.91	7.79 ± 1.39	8,46 ± 0,49	8.31 ± 0.50	8,29 ± 0,39	8.31 ± 0.50	8,88 ± 0,67[a]
White blood cells ($\times 10^{9}$/L)	4.15 ± 2.09	4.53 ± 1.59	3,58 ± 1,16	4.99 ± 0.67	3,73 ± 1,61	4.99 ± 0.67	4,00 ± 1,05
pH	7.35 ± 0.07	7.41 ± 0.04	7,38 ± 0,06	7.41 ± 0.03	7,39 ± 0,04	7.41 ± 0.03	7,37 ± 0,05
Sodium (mmol/L)	139,0 ± 1,0	140,0 ± 1,2	135.5 ± 2.6	140,3 ± 1,6	135.5 ± 3.2	140,3 ± 1,6	136.9 ± 2.6
Potassium (mmol/L)	3,50 ± 0,48	3,50 ± 0,2	3.74 ± 0.5	3,33 ± 0,21	3.5 ± 0.3	3,33 ± 0,21	3.9 ± 0.5
Calcium (mmol/L)	1,15 ± 0,05	1,1 ± 0,01	1.15 ± 0.06	1,14 ± 0,04	1.16 ± 0.06	1,14 ± 0,04	1.130.07

[a] statistically significant difference from the control group p<0,001

TABLE I – Hematological and biochemical parameters determined after a 30-day feeding period with low (LF) and half-fat (HF) milk

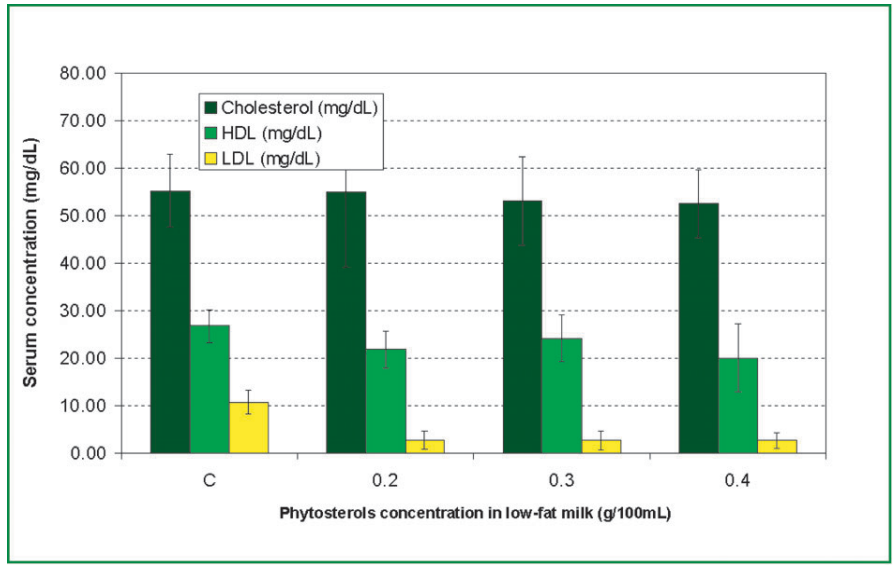


Figure 2. Mean values of total cholesterol, HDL and LDL concentrations in rat blood samples after a 30-day drinking period of low-fat milk supplied with phytosterols. The ingestion of milk without phytosterols leads to an increase in the LDL concentrations of about 54,5%, not affecting the cholesterol and HDL concentrations.

* Statistically significant difference from the control group p<0.001

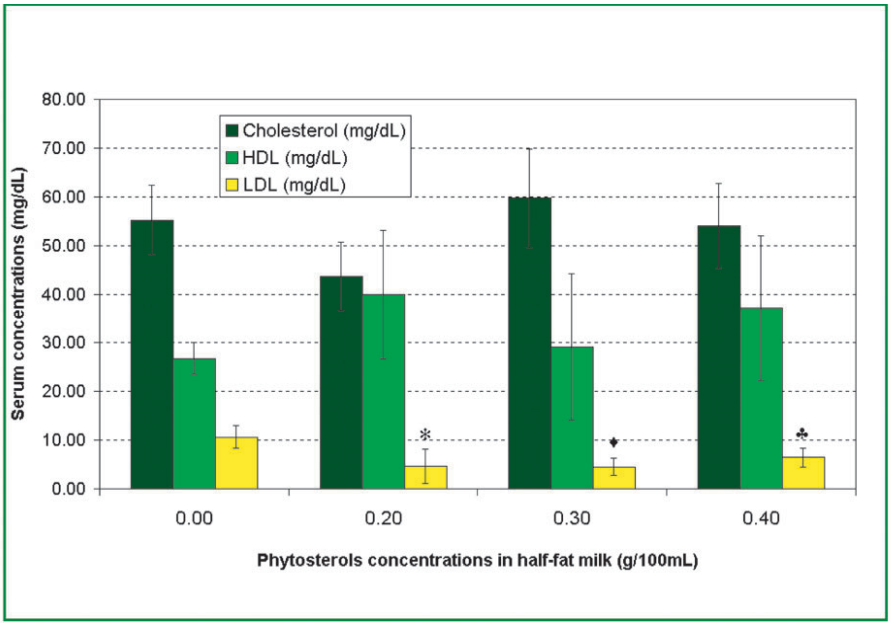


Figure 3. Mean values of total cholesterol, HDL and LDL concentrations in rat blood samples after a 30-day drinking period of half-fat milk supplied with phytosterols. Statistically significant difference from the control group *p<0.0003 ¨p<0.0001 §p<0.0005

There were no statistically significant differences in mean body weights, food and milk consumption between the control and the experimental groups, the minor statistically significant changes in some of the hematological parameters were considered to have no biological significance.

Relatively to the biochemical parameters, the following results were obtained:

- there were no significant diferences in total cholesterol and HDL levels
- there was a decrease in the LDL levels after the ingestion of both low and half-fat milk supplied with PE
- there is no proportional decrease in the LDL levels, with the increase of PE concentrations
- the decrease of LDL levels is higher with low-fat than with half-fat milk.

CONCLUSIONS

The phytosterols doses used did not produce any side effects on food or milk consumption, and there were no significant rat growth changes.

Phytosterols when added to milk did not lose its ability to lower cholesterol plasma concentrations.

APOIO

Mimosa

CNAM - Centro de Nutrição e Alimentação Mimosa

THE STUDY OF VELNACRINE MALEATE EFFECTS ON ENDOTHELIAL CELL SECOND MESSENGERS

F. A. Carvalho, J. Martins-Silva, C. Saldanha

Instituto de Biopatologia Química, Unidade de Biopatologia Vascular – Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, 1649-028 Lisboa, Portugal
menacarvalho@iol.pt

ABSTRACT

In the endothelial cell signal transduction pathways there are several receptors which have acetylcholine (ACh) as agonist. Acetylcholinesterase that hydrolyses ACh is also present. We studied the effect of ACh on the NO production and on the cAMP and cGMP concentration, on cultured HUVECs, in the absence or in the presence of velnacrine maleate (VM, acetylcholinesterase inhibitor). Cultured cells were allowed to stabilize for 30 minutes in PBS buffer. After that, we stimulated the HUVECs with ACh 10 mM in absence or in presence of VM 10 mM and monitorized the HUVECs NO production with a amperometric NO sensor. HUVECs stimulated with ACh (0, 0.01mM, 1 mM and 10 mM) in absence or in presence of VM 10 mM, were lysed and assayed for quantification of total cellular cAMP and cGMP. We observed that ACh induced an increase on NO production and this effect was significantly lowered in presence of VM ($P=0.009$, $n=5$). The cAMP and cGMP levels increased upon incubation with ACh. For ACh 10mM, the cAMP levels were of 16.7 fmol/well vs 4.1 fmol/well (control) and the cGMP levels were of 2.3 fmol/well vs 1.7 fmol/well (control), as an example. The VM slightly reduced those levels. This cAMP decrease was more evident for high ACh concentrations while the cGMP levels decrease was more relevant for low ACh concentrations.

In conclusion, these results revealed that the ACh activate the signal transduction mechanisms that leads to the NO, cAMP and cGMP production in HUVECs. The AChE-VM complex interfere with these mechanisms that so far are unknown

OBJECTIVE

The aim of this work was to study the *in vitro* effects of acetylcholine on nitric oxide production, cAMP and cGMP concentration by HUVECs in absence and presence of an acetylcholinesterase inhibitor, the velnacrine maleate (VM).

EXPERIMENTAL DESIGN

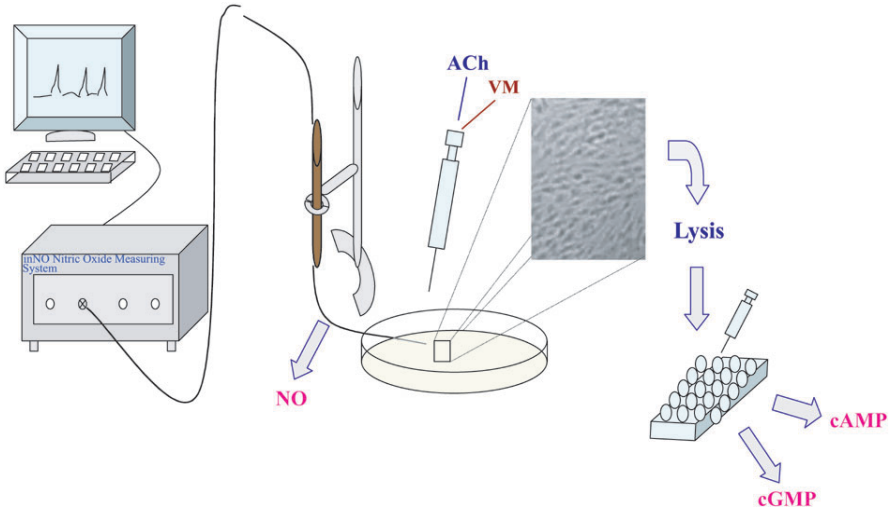


Figure 1. Endothelial cells were isolated from human umbilical cords obtained from St.Maria's Hospital Obstetrics Service. Cultured HUVECs were incubated for 30 minutes at room temperature, in PBS with immersion of an amiNO-IV sensor (*Innovative Instruments Inc. FL, USA*). After we stimulated the HUVECs with ACh 10 mM in absence or presence of VM 10 mM, we monitorized the HUVECs nitric oxide production. The total cellular cAMP and cGMP concentration are measured in supernatants, after 30 minutes of incubation at 37ºC in humidity air of 5 % CO_2 in presence of ACh (final concentrations of 0, 0.01 mM, 1 mM and 10 mM) and in absence or presence of VM 10 mM. After proceed with the HUVECs lysis, we quantified the cAMP and cGMP levels with the enzymeimmunoassay kit (*Amersham Pharmacia Biotech* (UK)). Before the lyses of the HUVECs, the cells concentration is $4x10^5$cells/sample.

RESULTS

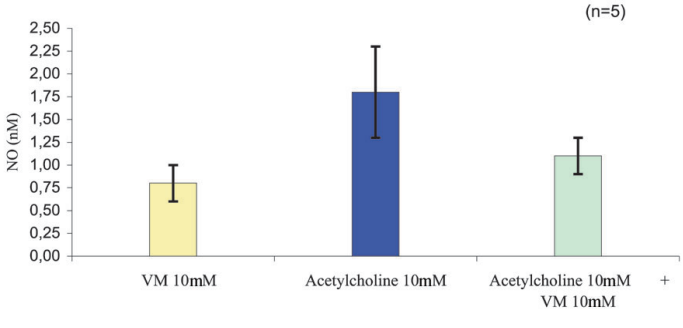


Figure 2. Changes on the NO production of HUVECs incubated with ACh 10mM in absence or resence of VM 10 mM (n=5). Significant value between the NO of HUVECs incubated with ACh and/or VM were of P=0.009.

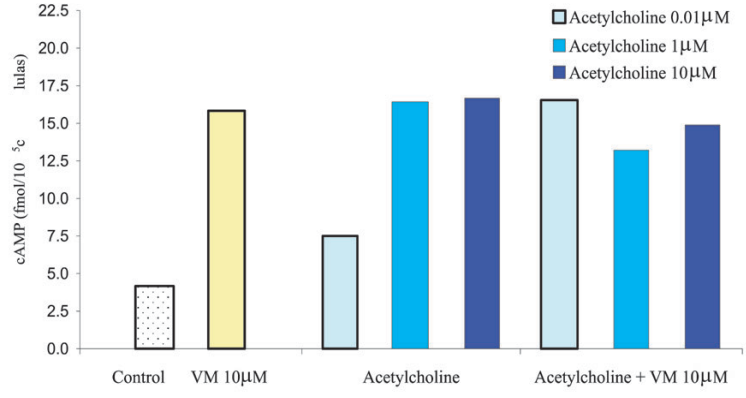


Figure 3. Changes on the cAMP values achieved on HUVECs with diferentes concentrations of ACh in absence or presence of VM (n=3).

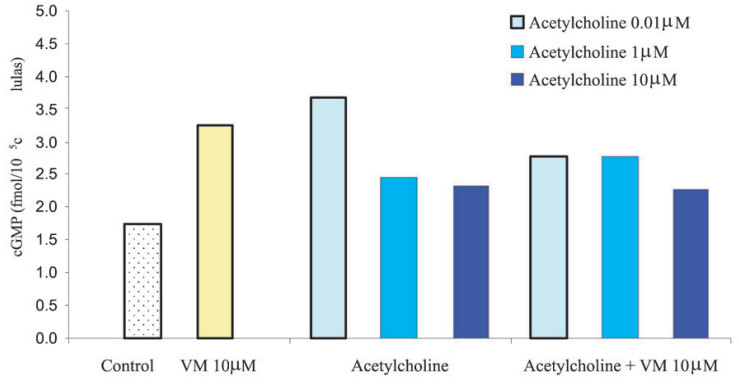


Figure 4. Changes on the cGMP values achieved on HUVECs with diferentes concentrations of ACh in absence or presence of VM (n=3).

CONCLUSIONS

- ACh increase NO production on endothelial cells.
- The presence of VM significantly decrease the NO levels achieved on endothelial cells incubated with ACh.
- ACh increase the cAMP and cGMP concentration on HUVECs.
- VM slightly decreased the cAMP values on HUVECs.
- VM decrease the cGMP values on HUVECs incubated with ACh at low concentrations.

This work reinforce the evidence that NO production by endothelial cells, and increase of intracellular cAMP and cGMP levels are involved in some of the different mechanisms of the signal transduction pathway.

The acetylcholinesterase inhibition, by velnacrine maleate,

leads to changes on NO production mechanisms but do not interfere with others pathways that could change the cAMP or cGMP levels.

ACKNOWLEDGMENTS

I would like to thank to St. Maria's Hospital Obstetrics Service (Prof. Doutor Luis Mendes Graça) who provided the Human umbilical cords for the endothelial cells culture used on this "in vitro" study.

The velnacrine maleate compound was given by *Hoechst Pharmaceutical Inc.* (New Jersey, USA).

APOIO FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA E DO ENSINO SUPERIOR Portugal

THE STUDY OF VELNACRINE MALEATE EFFECTS ON ERYTHROCYTES SECOND MESSENGERS

F. A. Carvalho, J. Martins-Silva, C. Saldanha

Instituto de Biopatologia Química, Unidade de Biopatologia Vascular – Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, 1649-028 Lisboa, Portugal
menacarvalho@iol.pt

ABSTRACT

Acetylcholine (ACh) has been detected in human blood. ACh receptors and acetylcholinesterase (AChE) are present in erythrocytes membranes. We tested the ACh effect on the nitric oxide production, the cAMP and cGMP concentration on erythrocytes suspensions, in absence or in presence of velnacrine maleate (VM, AChE inhibitor). We used human erythocytes suspensions in sodium chloride 0.9% pH 7 (haematocrit 0.05%) to measure the NO production with a amperometric NO sensor during

stimulation with ACh 10 mM in absence or in presence of VM 10 mM. For quantification of the total cellular cAMP and cGMP we incubate the erythrocytes suspensions (haematocrit 45%), for 15 minutes at 37ºC, with ACh (0, 0.01mM and 10 mM) in absence or in presence of VM 10 mM. After that we lysed them and used the supernatant for the enzyme immunoassay kits. We observed that ACh induce an increase on the erythrocytes NO production and the presence of VM reduced this increase (P=0.009, n=10). The erythrocytes ACh stimulation does not produced significant diferences in cAMP control levels and the presence of VM was also insignificant. Although cGMP levels increased with ACh and this fact is more relevant for low ACh concentrations. The VM significant decreased these levels and leads them to near the control values. These results demonstrated that ACh modulate the NO and the cGMP production on erythrocytes and that this effect could be mediated by unknown interactions with the membrane enzyme acetylcholinesterase. The signal transduction mechanisms that leads to cAMP production seams not to be related or activated by the presence of ACh on erythrocytes.

OBJECTIVE

The aim of this work was to study the *in vitro* effects of acetylcholine on nitric oxide production, cAMP and cGMP concentration on erythrocytes suspensions in absence or presence of velnacrine maleate (VM, acetylcholinesterase inhibitor).

EXPERIMENTAL DESIGN

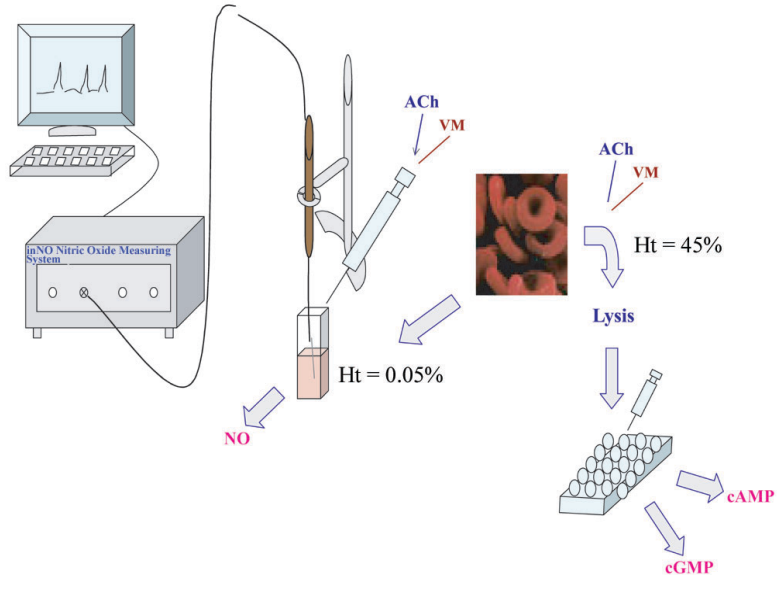


Figure 1: Venous blood samples were collected from ten healthy caucasian men for tubes with sodium heparin 10 UI/mL. The blood was centrifuged and plasma and buffy-coat was discarded. The erythrocyte suspensions were obtained adding sodium chloride 0.9% at pH 7.0 to the erythrocytes to reconstitute a hematocrit of 0.05%. For amperometric NO quantification we used the amiNO-IV sensor (*Innovative Instruments Inc. FL, USA*). The erythrocytes suspensions were incubated for 30 minutes at room temperature and after that, we stimulated the suspensions with ACh 10 mM in absence or presence of VM 10 mM and we monitorized the erythrocytes nitric oxide production. The total cellular cAMP and cGMP concentration are measured in supernatants, after 30 minutes of erytrocytes suspensions incubation at 37ºC with a hematocrit of 45%, in presence of ACh (final concentrations of 0, 0.01 mM, 1 mM and 10 mM) and in absence or presence of VM 10 mM. After proceed with the HUVECs lysis, we quantified the cAMP and cGMP levels with the enzymeimmunoassay kit (*Amersham Pharmacia Biotech* (UK)).

RESULTS

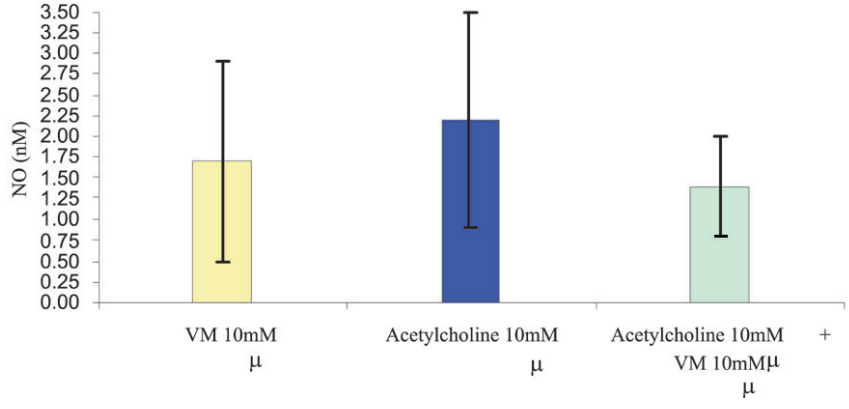


Figure 2. Changes on the NO production of erythrocytes suspensions incubated with ACh 10mM in absence or presence of VM 10 mM (n=10). Significant value between the NO of erythrocytes incubated with ACh and/or VM were of P=0.009.

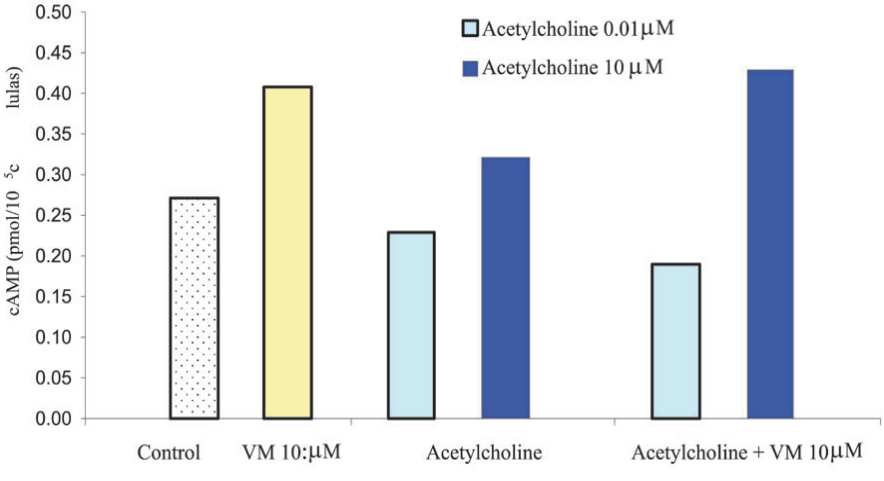


Figure 3. Changes on the cAMP values achieved on erythrocytes suspensions with diferentes concentrations of ACh in absence or presence of VM (n=3).

CONCLUSIONS

- ACh increase NO production on "*in vitro*" erytrocytes suspensions.
- The presence of VM significantly decrease the NO levels achieved on erythrocytes suspensions incubated with ACh.
- The cAMP levels do not have significant changes when ACh are present on erythrocytes suspensions.
- VM increased the cAMP values

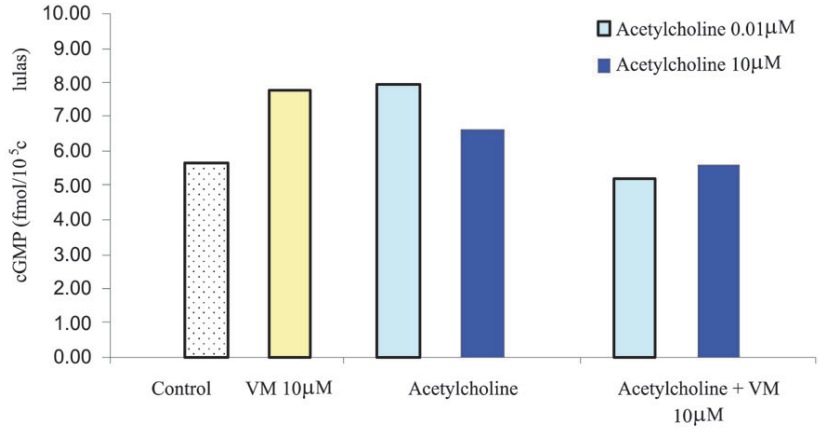


Figure 4. Changes on the cGMP values achieved on erythrocytes suspensions with diferentes concentrations of ACh in absence or presence of VM (n=3).

on erythrocytes suspensions with high ACh concentrations.

- Low concentration of ACh leads to an increase of cGMP erythrocytes levels.
- VM decrease the cGMP values, on erythrocytes suspensions incubated with ACh, to near control values.

With this study we may conclude that the NO and the cGMP production on erythrocytes are involved in some of the different mechanisms of the signal transduction pathway that are related with the membrane enzyme acetylcholinesterase.

The mechanisms that are involved with changes on cAMP erythrocytes levels, in presence of ACh, seems to be distinct of others that leads to changes on NO and cGMP levels.

ACKNOWLEDGMENTS

The velnacrine maleate compound was given by *Hoechst Pharmaceutical Inc.* (New Jersey, USA).

APOIO FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA E DO ENSINO SUPERIOR Portugal

STUDY OF NITRIC OXIDE PRODUCTION ON HEALTHY VERSUS SICK PERSONS

F. A. Carvalho, D. Soares, A.V. Maria[1], J. Guerra, M. Martins Prata, L. Caeiro[2], J. Martins-Silva, C. Saldanha, [1] V. Pimpão, G. Carvalho, A. Carreira e J. Azevedo
[2] J. M. Ferro, R. Albuquerque e M. C. Figueira

Instituto de Biopatologia Química, Unidade de Biopatologia Vascular -Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, 1649-028 Lisboa, Portugal
menacarvalho@iol.pt

ABSTRACT

The nitric oxide (NO) is an important vasodilator messenger interfering in a number of physiological and pathophysiological processes. In this study we stimulated human erythrocytes obtained from patients with different types of diseases (hypercholesterolemia, drepanocytosis, delirium and renal transplantation) with acetylcholine (ACh) and measured the NO production, comparing with the NO levels achieved on erythrocytes of healthy persons. We used human erythrocyte suspensions in sodium chloride 0.9% pH 7 (hematocrit 0.05%) to measure the NO production with a amperometric NO sensor during stimulation with ACh 10 mM. The erythrocytes NO levels, according to the different studied groups, were of 2.8 ± 0.8 nM (delirium; $P<0.001$, n=67), 2.5 ± 0.7 nM (hypercholesterolemia; P=0.018, n=17), 5.4 ± 0.8 nM (drepanocytosis; $P<0.001$, n=5) and 2.4 ± 1.2 nM (renal transplantation; n=15) against the 2.0 ± 0.8 nM (n=43) for the control values. We observed the most significant change on the NO production with drepanocytosis erythrocytes samples, which could be a positive factor for the compromised tissue oxigenation in this kind of anemia. In conclusion, human erythrocytes of different diseases have different physiological responses to ACh stimulation that leads to changes on NO mobilization mechanisms. The different erythrocytes NO values obtained after ACh stimulation, suggested a future target for vasodilate therapeutic action on a microcirculatory network, damaged by different sorts of stimulus.

OBJECTIVE

The aim of this work was to compare the *in vitro* effects of acetylcholine on nitric oxide production of healthy persons erythrocytes suspensions and of others with different types of human diseases (delirium, hypercholesterolemia, drepanocytosis and renal transplantation).

EXPERIMENTAL DESIGN

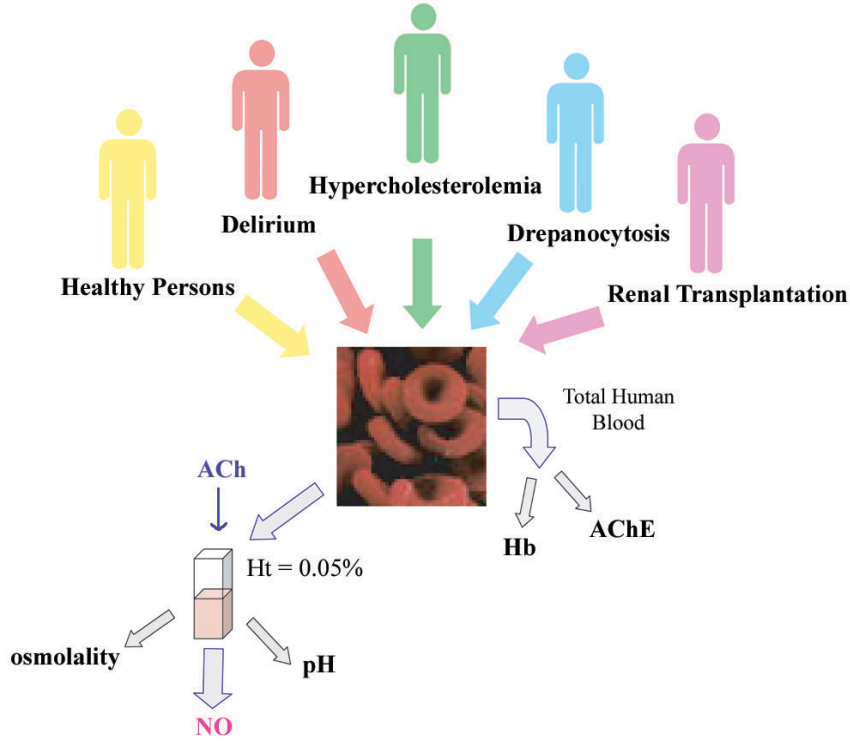


Figure 1. Venous blood samples were collected for tubes with sodium heparin 10 UI/mL, from healthy humans and from people who have different types of diseases such as, delirium, hypercholesterolemia, drepanocytosis and renal transplantation. We quantified the levels of hemoglobin and of acetylcholinesterase activity from the differents total blood samples. The blood was centrifuged and plasma and buffy-coat was discarded. The erythrocyte suspensions were obtained adding sodium chloride 0.9% at pH 7.0 to the erythrocytes to reconstitute a hematocrit of 0.05%. For amperometric NO quantification we used the amiNO-IV sensor (*Innovative Instruments Inc. FL, USA*). The erythrocytes suspensions were incubated for 30 minutes at room temperature and after that, we stimulated the erythrocytes suspensions with ACh 10 mM and monitorized the erythrocytes nitric oxide production. We also measured other parameters like osmolality and pH on 0.05% hematocrit samples before and after ACh stimulation.

RESULTS

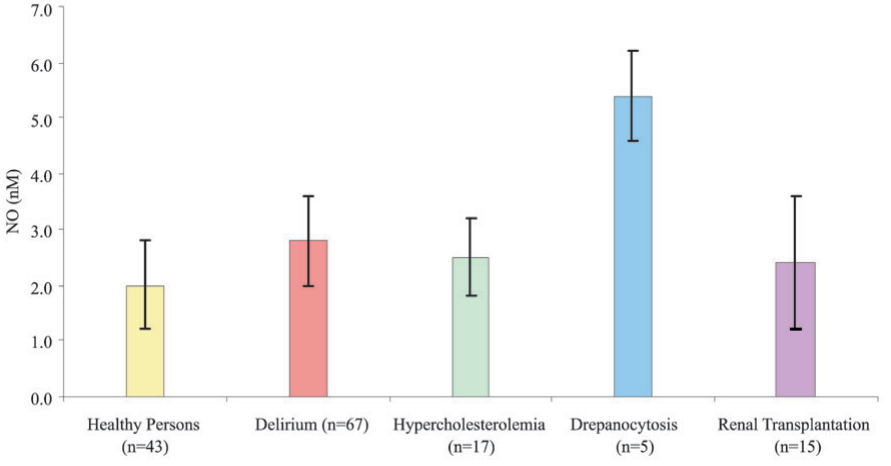


Figure 2. Changes on the NO production of erythrocytes suspensions incubated with ACh 10mM from healthy and different diseases persons, such as, delirium, hypercholesterolemia, drepanocytosis and renal transplantation. Significant value between the NO of healthy persons with others are of P <0.001 for delirium, P =0.018 for hypercholesterolemia, P <0.001 for drepanocytosis and for renal transplatation the values were not significants.

Parameters	Healthy Persons (n=43	Delirium (n=67)	Hypercholesterolemia (n=17)	Drepanocytosis (n=5)	Renal Transplantation (n=15)	
Hb (g/dL)	13.7	13.8	14.2	9.2	12.3	Total human blood values (Ht 40-45%)
AChE (UI/min.mgHb)	314.2	293.6	---	330.0	292.6	
pH	5.8 /5.9	5.9 /5.9	6.0 / 6.0	---	5.8 / 6.0	Human erythrocytes suspensions values (Ht 0.05%)
osmolality (osmol/Kg)	0.300 / 0.300	0.300 / 0.300	0.300 / 0.300	---	0.300 / 0.300	

Table 1. Mean values of hemoglobin (Hb), acetylcholinesterase activity, P50, pH, and osmolality from healthy persons and others who have delirium, hypercholesterolemia, drepanocytosis or renal transplantation diseases. The values indicated by _ / _ were those achived for measurements before / after ACh stimulation of erytrocytes suspensions.

CONCLUSIONS

- ACh increase NO production on "*in vitro*" erytrocytes of different diseases.
- Drepanocytosis is the disease that get the most increase of erythrocytes NO production.
- The hemoglobin levels increase with the studied diseases comparing to healthy persons, except for drepanocytosis that decrease.

- The acetylcholinesterase activity have different changes related with the different disorders.
- The pH and the osmolality maintain the same levels before and after ACh erythrocytes stimulation (hematocrit 0.05%).

With this study we may concluded that different diseases have different physiological responses to erythrocyte ACh stimulation that leads to changes on erythrocytes NO production. This fact could be related with changes on the membrane enzyme acetylcholinesterase activity or on hemoglobin concentration (*eg* drepanocytosis, an anemia type). The cellular interactions between ACh and erythrocytes membrane that leads to NO appearance are not related with changes on pH or osmolality of the medium.

All the results suggest a future target for vasodilate therapeutic action on a microcirculatory network, damaged by different sorts of stimulus.

ACKNOWLEDGMENTS

The velnacrine maleate compound was given by *Hoechst Pharmaceutical Inc.* (New Jersey, USA).

APOIO FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA E DO ENSINO SUPERIOR Portugal

GRAMICIDIN D, DITHIOTHREITOL AND CYTOCHALASIN B EFFECTS ON ERYTHROCYTE EXOVESICULATION

Nuno C. Santos, J. Martins-Silva, Carlota Saldanha

Instituto de Biopatologia Química and Unidade de Biopatologia Vascular - Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal
(nsantos@fm.ul.pt).

ABSTRACT

The use of diphenylhexatriene (DPH), trimethylamino-diphenylhexatriene (TMA-) or heptadecyl-hydroxycoumarin (C_{17}-HC) allows to, simultaneously and with the same molecule, to induce erythrocyte exovesiculation and label the released vesicles with the fluorescent probe. This method was used

to evaluate gramicidin D (a channel-forming peptide), dithiothreitol (a reducing agent) and cytochalasin B (a toxin that disrupts contractile microfilaments) effects on the human erythrocytes vesiculation process. The release of cholesterol and phospholipids in exovesicles at longer incubation times was only detectable in the presence of these effectors. When C_{17}-HC was used to induce the vesiculation, the presence of gramicidin or dithiothreitol lead to a drastic decrease on the [Phospholipids]/[Cholesterol] ratio. However, in the samples with dithiothreitol, this variation did not result in the expectable decrease of membrane fluidity. These effects can be related with the presence of lipid rafts, the transbilayer lipids reorientation induced by gramicidin or dithiothreitol, and the cholesterol-dependent gramicidin channels inactivation. At most of the experimental conditions under evaluation, cytochalasin B induced significantly increased cholesterol concentration and acetylcholinesterase (AChE) enzyme activity on the released vesicles.

1. INTRODUCTION

Erythrocyte membrane vesiculation occurs during differentiation, senescence and storage. ATP depletion, pH and temperature variations, or amphiphiles treatment also induce exovesicle release from erythrocytes. These microvesicles are enriched with acetylcholinesterase (AChE), a marker of erythrocyte membrane integrity.

As previously described (1, 2), the use of diphenylhexatriene (DPH), trimethylamino-diphenylhexatriene (TMA-DPH) or heptadecyl-hydroxycoumarin (C_{17}-HC) allows to induce erythrocyte exovesiculation and label the released vesicles with the fluorescent probe (Figure 1).

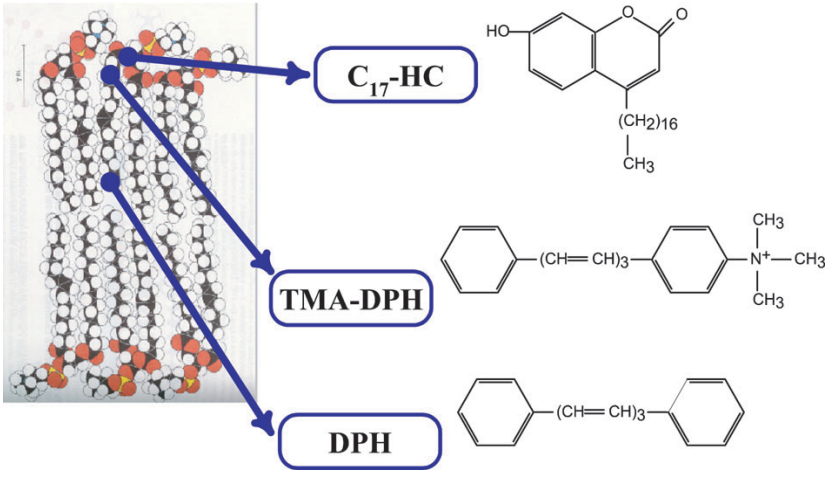


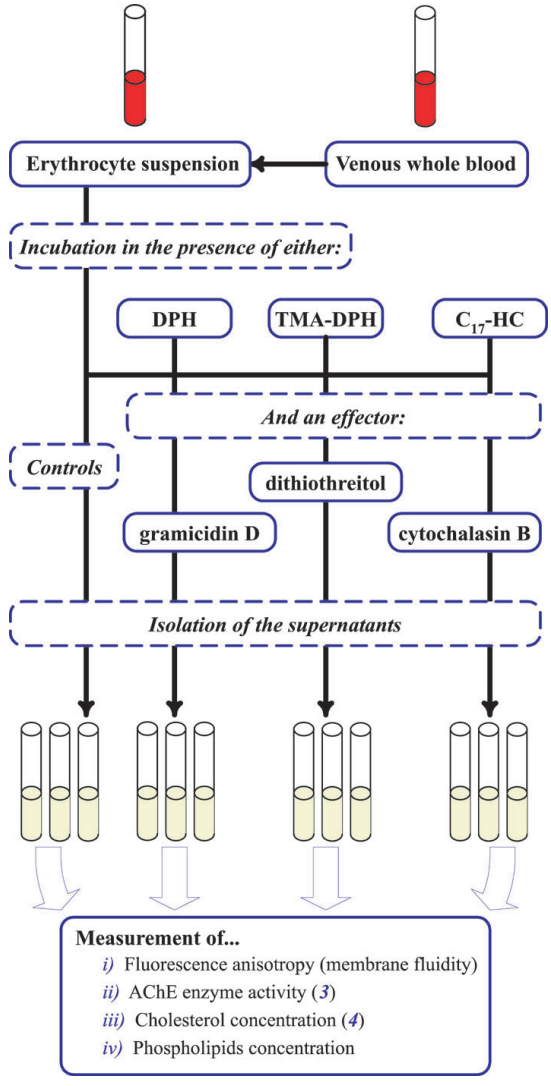
Figure 1. Molecular structures of DPH, TMA-DPH and C_{17}-HC, together with the location of the fluorophores in the membrane.

2. AIMS

- To use the previously developed method to evaluate the effects on the human erythrocytes vesiculation process of:
  - Gramicidin D (a channel-forming peptide)
  - Dithiothreitol (a reducing agent)
  - Cytochalasin B (a toxin that disrupts contractile microfilaments)

- The effects on the exovesiculation process were assessed by:
  i) comparing the exovesicle membrane fluidity at different depths;
  ii) determining the cholesterol content released on the exovesicles;
  iii) determining the phospholipids content of the same vesicles;
  iv) determining the AChE enzyme activity on the exovesicles.

3. MATERIALS AND METHODS



4. RESULTS

Table 1 – Cholesterol concentrations determined on the supernatants of centrifugations carried out 1 h (t_1), 24 h (t_{24}) and 48 h (t_{48}) after erythrocyte suspensions incubation with DPH, TMA-DPH or C_{17}-HC (C, control; G, gramicidin; D, dithiothreitol; cB cytochalasin B). There is a significant ($p < 0.0005$) decrease of the cholesterol content with time. Independently of the incubation time, the controls cholesterol concentration decreases following the trend $[C]_{TMA\text{-}DPH} > [C]_{DPH} > [C]_{C17\text{-}HC}$ ($p < 0.0005$). For samples with gramicidin or dithiothreitol, the trend changes to $[C]_{C17\text{-}HC} > [C]_{TMA\text{-}DPH} > [C]_{DPH}$ ($p < 0.04$).

SAMPLE		[Cholesterol] (mM)		
		DPH	TMA-DPH	C_{17}-HC
Supernatant t_1	C	1.53 ± 0.13	1.73 ± 0.21	0.53 ± 0.07
	G	1.01 ± 0.10	1.25 ± 0.19	1.56 ± 0.19
	D	0.98 ± 0.19	1.44 ± 0.21	1.86 ± 0.13
	cB	1.62 ± 0.18	2.00 ± 0.23	1.56 ± 0.11
Supernatant t_{24}	C	1.43 ± 0.14	1.60 ± 0.22	0.39± 0.09
	G	0.76 ± 0.11	1.01 ± 0.24	1.30 ± 0.23
	D	0.74 ± 0.19	1.12 ± 0.17	1.55 ± 0.16
	cB	1.05 ± 0.13	1.31 ± 0.07	1.05 ± 0.91
Supernatant t_{48}	C	≈ 0	≈ 0	≈ 0
	G	0.32 ± 0.19	0.49 ± 0.31	0.60 ± 0.30
	D	0.20 ± 0.11	0.45 ± 0.14	0.31 ± 0.18
	cB	0.73 ± 0.10	0.94 ± 0.09	0.96 ± 0.08

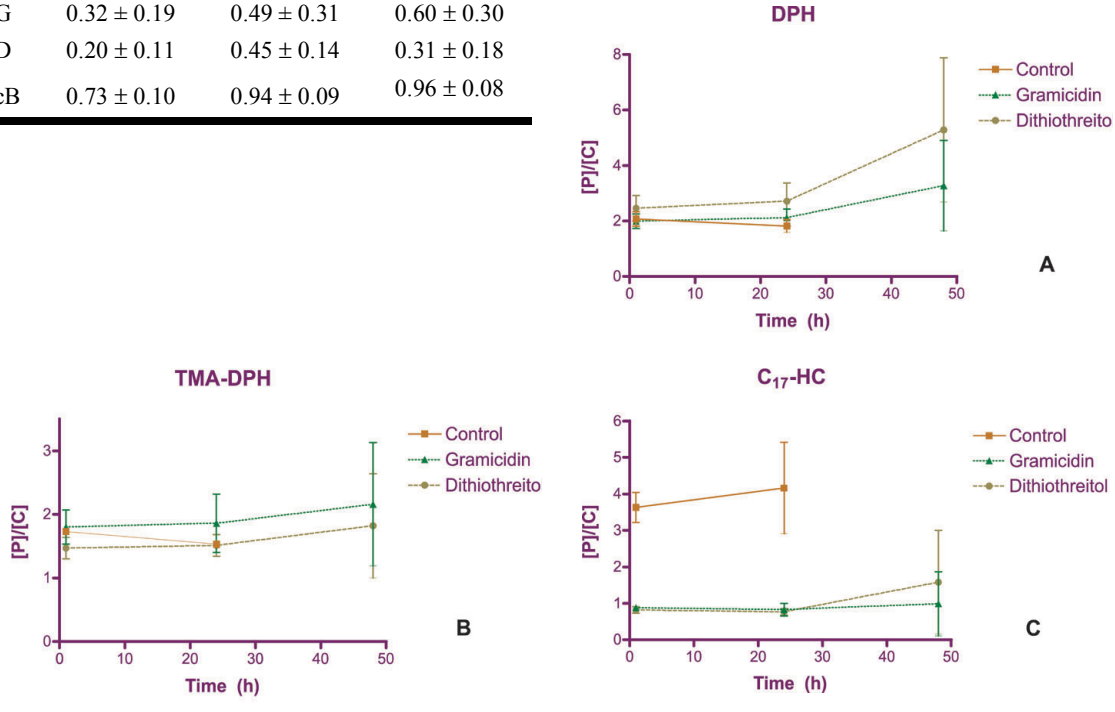


Figure 2. Ratios between the phospholipids and cholesterol molar concentrations ([P]/[C]) obtained after different times of erythrocyte suspensions incubation with DPH (**A**), TMA-DPH (**B**) and C_{17}-HC (**C**), in the absence and presence of gramicidin D or dithiothreitol. For the controls, the ratios obtained for the different probes follow the trend $[P]/[C]_{C17\text{-}HC} > [P]/[C]_{DPH} > [P]/[C]_{TMA\text{-}DPH}$ ($p < 0.008$). In the presence of gramicidin or dithiothreitol the trend changes to $[P]/[C]_{DPH} > [P]/[C]_{TMA\text{-}DPH} > [P]/[C]_{C17\text{-}HC}$. These ratios were significantly ($p < 0.05$) different from their controls.

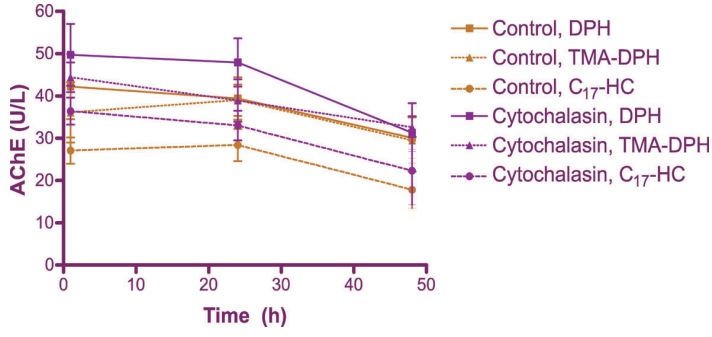


Figure 3. AChE activities measured in the supernatants of aliquots centrifuged at different times after erythrocyte incubation with DPH, TMA-DPH or C_{17}-HC, in the absence and presence of cytochalasin B. This contractile microfilaments disrupter led to increased AChE activity.

5. CONCLUSIONS

- AChE enzyme activities of the supernatants confirm the presence of exovesicles released from erythrocyte membrane labeled with the fluorescent probes DPH, TMA-DPH and C_{17}-HC, indicating that these probes can be used to induce erythrocyte vesiculation.
- Contractile microfilaments disruption by cytochalasin B facilitates the vesiculation process, as shown by the increased AChE activity and by the increased cholesterol release.
- When (TMA-)DPH is used to induce vesiculation, erythrocytes incubated with gramicidin present a slower release of cholesterol and phospholipids (compared with control), justifiable by a decreased cholesterol availability due to its interaction with gramicidin.
- When C_{17}-HC is used to induce vesiculation, the presence of gramicidin leads to larger cholesterol release, without a concomitant variation on the phospholipids release. This increased cholesterol release can be due to some gramicidin-induced phenomena, namely: decrease on ATP content, promotion of inverted hexagonal HII non-lamellar lipid phases and/or enhancement of transbilayer reorientation of lipids in erythrocytes (all these effects can lead to a more intense vesiculation).
- Both gramicidin or dithiothreitol in samples with C_{17}-HC led to identical reductions of [P]/[C]. For gramicidin, this decrease is in agreement with the expectable increase on fluorescence anisotropy (lower membrane fluidity), due to a higher percentage of cholesterol. However, for dithiothreitol a decrease on anisotropy (higher membrane fluidity) is observed.

- Gramicidin, dithiothreitol and cytochalasin B change: *i)* the variation of [P]/[C] *vs.* time induced by any of the three fluorescent probes, and *ii)* the composition of the exovesicles released with C_{17}-HC (specially relevant at t_{48}).
- Erythrocyte vesiculation may be nonspecifically triggered by most of the alterations on the native erythrocyte membrane composition, such as the incorporation of exogenous molecules or the impairment of the native phospholipid asymmetry between inner and outer monolayers.

6. REFERENCES

1. Saldanha *et al.* (2002) *J. Membr. Biol.*, **190**, 75-82.
2. Saldanha *et al.* (2004) *Biochem. Mol. Biol. Educ.*, **32**, 250-253.
3. Ellman *et al.* (1961) *Biochem. Pharmacol.*, **7**, 88-95.
4. Ott *et al.* (1982) *Biochim. Biophys. Acta*, **685**, 211-213.

APOIO FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA E DO ENSINO SUPERIOR Portugal