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ORIGINAL ARTICLE

Development and characterization of multilamellar liposomes containing pyridostigmine

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Abstract

Pyridostigmine has cardioprotective activity in both free and liposomal forms. This study aimed to develop and characterize liposomal formulations of pyridostigmine. For this, a spectrophotometric ultraviolet (UV) analytical method, at 270 nm, was developed and validated to quantify liposomal pyridostigmine. The method was linear in ranges from 0.02 to 0.09 mg/mL. The accuracy of this method was determined intra- and inter-day; the results of coefficient of variation were of 1.73–2.72% and 0.32–2.32%, respectively. The accuracy ranged between 99.45% and 101.12%. The method has not changed by influence of liposomal matrix and demonstrated being able to quantify pyridostigmine in liposomes. Two liposomal multilamellar formulations were developed: a constituted by dystearoyl-phosphatidylcholine (DSPC) and cholesterol (CHOL) other by dioleil-phosphatidylcholine (DOPC) and CHOL. The encapsulation efficiency was determined as 23.4% and 15.4%, respectively. Analyses of size and release of pyridostigmine from the formulations were made and the results showed that the formulations are viable for future studies *in vivo*.

Keywords

Liposomal matrix, pyridostigmine, UV spectrophotometric method

History

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Introduction

Pyridostigmine (3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide) is a drug indicated for the treatment of myasthenia gravis because it increases the concentration of acetylcholine at the motor endplate, increasing muscle strength and tone. Previous studies in normal rats and humans evaluated the therapeutic potential of pyridostigmine as cardioprotection drug[1–3]. The pyridostigmine in healthy humans, reduces heart rate without depressing cardiac function[4,5], inhibits the increases of rate pressure product, an indirect index of cardiac oxygen consumption, and increases the diastolic function in the left ventricle[3] induced by mental stress[6], inhibits the chronotropic response to exercise without loss of functional capacity[7] and decreases the QT interval dispersion of ECG at rest[8]. In patients with coronary artery disease, administration of pyridostigmine protects against myocardial ischemia induced by effort[9], prevents the impairment of myocardial function caused by mental stress[10] and improves autonomic and hemodynamic profiles during exercise[11]. A single oral dose of pyridostigmine in rats was able to attenuate the excitatory cardiovascular response induced by central glutamate, as dP/dt_{max}, rate pressure product and triple product, indicating its ability to prevent increases in cardiac contractility and oxygen consumption[2]. Despite the clear demonstration of the pyridostigmine as a potential therapeutic option for cardiovascular disease[12], its short half-life and the incidence of adverse effects[13,14] are factors that may limit its current use. This way, it was recently proposed for the first time the pyridostigmine encapsulation in unilamellar liposomes and the cardioprotective capacity was maintained until 6 h after its intravenous administration[15]. Liposomes are nanometric and micrometric vesicles presenting the property of conveying hydrophilic or lipophylic substances, that may confer differentiate drug release, body distribution and organism interactions[16], aspect that can thereby increase the drug effects and/or reduce the toxicity.[17,18]

Liposome preparation requires the quantification of drug encapsulation and in order to attest the efficiency of pyridostigmine encapsulation, the development and validation of an analytical method was necessary that is not influenced by the lipid matrix[19]. The pyridostigmine concentration and/or its metabolites has been already measured in biological material by radioisotope method[20–22], gas chromatography technique[23], high performance liquid chromatography (HPLC)[24–27], radioimmunoassay[28,29] and by electrochemical method in pills[30]. The techniques using spectroscopy detection ultraviolet (UV) can be regarded as less expensive, more easily feasible in the quality control of raw materials and pharmaceuticals and kinetic studies of drug release[31]. Additionally, the quantification method for pyridostigmine raw material is also described in European Pharmacopoeia (2004) and in North American Pharmacopoeia (USP) (2005), by HPLC with the detection by spectrophotometry at 220 nm[32] and by infrared absorption and UV[33]. Hegazy et al.[34] determined the content of pyridostigmine in microparticles using the spectrophotometry at 270 nm. However, a method for pyridostigmine quantification in liposomal form is not yet described.

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Thus, in order to prolong the cardioprotection effect of pyridostigmine and reduce its side effects, the aim of present work was to develop and characterize formulations of conventional liposomes multilamellar of pyridostigmine to subcutaneous administration, composed of dystearoyl-phosphatidylcholine (DSPC) and cholesterol (CHOL) or dioleil-phosphatidylcholine (DOPC) and CHOL. So, a simple and effective analytical spectrophotometric method was validated allowing quantifying the pyridostigmine in liposomal matrix.

Materials and methods

Drug, lipids and reagents

Pyridostigmine and CHOL were purchased from Sigma (St. Louis, MO). The lipids DSPC, CHOL and DOPC were purchased from Lipoid GmbH (Ludwigshafen, Germany). The solvents were of analytical grade and all other chemicals were commercially available. Water was purified by reverse osmosis (185Symplicity System, Millipore, Billerica, MA).

Multilamellar liposomes

The multilamellar liposomes were composed of DOPC or DSPC and CHOL which were prepared by freezing and thawing method proposed by Nayar et al.[35] Briefly, lipids were solubilized in chloroform and the residue was taken with the aid of a rotary evaporator (Laborota4000®, Instruments Heidolph, Schwabach, Germany) at 70 rpm, 60 °C. The lipid film was hydrated with pyridostigmine bromide solution isosmotic related to the plasma to obtain multilamellar liposomes containing the active substance, or tamponated saline solution (phosphate-buffered saline (PBS) – 0.15 M NaCl, 0.01 M phosphate, pH 7.2) to obtain empty liposomes. The emulsions obtained were then homogenized for 5 min at a rotary vacuum evaporator and subjected to 10 freeze and thaw cycles using liquid nitrogen and bath at 60 °C, leading to the encapsulation of active hydrophilic substance into the aqueous liposome compartment. Finally, the encapsulated pyridostigmine was separated from the non-encapsulated pyridostigmine by centrifugation at 14 000 g (Quimis®centrifugeQ222E12, Diadema, Brazil).

Spectrophotometric analytical method development

To establish the analytical conditions, methanol was used as solvent and readings of the samples were evaluated at wavelength range between 240 and 300 nm. All quantitative analyses of the pyridostigmine assay were performed on Micronal spectrophotometer, Model B582 (Sao Paulo, Brazil), using quartz cuvettes.

Standard solutions

To prepare the standard stock solution, 25.0 mg of pyridostigmine were solubilized at 25.0 mL of methanol. The final concentration of pyridostigmine was 1.0 mg/mL, which were pipetted from the stock standard solution aliquots of pyridostigmine 200, 300, 400, 500 and 600 µL being diluted in methanol to 10.0 mL and 350, 400 and 450 µL being diluted to 5.0 mL in methanol to yield final concentrations of 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08 and 0.09 mg/mL.

Validation of the analytical method to quantify pyridostigmine

Before a completely new analytical method to be implemented in routine, it must be preliminarily validated to demonstrate that it is appropriate for its purpose[34–36]. The procedures were performed according to ANVISA (National Health Surveillance Agency) Resolution RE No. 899 of 29/05/2003[37] and criteria established by the International Conference on Standardization of Technical Requirements for Registration of Pharmaceuticals for Human Use (International Conference on Harmonization, ICH of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH, 2005). The validation parameters evaluated in this study are described below and all the analyses were performed on quintuplicates.

Specificity

In order to identify possible interferences from the sample matrix of liposomes, samples with or without pyridostigmine were analyzed using the developed method.

Linearity

It was established by statistical analysis of five standard curves, elaborated from the quantification of the diluted standard solution concentrations between 0.02 and 0.09 mg/mL by UV spectrophotometry at a wavelength of 270 nm.

Precision

The repeatability (intra-day) and intermediate precision (inter-day) were evaluated by determining three concentrations of pyridostigmine (0.03, 0.06 and 0.09 mg/mL) each one in quintuplicate. Analyses were performed on two different days, by two analysts, being determined different values of coefficient of variation (CV%) of each result group.

Accuracy

It was evaluated by the recovery of known amounts of pyridostigmine in solution at low, medium and high concentrations: 0.03, 0.06 and 0.09 mg/mL, respectively. The percentage of recovery was used and the accuracy is expressed as the ratio between the average concentration determined experimentally and the corresponding theoretical concentration.

$$\text{Accuracy} = \frac{\text{Experimental concentration}}{\text{Theoretical concentration}} \times 100$$

Limit of quantification (LOQ)

It was obtained using the analysis of solutions containing decreasing concentrations of pyridostigmine to the lowest determinable level with acceptable precision and accuracy.

Encapsulation efficiency (EE)

The EE of pyridostigmine in multilamellar liposomes was determined using the quantification methodology validated in the present study, using the standard reference curve of pyridostigmine at the concentration range from 0.02 to 0.09 mg/mL. After separation of pyridostigmine encapsulated from the non-encapsulated by centrifugation at 14 000 g, the pyridostigmine content present in liposomes was obtained using the equation following:

$$\%\text{EE} = \frac{\text{Concentration of pyridostigmine in liposomes}}{\text{Total content of pyridostigmine}} \times 100$$

Liposomes size and zeta-potential

The particles size and the size polydispersity index of the liposomes were determined using the HS Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). Pyridostigmine in liposomes or liposomes without drug were diluted in PBS (1:250) and three readings were performed for each sample.

The surface charge of the liposomes was obtained using the zeta-potential, determined by measuring the electrophoretic mobility at the HS Zetasizer 3000. For this, the liposomal preparations containing pyridostigmine or PBS were diluted in PBS solution (1:200) determination in triplicate.

In vitro release profile

In order to assess the ability of liposomes retain the pyridostigmine under physiological conditions, a 1:5 dilution of the liposomal formulation was made in PBS solution to ensure the sink condition. Samples were then incubated at 37 °C and 0, 2, 4, 6, 12, 24, 48 and 72 h after, 200 µL aliquots were centrifuged at 14 000 g for 120 min to separate the pyridostigmine released at different times. The supernatant samples were then submitted for determination of the content of pyridostigmine released, by UV spectrophotometric method, in order to determine the *in vitro* release profile of the drug from the liposomes.

Results and discussion

Development of analytical method

The development of analytical methods is the search and evaluation of different experimental conditions that allow the quantitative determination of the analyte with reliability, speed, low cost and with minimum generation of waste[19]. The ultraviolet spectrophotometry meets these objectives and the scan range of pyridostigmine in methanol showed maximum absorbance at the wavelength 270 nm as described before[32]. So, this wavelength was selected.

Specificity

The sample analysis containing only the liposomal matrix produced data coincident to the baseline of spectrum showing that the matrix does not interfere in the quantification of pyridostigmine in liposomes at a wavelength 270 nm.

Linearity

Data from analytical curves, resulting from the average of five standard curves, were fitted by linear regression analysis (Table 1). The line equation obtained showed absorbance = 16.398 (pyridostigmine bromide) mg/mL + 0.0351 with correlation coefficient $r = 0.9993$. The average concentration and the relative standard deviation calculated were within the acceptable limits.

Precision

The precision was assessed by repeatability studies and the variation coefficient was from 1.73% to 2.72% and the intermediate precision was from 0.32% to 2.32%. These results are lower than the maximum value required of 5% (Tables 2 and 3).

Table 1. Data used for the curve linearity determination from eight concentrations of pyridostigmine.

Concentration of pyridostigmine (mg/mL)	Mean absorbance (±SD)[a]	Calculated concentration of pyridostigmine (mg/mL)
0.02	0.3430 ± 0.027	0.0188
0.03	0.5325 ± 0.0135	0.0303
0.04	0.7047 ± 0.0215	0.0408
0.05	0.8661 ± 0.01	0.0507
0.06	1.0163 ± 0.0170	0.0598
0.07	1.1853 ± 0.0166	0.0701
0.08	1.3452 ± 0.0222	0.0799
0.09	1.5028 ± 0.0351	0.0895

[a]$n = 5$.

Accuracy

The experimental data showed recovery percentage ranging from 99.45% to 101.12% (Table 4). The accuracy study results demonstrate that small variations in concentration of pyridostigmine can be promptly quantified by the method and there is no interference of the excipients of the liposomal formulation at the concentration determination of the final product.

LOQ

The value of the LOQ, established experimentally, that presented acceptable precision and accuracy of analysis, was 0.02 mg/mL (Table 5), demonstrating that the method is sensitive to low concentrations.

Table 2. Intra-day pyridostigmine concentration for the precision determination[a].

Theoretical concentration (mg/mL)	Experimental concentration in quintuplicate (mg/mL)					SD	CV (%)
0.03	0.030	0.032	0.030	0.030	0.031	0.001	2.72
0.06	0.061	0.061	0.060	0.060	0.058	0.001	1.73
0.09	0.090	0.092	0.088	0.091	0.087	0.002	2.39

CV: coefficient of variation.
[a]$n = 5$.

Table 3. Inter-day pyridostigmine concentration for the precision determination[a].

Concentration (mg/mL)	Mean absorption (nm)		Calculated concentration		Mean of calculated concentration (±SD)	CV (%)
	Day 1	Day 2	Day 1	Day 2		
0.03	0.533	0.530	0.030	0.031	0.031 ± 0.0007	2.32
0.06	1.016	1.039	0.060	0.061	0.060 ± 0.0008	1.41
0.09	1.503	1.558	0.090	0.090	0.090 ± 0.0002	0.32

CV: coefficient of variation.

Table 4. Accuracy determination obtained from the analysis of three increasing concentrations of pyridostigmine[a].

Theoretical concentration (mg/mL)	Real concentration in quintuplicate (mg/mL)					Mean	SD	Accuracy (%)
0.03	0.030	0.030	0.032	0.030	0.030	0.031	0.001	101.12
0.06	0.061	0.060	0.061	0.060	0.060	0.058	0.001	99.72
0.09	0.090	0.090	0.092	0.088	0.091	0.087	0.002	99.45

Table 5. Limit of quantification determinate using decreasing concentrations of pyridostigmine.

Theoretical concentration (mg/mL)	Experimental concentration in triplicate (mg/mL)			SD	CV	Accuracy (%)
0.01	0.008	0.008	0.008	0.00	4.69	78.58
0.02	0.020	0.020	0.019	0.001	4.81	99.08

CV: coefficient of variation.

EE

The values of the yield of encapsulation of pyridostigmine (Table 6) are within acceptable and expected values for the methodology used in the preparation of liposomes[35]. Vidal et al.[15] had 15.5% for the encapsulation of pyridostigmine bromide using DSPC lipids as to be greater, indicating that the values of EE obtained in this study are acceptable. One factor that may influence the EE is the formation of lipid bilayer in liposomes. Different combinations of lipids can result in different characteristics of the membranes and values of encapsulation, since each lipid has singular characteristics. CHOL, for example, can stiffen the lipid membrane depending on the main lipid associated to it[38], by altering the characteristics of the liposomes.

Liposomes size

Liposomes in general consists of a single lipid bilayer or multiple bilayers around its inner compartment, being graded low unilamellar vesicles and multilamellar vesicles (MLVs), respectively[18]. Liposomes classified as MLV may present the diameter up to 5000 nm[39]. The liposomal formulations developed in the present study were classified as MLV, since the liposomes obtained showed size ranging from 2074 ± 399.88 to 3022 ± 824.80 (Table 6)[39]. Additionally, it is known that trapped volume can be used as an indicator of vesicle lamellarity. In this study, the liposomes average volume trapped was calculated as 3.29 μL/μg, equivalent to the values proposed by Mayer et al.[40] (1.8–5.27 μL/μg) and Nayar et al.[35] (3.5–6 μL/μg) for MLVs; so, can be inferred that MLVs are appreciable proportion of the vesicles.

The polydispersity index reflects the size uniformity of the liposomal vesicles, and the values below 0.3 indicates a monodispersed formulation.[41] Here, the objective was to obtain a formulation of large MLVs for subcutaneous administration of pyridostigmine which can be used as a means of slow release depot of the drug. Since obtaining liposomes with calibrated size and monodispersion was not the objective, the data are as expected for both liposomal formulations.

Ratio pyridostigmine/lipid

The ratio of drug/lipid is an important parameter in formulation characterization and the ideal is that this factor is maximized, so that the amount of lipids administered to the patient would be less, resulting in a minimization of adverse effects associated with the lipid formulation[39]. The ratio of drug/lipid in the formulation prepared with DOPC:CHOL is greater than that found for the formulation composed of DSPC:CHOL (Table 6). However, both are acceptable to the method used.

Surface charge of liposomes

The surface charge of the liposomes is also known as the zeta potential. In module, a high value of the surface charge indicates good stability of formulations, since it leads the particles to move away from each other, preventing collisions and aggregation between the vesicles that could result in formulation loss. Thus, the electrostatic repulsion between the vesicles is an important factor for the stability of the formulation[42]. The DSPC:CHOL formulation showed higher stability compared to the DOPC:CHOL formulation and both showed negative surface charge (Table 6).

In vitro release profile

The *in vitro* release profile shows the ability of liposomes releasing the active substance over the time. The DOPC is a lipid which has an amphoteric charge, phase transition temperature below room temperature and generates a fluid bilayer with a big movement of the lipid chains.

This led to the bigger permeation of pyridostigmine that the DSPC formulation during the 72 h period was evaluated. On the other hand, the DSPC produces more rigid bilayer due to its phase transition temperature greater than 60 °C, with more static chains, resulting in lower permeability of active encapsulated substances. The CHOL was added to the formulation containing DOPC to decrease its fluidity of bilayer. However, in the formulation containing DSPC, it has the opposite effect, increasing the fluidity of the bilayer and avoiding that it becomes extremely rigid. The CHOL is also used to decrease the deformation of the membranes[39,43].

Moreover, depending on the phase transition temperature (T_c) of the lipids, the liposomes will have a state of membrane more fluid or more rigid, significantly changing the values of release of the encapsulated substance. In general, a greater flow and release of substances occurs in the membranes in liquid-crystalline (fluid) in relationship the gel state (solid), because the movement of the components of the bilayer facilitates the drug permeability. Thus, the DSPC having a bigger T_c, forms a more rigid liposome, with a slower drug release compared to the DOPC, which have a lower T_c and form a more fluid liposome, with faster release of the drug[39,43].

In this context, the DOPC:CHOL formulation released a concentration of pyridostigmine slightly larger (75.4%), during the evaluation period, compared to the formulation composed of DSPC:CHOL (65.6%; Figure 1). This fact results of the physical–chemical characteristics of these lipids that interfere with the higher or lower fluidity of the lipid bilayer formed, which interferes in drug permeability.

Conclusion

This work showed that the proposed method provides specificity, linearity, precision, LOQ and accuracy appropriate, being valid for the quantification of pyridostigmine in liposomal formulations. The spectrophotometric analytical method at the UV region is, therefore, a tool that is useful, inexpensive and easy to perform in the routine to the pyridostigmine quality control as raw material and into pharmaceutical formulations such as liposomes.

According to the results obtained, it can be concluded that the two liposomal formulations made are suitable for subcutaneous administration *in vivo*. It is worth noting that the development and

Table 6. Parameters to liposomal formulations characterization.

Formulation	Encapsulation efficiency (%)	Mean size (nm)	Polydispersity	Zeta potential	Ratio drug/lipid (p/p)
DOPC (W)	–	2074 ± 399.88	0.196 ± 0.024	-3.73 ± 0.24	–
DOPC (PIR)	23.38 ± 3.43	2631 ± 479.45	0.390 ± 0.322	-3.68 ± 0.76	0.151
DSPC (W)	–	2885 ± 656.79	0.105 ± 0.042	-4.90 ± 0.24	–
DSPC (PIR)	15.43 ± 0.38	3022 ± 824.80	0.655 ± 0.160	-4.55 ± 0.40	0.095

W: white; PIR: pyridostigmine.

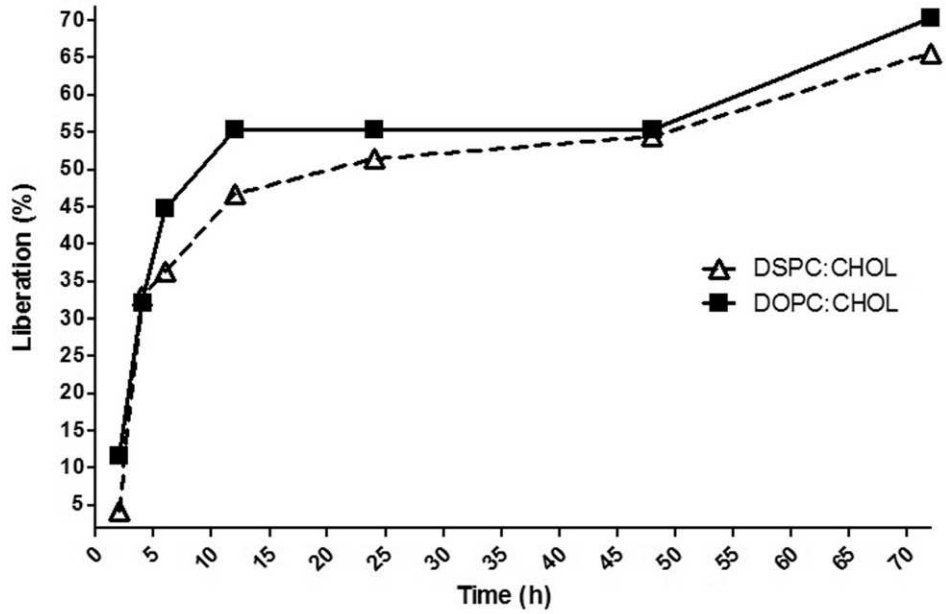

Figure 1. Pyridostigmine release profile of formulations DSPC:CHOL and DOPC:CHOL.

characterization of these new liposomal formulations containing pyridostigmine can be administered by alternative route at the intravenously and allow a gradual and sustained release of the drug to the blood compartment. So, the consequence will be the prolonged pharmacological activity and reduced side effects of the pyrisdostigmine and a more efficient therapy to ischemic heart disease, with greater convenience and security.

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Declaration of interest

The authors report no declaration of interest.

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Prolonged cardioprotective effect of pyridostigmine encapsulated in liposomes

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ABSTRACT

Aims: The purpose of the present work was to investigate the ability of pyridostigmine encapsulated in long-circulating liposomes, to protect against ECG (electrocardiogram) alterations induced by sympathetic stimulation in rats.

Main methods: The encapsulation of pyridostigmine was carried out by freeze–thaw and extrusion. Blood pressure and ECG (limb lead II) were monitored in anaesthetized male Wistar rats. The formulation containing pyridostigmine was intravenously administrated in 0.1, 0.3 and 1.0 mg/kg doses, and sympathetic stimulation was conducted by administration of 1 or 3 μg of noradrenaline (NA) after 1, 2, 4 or 6 h. The obtained cardiovascular parameters were compared to animals that received intravenous injection of pyridostigmine in free form or saline.

Key findings: After saline, NA induced a significant increase in QT interval (22.3% after 3.0 μg). Previous administration of free pyridostigmine significantly prevented the increase of QT interval after sympathetic stimulation and the most prominent effect was observed after 1 h for the dose of 0.3 mg/kg (6.8% after 3.0 μg of NA) and was no longer observed after 2 h of the treatment. On the other hand, the maximum effect of pyridostigmine in liposomal formulation preventing QT interval increase was observed 2 h after treatment (9.7% after 3.0 μg of NA) and was still present until 6 h when 1 mg/kg was previous administrated.

Significance: The results of the present study, beyond to confirm the cardioprotective action of pyridostigmine, suggest that liposomal pyridostigmine may be a potential therapeutic alternative to prevent cardiovascular disturbances resulting from sympathetic hyperactivity.

Introduction

Several studies had demonstrated the relationship between the autonomic nervous system (ANS) and cardiovascular diseases (Francis 1988; Porter et al. 1990; Tai et al. 2002; Hoyer et al. 2008). The misbalance of this system, characterized by an increase of sympathetic and depression of parasympathetic activities, may lead to arrhythmias and sudden death, mainly in patients with myocardial infarction and heart failure (Porter et al. 1990; Hoyer et al. 2008). Among the changes caused by ANS on cardiovascular system, the influence on QT interval of electrocardiogram (ECG) has particular importance (Ahnve and Vallin 1982; London et al. 1998; Magnano et al. 2002). In fact, prolongation of QT interval is an independent risk factor for sudden death due to cardiac arrest (Schwartz and Wolf 1978; Algra et al. 1991; Schouten et al. 1991).

The effectiveness of adrenergic blockade in preventing fatal arrhythmias, by reducing the sympathetic effects, has been extensively characterized (Hjalmarson 1997; Catelli et al. 2003; Taggart et al. 2003). Although the benefits of parasympathetic stimulation in the prevention of arrhythmias have been defined (De Ferrari et al. 1992), there is not a pharmacological option available. In this context, the pyridostigmine bromide, a reversible cholinesterase inhibitor, has been demonstrated as a promissory agent on cardiac ischemic disease (Grabe-Guimarães et al. 1999; Sant'anna et al. 2003). Patients with exercise induced myocardial ischemia (Castro et al. 2004) or heart failure (Serra et al. 2009) presented improvements in autonomic and hemodynamic profiles during exercise after oral administration of pyridostigmine. However, its half-life is short (Aquilonius et al. 1980), the side effects related to cholinergic stimulation are frequent and a limitation for safety clinical use.

The importance of liposomes to target the cardiovascular system is widely studied (Torchilin 1995). It was shown that liposomes coated with polyethylene glycol (PEG) prolong its life time in the circulation (Klibanov et al. 1990). Additionally, PEGylated liposomes were found to accumulate in infarcted myocardium (Torchilin et al. 1992) and are useful to protect the myocardium from the ischemia damage (Verma et al. 2005).

The main goal of the present work was to investigate the ability of pyridostigmine encapsulated in long-circulating liposomes to prolong its cardioprotective action, by analysing ECG parameters, particularly

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QT interval, and arterial blood pressure changes in anaesthetized Wistar rats, followed by IV (intravenous) injection of a single dose of noradrenaline (NA).

Materials and methods

Drugs and reagents

Pyridostigmine bromide and cholesterol were purchased from Sigma (USA). L-α-Distearoylphosphatidylcholine (DSPC) and PEG (2000)-distearoylphosphatidyl-ethanolamine (DSPE-PEG) were purchased by Lipoid GmbH (Ludwigshafen, Germany). The solvents were of analytical grade and all other chemicals were commercially available. Water was purified by reverse osmosis (Symplicity System 185, Millipore, USA).

PEGylated liposomes preparation

The liposome system consisted of DSPC, cholesterol and DSPE-PEG at a molar ratio of 5:4:0.3. Pyridostigmine encapsulation was carried out by freeze–thaw and extrusion (Nayar et al. 1989). Control liposomes were similarly prepared, using only PBS (150 mM NaCl, 10 mM phosphate, pH 7.2).

Liposomes characterization

Pyridostigmine content of the liposomes was measured by UV ($\lambda = 270$ nm) (Hegazy et al. 2002) against a pyridostigmine-solution standard curve. Particle size, polydispersity index and zeta potential of the liposome population by photon correlation spectrometry were carried out using Zetasizer 3000 HS (Malvern Instruments, UK).

Animals

Male Wistar rats were supplied by Universidade Federal de Ouro Preto and maintained with water and food ad libitum at constant humidity and temperature with a light/dark cycle of 12 h. Six animals were used in each experimental group. All procedures related to the use of animals in these studies were reviewed and conform to the Ethical Principles of Animal Experimentation (Brazilian College of Animal Experimentation) and were approved by the UFOP Ethics Committee under number 11/2009.

Determination of in vivo cardiovascular parameters

Experimental procedures

Rats were anaesthetized with intraperitoneal sodium pentobarbital (60 mg/kg). Intravenous (IV) injections of free pyridostigmine, pyridostigmine in liposomes (Lipo-Pyr) or empty liposomes were performed via a catheter inserted into the femoral vein. Free pyridostigmine and pyridostigmine in liposomes were dissolved in saline to give the desired dose (0.1, 0.3 or 1.0 mg/kg). All formulations were administered in bolus, at maximum volume of 0.2 ml. Control rats received a corresponding volume of saline or empty liposomes. In order to simulate an adrenergic discharge, the animals received a single in bolus dose of NA solution (1.0 or 3.0 μg), 1, 2, 4 or 6 h after the treatment.

Arterial blood pressure (AP) was continuously recorded using a polyethylene catheter, filled with heparinised saline, inserted into femoral artery and joined to a disposable pressure transducer (TruWave; Edwards Life Sciences); the pressure transducer was connected to a bridge amplifier. Limb lead II ECG was continuously recorded using subcutaneous stainless steel needle electrodes connected by a shielded cable to a biopotencial amplifier with a band-pass of 0.5–100 Hz. These signals from amplifiers were simultaneously sampled at a rate of 1200 Hz per channel, with an A/D conversion board of 16 bits resolution (DaqBoard/2001, IOtech).

Data analyses

The ECG parameters measured were QT (interval between the beginning of the *Q*-wave and the end of the *T*-wave), RR (interval between two successive *R*-waves and used to obtain the heart rate: HR = 60/RR), PR (interval between the beginning of the *P*-wave and the end of the *R*-wave) and QRS (interval from the beginning of the *Q*-wave to the end of the *S*-wave) intervals.

As the QT interval on standard ECG is influenced by a variety of physiological and pathological factors, such as heart rate, autonomic nervous system activity, day time, age, gender, drugs, hormone concentrations, electrolyte variations, heart disease or ventricular dysfunction (Hodges 1997), several mathematical formulae have been proposed to derive a heart rate corrected QT interval (QTc), or at least minimize their dependence on heart rate. Simonson et al. (1962) enumerate 9 formulae for these purpose, but the best known are Bazett's ($QTc = QT/RR^{1/2}$) (1920) and Fridericia's ($QTc = QT/RR^{1/3}$) (1920) formulae, commonly used in clinical trials and pre-clinical studies. There is an almost centenary unsolved controversy about the rate adjustment methods of QT intervals (Puddu et al. 1988; Hayes et al. 1994; Malik et al. 2002; Koga et al. 2007). Embedded in a plethora of formulae and methods on the strength of this controversy, we based our choice on the work of Abernethy et al. (2001), who suggest the Fridericia correction for the QTc for the cases of RR < 500 ms, as was consistently observed in our experiments.

The systolic (SAP) and diastolic arterial pressure (DAP) were also determined from the same segments. These parameters were measured before and after injection of pyridostigmine formulations followed by adrenergic stimulation at different times.

Statistical analysis

The Kolmogorov–Smirnov method was used to determine whether continuous variables were normally distributed. Results are expressed as mean ± standard error of the mean (S.E.M.). Statistical comparisons were made using ANOVA and Tukey post-hoc test. Significance was accepted when $P < 0.05$.

Results

Particle size, polydispersity index and zeta potential

The freezing thawing/extrusion method resulted in vesicles with a calibrated size smaller than 200 nm, an encapsulation efficiency of 15.5%, and narrow size distribution for both empty and pyridostigmine in liposomes. Zeta-potential values were similar between both formulations (Table 1).

Determination of cardiovascular parameters

Tables 2 and 3 reports in details the absolute values of ECG parameters measured at different times after treatment with saline, free pyridostigmine or liposomal pyridostigmine. The IV administration of 1.0 μg NA in Wistar rats that previously received saline, did not caused significant increase of PR and QRS intervals of ECG when compared to the control period. A slight increase of these parameters was caused by 3.0 μg of NA, such 5.7% and 6.0% of increase to PR and QRS, respectively. The analysis of QT interval showed increases of 20.1% and 22.3% after 1.0 and 3.0 μg of NA, respectively. The pre-

Table 1
Characterization of liposomes formulations.

	Diameter (nm)	Polydispersity index	Zeta potential (mV)
Lipo-Pyr	174.3 ± 6,01	0.061 ± 0.0127	−50.6 ± 2.5
Empty liposomes	158.5 ± 4,42	0.070 ± 0.0133	−41.2 ± 0.9

Each data represents the mean ± e.s.m. of three preparations.

Table 2
Means of absolute values of ECG parameters measured before and after IV injection of NA 1 μg to animals previously treated with free or liposomal pyridostigmine at different times.

Experimental groups		Parameter											
		PR				QRS				QT			
		Time after NA (seconds)				Time after NA (seconds)				Time after NA (seconds)			
		0	15	30	180	0	15	30	180	0	15	30	180
Control		49.4 ± 0.51	49.8 ± 0.34	50.4 ± 0.28	49.1 ± 0.42	24.1 ± 0.80	23.8 ± 0.74	24.2 ± 0.60	24.0 ± 0.76	63.4 ± 1.99	76.0 ± 2.87	71.4 ± 1.69	64.8 ± 2.17
FreePyr	1 h	47.1 ± 1.56	47.1 ± 1.69	46.9 ± 1.36	46.9 ± 1.51	22.0 ± 0.39	22.1 ± 0.48	22.0 ± 0.43	21.9 ± 0.41	65.1 ± 1.46	71.1 ± 2.07	70.8 ± 2.03	65.3 ± 1.54
0.1 mg/kg	2 h	46.4 ± 1.60	46.6 ± 1.66	46.2 ± 1.49	46.3 ± 1.64	21.4 ± 0.84	21.4 ± 0.97	21.3 ± 0.85	21.3 ± 0.87	62.0 ± 1.32	70.3 ± 2.24	71.6 ± 1.90	63.5 ± 1.65
FreePyr	1 h	44.9 ± 1.48	45.0 ± 1.42	44.9 ± 1.53	45.0 ± 1.43	23.1 ± 2.19	23.3 ± 2.22	23.2 ± 2.12	23.1 ± 2.32	71.7 ± 1.10	76.3 ± 1.18	75.6 ± 1.02	72.1 ± 1.24
0.3 mg/kg	2 h	49.9 ± 1.88	50.0 ± 1.89	49.7 ± 1.91	49.9 ± 1.91	22.1 ± 0.50	22.2 ± 0.32	22.2 ± 0.35	21.9 ± 0.36	69.5 ± 0.95	77.6 ± 1.31	77.7 ± 0.93	70.6 ± 0.70
LipoPyr	1 h	46.2 ± 1.33	46.5 ± 1.32	46.3 ± 1.28	46.2 ± 1.35	21.7 ± 0.45	21.9 ± 0.39	22.0 ± 0.46	21.6 ± 0.41	67.6 ± 1.31	76.8 ± 2.52	78.0 ± 1.34	69.0 ± 1.62
0.1 mg/kg	2 h	53.4 ± 0.93	53.5 ± 1.19	53.1 ± 1.12	53.1 ± 0.92	25.8 ± 0.98	25.6 ± 0.89	25.7 ± 0.77	25.4 ± 0.82	67.2 ± 1.30	72.7 ± 1.34	72.5 ± 1.97	68.1 ± 1.46
	4 h	47.0 ± 1.43	47.1 ± 1.45	47.3 ± 1.40	47.0 ± 1.44	21.9 ± 0.35	22.1 ± 0.33	22.2 ± 0.35	22.0 ± 0.38	64.9 ± 1.82	73.5 ± 3.04	75.5 ± 1.92	65.7 ± 2.23
	6 h	45.5 ± 1.51	45.9 ± 1.54	45.4 ± 1.43	45.6 ± 1.52	22.3 ± 0.35	22.5 ± 0.24	22.7 ± 0.31	22.3 ± 0.38	66.4 ± 1.61	77.3 ± 1.92	76.6 ± 1.62	67.6 ± 1.81
LipoPyr	1 h	53.8 ± 0.96	53.9 ± 0.86	54.1 ± 0.70	54.0 ± 1.03	23.6 ± 0.17	23.1 ± 0.14	23.1 ± 0.13	23.1 ± 0.18	67.9 ± 1.02	74.1 ± 1.80	78.4 ± 1.66	68.4 ± 0.95
0.3 mg/kg	2 h	54.6 ± 1.67	54.0 ± 1.91	53.6 ± 1.90	53.4 ± 1.21	24.7 ± 0.65	24.7 ± 0.51	24.8 ± 0.36	25.1 ± 0.44	62.7 ± 1.77	65.8 ± 1.51	67.6 ± 1.50	63.8 ± 2.06
	4 h	47.2 ± 1.18	47.5 ± 1.07	47.0 ± 0.99	47.3 ± 1.18	21.8 ± 0.25	22.1 ± 0.25	22.2 ± 0.25	21.9 ± 0.27	65.4 ± 2.05	73.7 ± 3.04	75.3 ± 1.94	65.7 ± 2.25
	6 h	45.4 ± 0.99	45.2 ± 1.10	45.4 ± 0.89	45.2 ± 1.01	21.0 ± 0.79	20.9 ± 0.86	21.0 ± 0.81	21.0 ± 0.80	64.7 ± 1.39	69.5 ± 1.92	70.7 ± 1.77	64.5 ± 1.41
LipoPyr	1 h	46.8 ± 1.44	46.8 ± 1.36	46.4 ± 1.29	46.9 ± 1.44	21.4 ± 0.47	21.7 ± 0.46	21.6 ± 0.50	21.5 ± 0.41	66.2 ± 1.77	73.3 ± 2.34	73.2 ± 2.06	66.6 ± 2.00
1.0 mg/kg	2 h	42.4 ± 0.88	42.4 ± 0.95	42.8 ± 0.77	42.4 ± 0.90	22.8 ± 0.24	23.1 ± 0.24	23.2 ± 0.23	22.9 ± 0.23	66.9 ± 1.47	72.5 ± 1.59	72.2 ± 1.45	67.3 ± 1.63
	4 h	46.1 ± 1.32	45.9 ± 1.40	46.5 ± 1.46	46.2 ± 1.39	21.1 ± 0.36	20.9 ± 0.31	20.9 ± 0.36	21.0 ± 0.32	68.9 ± 0.30	76.1 ± 0.49	76.4 ± 0.80	69.2 ± 0.33
	6 h	45.0 ± 1.68	44.8 ± 4.67	44.6 ± 1.51	44.9 ± 1.68	21.3 ± 0.88	21.5 ± 0.92	21.5 ± 0.92	21.4 ± 0.90	62.6 ± 1.48	67.0 ± 1.79	67.1 ± 1.63	63.2 ± 1.69

Each value represents the mean ± e.s.m. of six animals.

treatment with free pyridostigmine at 0.1 and 0.3 mg/kg and with the liposomal form at 0.1, 0.3 and 1.0 mg/kg did not change the baseline values, but attenuated significantly the increases of the ECG parameters produced by IV injection of NA compared to the control group. After previous treatment with pyridostigmine, changes in PR and QRS intervals caused by 1.0 μg of NA were also not observed. All formulations prevented the increase of these parameters caused by 3.0 μg of NA. Free pyridostigmine and liposomal pyridostigmine in all used doses inhibited the QT interval increase after NA injection. The maximum effect of free pyridostigmine to inhibit the QT interval increase was observed after 1 h for the dose of 0.3 mg/kg (6.5% and 6.8% after 1.0 and 3.0 μg of NA, respectively) but it was not observed after 2 h. The maximum effect of QT interval after liposomal pyridostigmine was observed after 2 h (8.5% and 9.7% after 1.0 and 3.0 μg of NA, respectively) and this effect was also observed after 4 and 6 h of its administration at 1.0 mg/kg. Fig. 1 shows the percentage variation of ECG parameters induced by NA after the administration of each formulation of pyridostigmine. The maximum effect of NA to increase all parameters was observed between 15 and 25 s after its administration and the return to baseline values occurred after about 3 min. Fig. 2 shows representative ECG segments of animals that received saline, free pyridostigmine or liposomal pyridostigmine, before and after 3.0 μg NA.

Fig. 3 shows the percentage variation of AP and HR induced by NA after the administration of each formulation of pyridostigmine. The SAP and DAP were both similarly increased, about 55% after 1.0 μg and 70% after 3.0 μg NA. The administration of free pyridostigmine and liposomal pyridostigmine did not change the baseline AP values and was not able to inhibit the increases of AP. The maximum effect of NA to increase AP was observed between 10 and 25 s after administration. The IV administration of 1.0 μg of NA did not cause significant changes of HR when compared to the control period. The dose of 3.0 μg of NA decreased 18% the HR. Previous treatment with free pyridostigmine or liposomal pyridostigmine did not change HR.

In animals subjected to sympathetic stimulation with 1.0 μg of NA, as no relevant changes were observed in HR, the QTc index showed similar profiles compared to the QT interval. Thus, significant decreases of QTc prolongation were observed in animals treated 1 h

Table 3
Means of absolute values of ECG parameters measured before and after IV injection of NA 3 μg to animals previously treated with free or liposomal pyridostigmine at different times.

Experimental groups		Parameter											
		PR				QRS				QT			
		Time after NA (seconds)				Time after NA (seconds)				Time after NA (seconds)			
		0	15	30	180	0	15	30	180	0	15	30	180
Control		46.7 ± 0.92	49.4 ± 0.99	48.6 ± 1.07	47.0 ± 0.89	22.4 ± 0.87	23.5 ± 0.86	23.6 ± 1.02	22.5 ± 0.84	71.2 ± 0.90	84.5 ± 1.68	85.0 ± 1.52	73.3 ± 1.20
FreePyr	1 h	45.6 ± 1.52	45.4 ± 1.60	45.8 ± 1.61	45.3 ± 1.50	23.1 ± 0.93	23.1 ± 0.82	23.3 ± 0.89	23.1 ± 0.98	70.9 ± 0.81	80.7 ± 1.49	78.5 ± 1.54	71.3 ± 1.04
0.1 mg/kg	2 h	45.8 ± 2.02	46.3 ± 2.12	45.8 ± 1.89	45.3 ± 1.88	24.8 ± 1.24	25.0 ± 1.41	24.9 ± 1.25	24.6 ± 1.26	71.4 ± 0.98	84.9 ± 1.60	80.4 ± 1.03	72.4 ± 1.06
FreePyr	1 h	46.0 ± 1.57	46.0 ± 1.37	46.0 ± 1.39	46.1 ± 1.54	21.5 ± 0.52	21.9 ± 0.51	21.8 ± 0.63	21.5 ± 0.46	69.8 ± 1.35	76.1 ± 1.61	75.8 ± 1.46	70.0 ± 1.36
0.3 mg/kg	2 h	45.4 ± 1.82	45.6 ± 1.76	45.0 ± 1.84	45.2 ± 1.80	22.5 ± 0.35	22.6 ± 0.41	22.7 ± 0.38	22.4 ± 0.30	68.3 ± 1.18	74.5 ± 2.04	76.2 ± 1.37	68.9 ± 1.92
LipoPyr	1 h	47.4 ± 1.50	47.6 ± 1.47	47.1 ± 1.41	47.4 ± 1.61	21.9 ± 0.40	22.0 ± 0.40	21.8 ± 0.46	21.9 ± 0.37	65.4 ± 1.69	72.8 ± 2.34	73.3 ± 1.84	65.8 ± 1.79
0.1 mg/kg	2 h	46.0 ± 1.91	45.9 ± 1.96	46.3 ± 1.82	45.9 ± 1.94	22.1 ± 0.46	22.2 ± 0.47	22.2 ± 0.44	22.2 ± 0.46	66.8 ± 2.08	75.4 ± 2.69	76.2 ± 2.92	67.0 ± 1.89
	4 h	46.0 ± 1.33	46.4 ± 1.39	46.8 ± 1.47	46.2 ± 1.37	22.1 ± 0.37	22.1 ± 0.22	22.6 ± 0.32	22.1 ± 0.38	67.9 ± 1.34	77.8 ± 2.46	78.1 ± 1.24	69.1 ± 1.65
	6 h	47.8 ± 0.97	48.5 ± 0.60	48.0 ± 0.77	48.0 ± 1.02	21.6 ± 0.31	21.8 ± 0.16	21.8 ± 0.24	21.6 ± 0.18	65.7 ± 1.88	74.6 ± 2.54	78.5 ± 2.31	66.6 ± 2.32
LipoPyr	1 h	45.7 ± 1.89	45.8 ± 1.87	46.0 ± 1.75	45.6 ± 1.87	23.3 ± 0.76	23.3 ± 0.84	23.2 ± 0.73	23.2 ± 0.69	67.0 ± 2.28	76.9 ± 3.02	76.3 ± 2.94	67.8 ± 2.29
0.3 mg/kg	2 h	46.3 ± 1.69	46.2 ± 1.68	45.9 ± 1.58	45.9 ± 1.62	21.9 ± 0.43	22.3 ± 0.34	22.2 ± 0.40	21.8 ± 0.42	66.9 ± 1.65	75.9 ± 2.62	75.1 ± 1.97	67.0 ± 1.82
	4 h	46.1 ± 1.95	45.9 ± 1.92	46.3 ± 1.84	45.9 ± 1.95	22.2 ± 0.44	22.3 ± 0.48	22.3 ± 0.46	22.2 ± 0.45	66.3 ± 1.80	74.2 ± 2.81	75.7 ± 2.40	66.7 ± 1.93
	6 h	46.9 ± 1.45	47.2 ± 1.47	46.7 ± 1.26	46.9 ± 1.45	21.9 ± 0.35	22.1 ± 0.35	22.3 ± 0.33	21.9 ± 0.37	65.1 ± 2.11	72.9 ± 3.18	75.3 ± 1.90	65.8 ± 2.22
LipoPyr	1 h	46.6 ± 1.38	47.1 ± 1.45	47.2 ± 1.34	46.9 ± 1.43	21.9 ± 0.39	22.1 ± 0.33	22.4 ± 0.35	21.8 ± 0.42	69.0 ± 0.82	77.2 ± 1.37	78.8 ± 0.78	69.5 ± 0.91
1.0 mg/kg	2 h	46.6 ± 1.39	46.5 ± 1.41	46.2 ± 1.24	46.9 ± 1.40	21.4 ± 0.52	21.7 ± 0.46	21.5 ± 0.52	21.5 ± 0.50	67.9 ± 1.26	73.9 ± 1.58	73.6 ± 1.53	68.1 ± 1.28
	4 h	44.6 ± 0.67	44.3 ± 0.79	44.5 ± 0.74	44.4 ± 0.71	20.8 ± 0.75	20.8 ± 0.86	21.0 ± 0.79	20.8 ± 0.77	64.7 ± 0.88	70.5 ± 1.27	71.1 ± 0.91	65.0 ± 1.17
	6 h	47.4 ± 1.42	47.4 ± 1.50	48.1 ± 1.51	47.3 ± 1.44	22.3 ± 0.32	22.3 ± 0.29	22.4 ± 0.31	22.3 ± 0.26	65.9 ± 1.96	72.5 ± 2.38	75.6 ± 1.60	65.8 ± 1.81

Each value represents the mean ± e.s.m. of six animals.

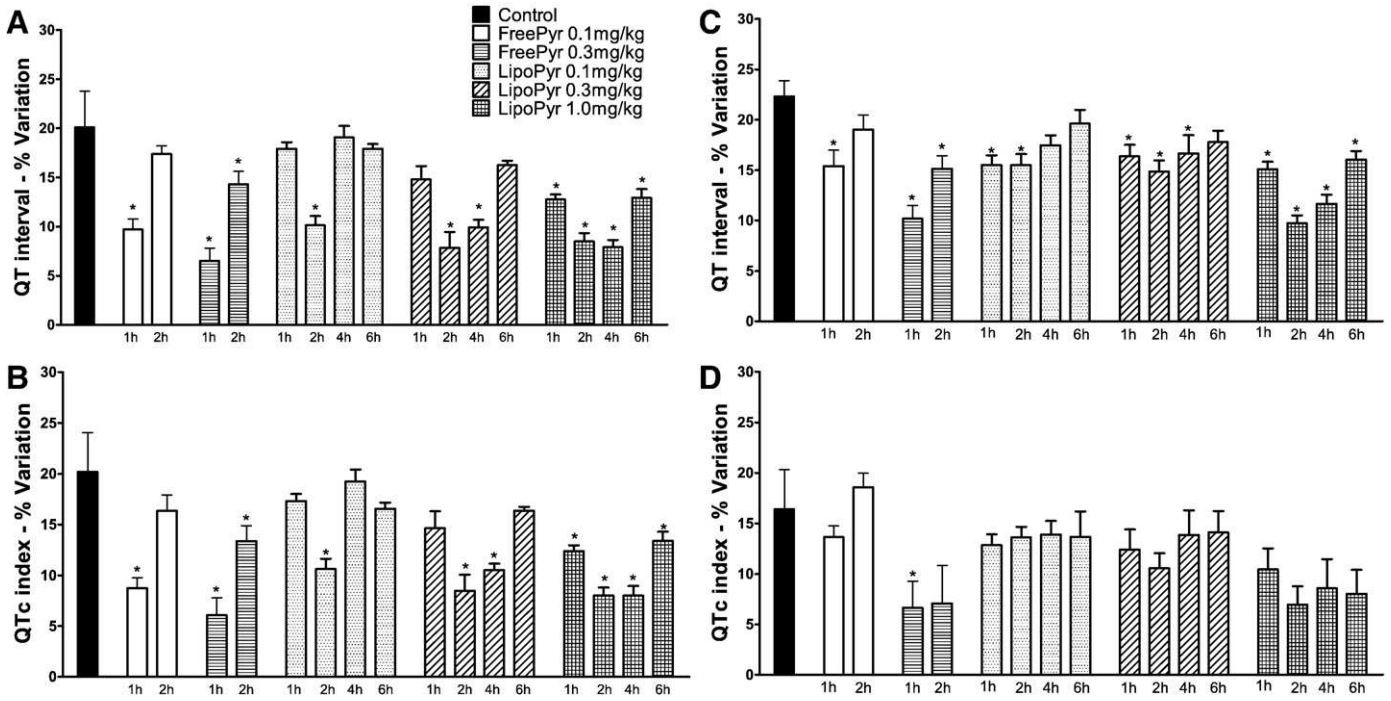

Fig. 1. Percentage variation of QT interval and QTc index measured after the sympathetic stimulation with NA in animals pre-treated with saline, free or liposomal pyridostigmine at different times. (A) and (B) changes after 1 μg of NA. (C) and (D) changes after 3 μg of NA. FreePyr: free form of pyridostigmine; LipoPyr: liposomal form of pyridostigmine. Each value represents the mean of the maximum variation of six animals, compared to the control group. *$P<0.05$ — ANOVA, Tukey post-hoc test.

before sympathetic stimulation with free pyridostigmine in doses 0.1 and 0.3 mg/kg. For liposome formulations, significant differences were observed at 2 h of treatment with doses 0.3 and 1.0 mg/kg, and these effects occurred up to 6 h after treatment with the dose of 1.0 mg/kg. The significant decrease in HR caused by 3.0 μg NA changed the profile of variation of QTc index for all protocols of treatment. No significant differences for this parameter between groups untreated and treated with pyridostigmine were observed. The alterations of AP and ECG intervals induced by NA in animals that previously received saline were similar to the animals that received empty liposomes (data not shown).

Discussion

Our findings suggest that encapsulation of pyridostigmine in liposomes was able to extend its protective effects under sympathetic hyperactivity. Several studies showed that the autonomic misbalance with adrenergic hyperactivity could be accompanied by vagal hypoactivity (Goldstein et al. 1975; Porter et al. 1990; Padley et al. 2005; Lahiri et al. 2008). It was identified the benefits of electrical vagal stimulation in dogs (Henning et al. 1990), cats (Zuanetti et al. 1987), rats (Li et al. 2004) and in patients with cardiovascular diseases (Zamotrinsky et al. 2001), thus encouraging the search for alternative therapies that could modulate the parasympathetic system, particularly enhancing the acetylcholine activity. Taking into account that cholinergic agonists, as oxitremorine, have characteristics of cardiac protection (De Ferrari et al. 1992, 1993), the effect of transdermal scopolamine in patients with advanced congestive heart failure was evaluated and the main observed beneficial effect was the increase of heart rate variability (Casadei et al. 1996; Venkatesh et al. 1996). This effect, however, was only observed with low doses of the drug and, at high concentrations, the scopolamine acts as a cholinergic blocker, limiting the performance of prolonged studies (Hayano et al. 1999). The pyridostigmine is a reversible cholinesterasic inhibitor that increases the concentration of endogenous acetylcholine. It is clinically used in patients with myasthenia gravis by increasing the concentration of acetylcholine at the synaptic cleft, reducing the deficit in muscle strength. Its cardiovascular action is usually considered a side effect (Castro et al. 2000). Previous studies in normal rats (Grabe-Guimarães et al. 1999) and in humans (Nóbrega et al. 1996) evaluated the therapeutic potential of pyridostigmine as a cardioprotective drug and its potential use in congestive heart failure (Androne et al. 2003) and in neurogenic orthostatic hypotension (Singer et al. 2006). Although the advantages of pyridostigmine has been shown both in health (Nóbrega et al. 1996, 1999; Castro et al. 2000; Sant'anna et al. 2003) and in patients with heart failure (Castro et al. 2002, 2004, 2006; Nóbrega et al. 2008; Serra et al. 2009), side effects characterized mainly by intestinal distress were observed with a daily oral dose of 5 mg/kg for 3 months, and the dose of 20 mg/kg was lethal to dogs when given for up to 14 days (Kluwe et al. 1989). Furthemore, combined exposure of mice to 10 mg/kg/day of pyridostigmine bromide and shaker stress for 7 days resulted in neurobehavioral changes such as sensorimotor alterations and decreased locomotor activity (Dubovicky et al. 2007). That same dose of pyridostigmine caused to male mice adverse influence on cardiac growth and vascular structure, specifically reduction of the aortic wall thickness/diameter ratio and reduced relative heart weight (Bernátová et al. 2006). In our experiments, the administration of free pyridostigmine at 1.0 mg/kg caused toxic effects characteristic of cholinergic hyperstimulation, and for this reason, we discontinued the study for this dose on free form. As expected, it was not observed the toxic effects for the same dose of liposomal pyridostigmine. This finding suggests a potential ability of liposomes to reduce the incidence of adverse effects of pyridostigmine, which should be further studied.

The most important finding of the present study is the cardioprotective effect of pyridostigmine by inhibiting the increases of the QT interval caused by sympathetic hyperstimulation in rats. The utility of the QT interval measurement as a tool to evaluate the cardiotoxic activity of drugs was previously demonstrated in our laboratory (Leite et al. 2007). The autonomic tone has its signature on the QT interval, which is primarily determined by the parasympathetic branch, since the cholinergic blockade was impressively related to QT prolongation (Ahnve and Vallin 1982). In coronary disease, when the vagal activity is reduced, the QT interval prolongation is a predictor of arrhythmias (Zuanetti et al. 1987; London et al. 1998) and sudden death (Schwartz

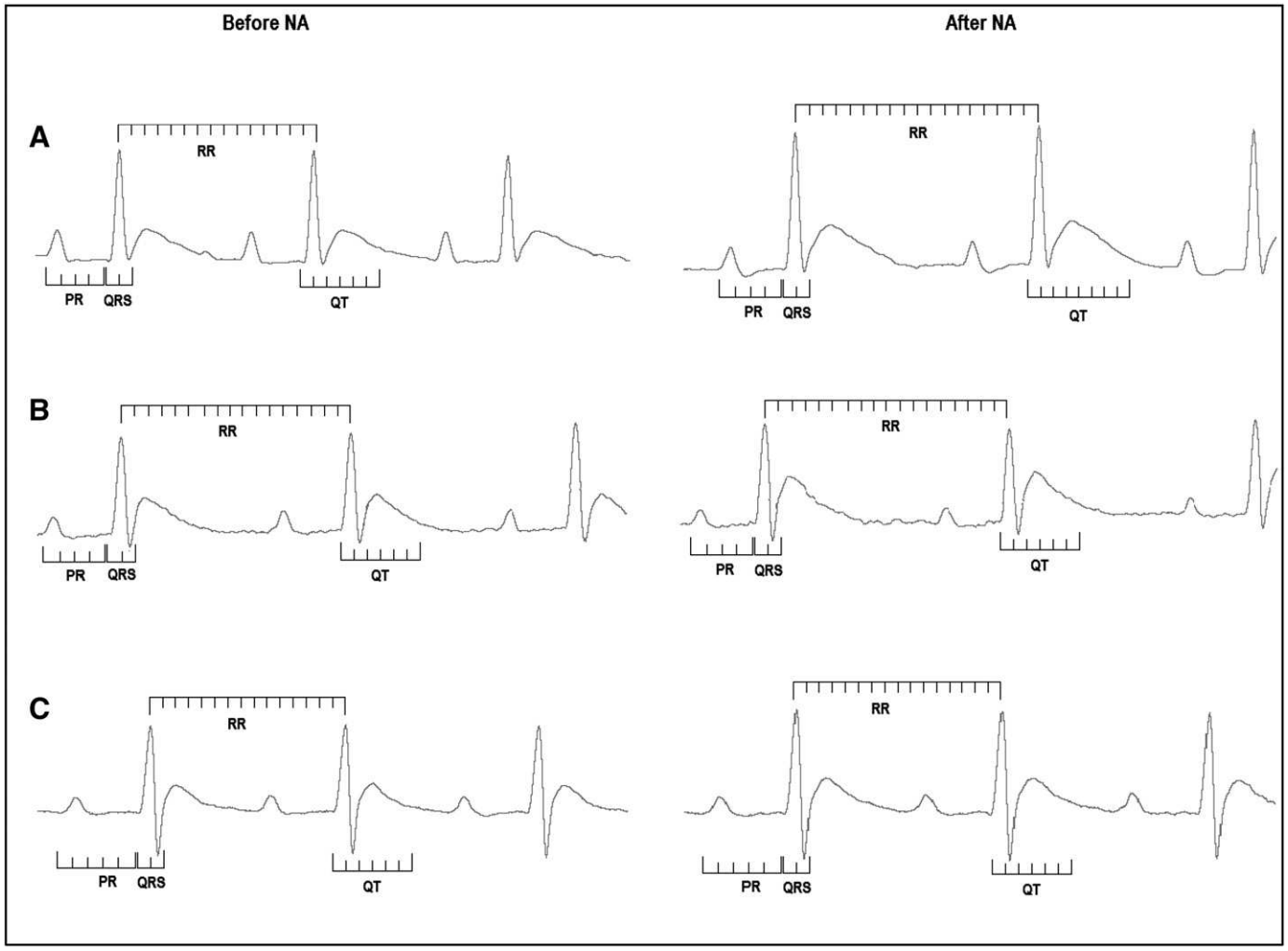

Fig. 2. Representative traces of ECG showing the effects of pre-treatment with pyridostigmine to prevent the alterations of the QT interval induced by 3 μg of NA, compared to the group that received saline.

and Wolf 1978; Ahnve 1991). Therefore, pyridostigmine at the doses used (0.3 and 1.0 mg/kg), presented potential cardioprotective effect in regard to its ability to prevent increases in the QT interval induced by NA. As expected, PEGylated liposomes were able to prolong (Takahama et al. 2009) the circulation of pyridostigmine, augmenting its cardioprotective effects. Thus, the use of pyridostigmine, especially in liposomal form, could prevent the occurrence of ventricular arrhythmias and sudden death, as the encapsulation was capable of promoting its slow release prolonging its protective effects on the QT interval up to 6 h after administration, providing maintenance of acetylcholine in the synaptic cleft. Furthermore, the density or affinity of muscarinic receptors are increased on the atria in rats with sinoaortic denervation (Soares et al. 2006) and dogs with heart failure (Dunlap et al. 2003), indicating a natural mechanism to control the decrease on the parasympathetic activity. In this context, pyridostigmine encapsulated in PEGylated liposomes may be a powerful formulation to release the drug into the ischemic heart (Torchilin 1995; Lukyanov et al. 2004).

The administration of the higher dose of NA caused significant bradycardia, a factor that interfered with the evaluation of the beneficial effects of pyridostigmine when using the Fridericia's correction formula (QTc). According to Indik et al. (2006), this calculation may underestimate the values of the QT interval when HR decreased, as observed in our experiments. Moreover, studies showed that drugs that modulate the ANS can alter the absolute values of the QT interval independently of HR (Browne et al. 1982), and the autonomic conditions that directly affect the ventricular myocardium of healthy subjects, causing variations in QT are also independent of HR (Magnano et al. 2002).

The absence of cardiodepression, characterized by the normal baselines values observed after administration of IV pyridostigmine in free and liposomal forms is in conformity with previous studies (Soares et al. 2004). These observations favor its use in patients with cardiovascular diseases. Cholinergic substances may slow the conduction of the cardiac electrical impulse, inducing bradycardia (Pontes et al. 1999), what was not observed in the present study in anesthetised rats. In this study, it was shown that the cardiac protection by IV administration of pyridostigmine in rats involves the modulation of the QT interval under conditions of sympathetic hyperactivity. Moreover, despites of the definition of probable mechanisms of cardioprotection promoted by pyridostigmine, it is known that increased vagal tone is related to the good prognosis in ischemic heart disease and heart failure (Osterziel and Dietz 1996), since both diseases are characterized by an increase in sympathetic tone and a decrease in cardiac vagal activity.

Conclusion

The most important result emerging from this work is the ability of a liposomal system to prolong the cardioprotective effect of pyridostigmine when compared to the free drug, mainly by its ability to prevent the prolongation of the QT interval under conditions of sympathetic hyperactivity. It can be speculated that pyridostigmine in liposomes

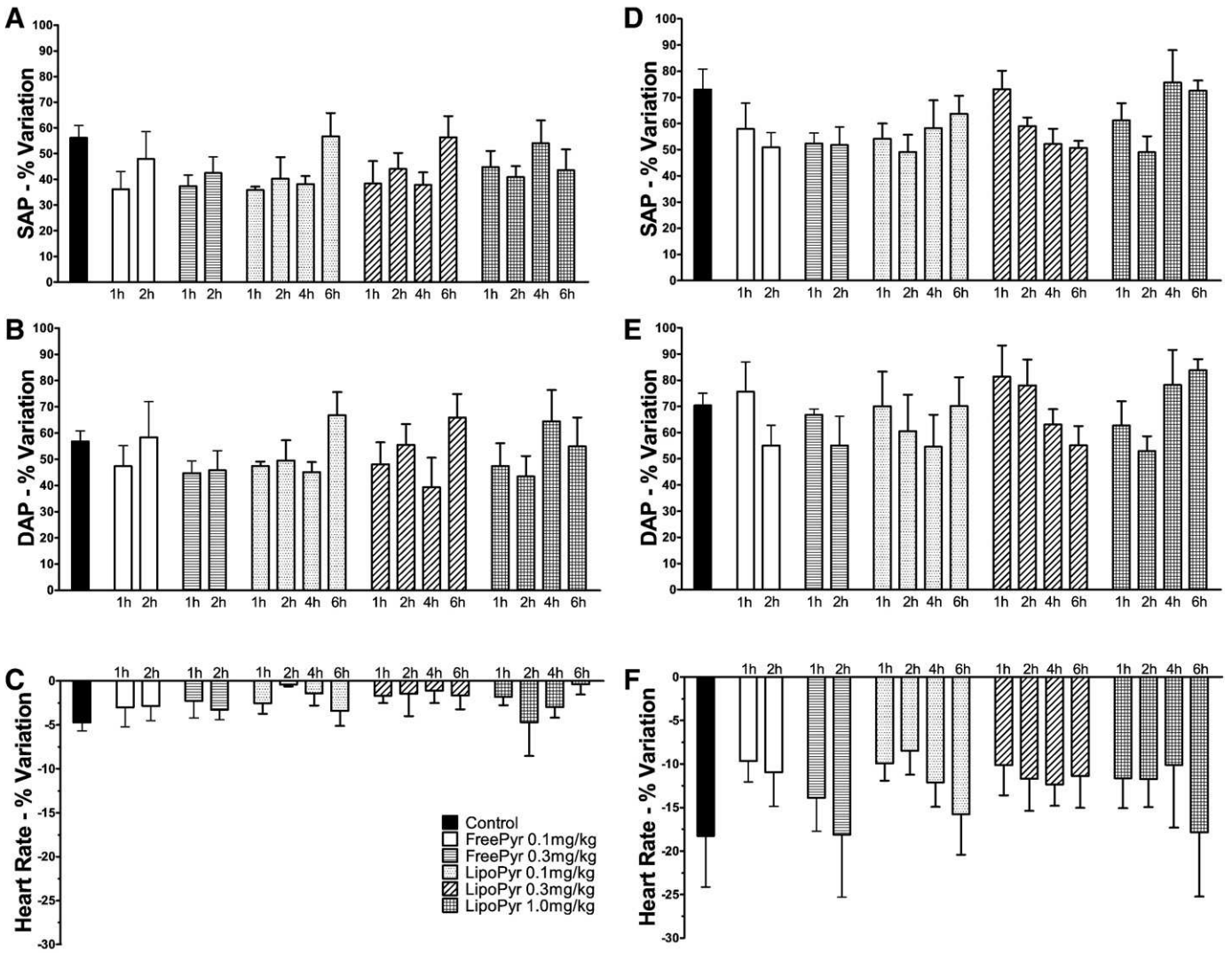

Fig. 3. Percentage variation of SAP, DAP and HR measured after the sympathetic stimulation with NA in animals pre-treated with saline, free or liposomal pyridostigmine at different times. (A), (B) and (C) after 1 μg of NA. (D), (E) and (F) after 3 μg of NA. FreePyr: free form of pyridostigmine; LipoPyr: liposomal form of pyridostigmine. Each value represents the mean of the maximum variation of six animals, compared to the control group.

may be a potential therapeutic alternative to prevent cardiovascular disturbances resulting from sympathetic hyperactivity in patients with ischemic heart disease.

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