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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.

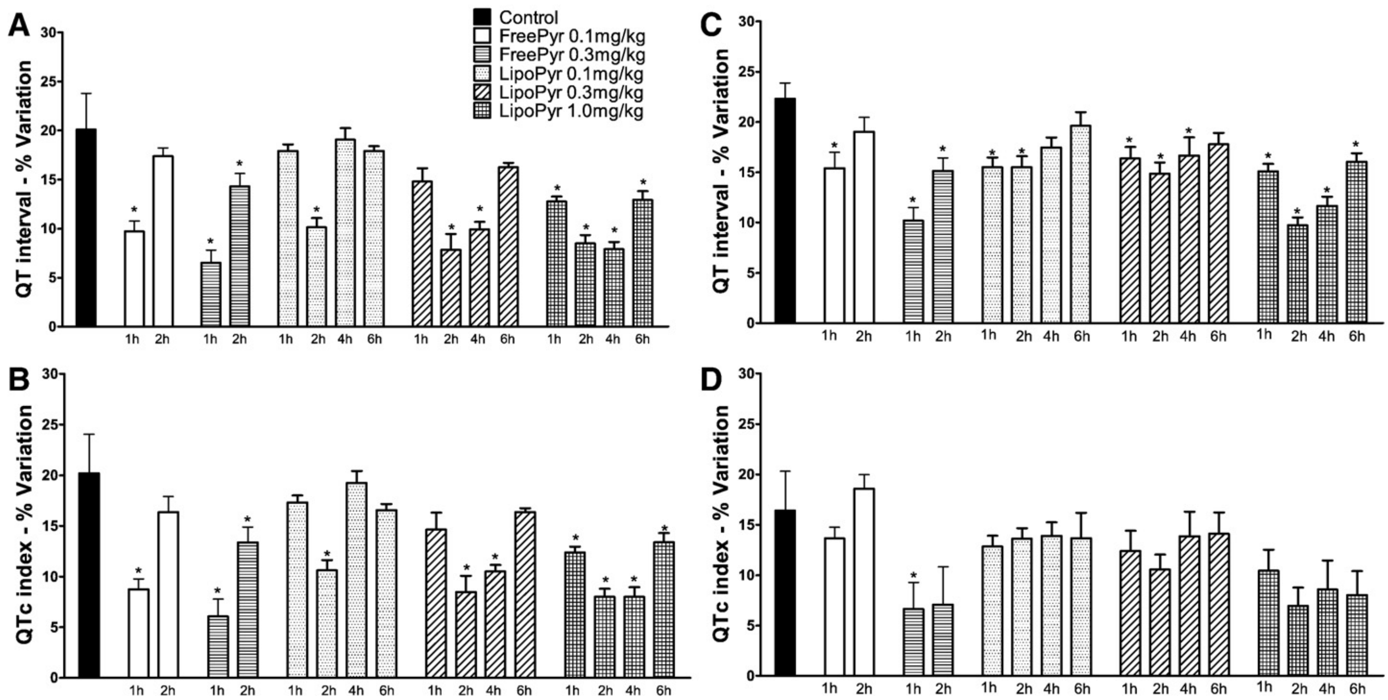

Fig. 1. Percentage variation of QT interval and QTc index measured after the sympathetic stimulation with NA in animals pre-treated with saline, free or liposomal pyridostigmine at different times. (A) and (B) changes after 1 µg of NA. (C) and (D) changes after 3 µg of NA. FreePyr: free form of pyridostigmine; 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

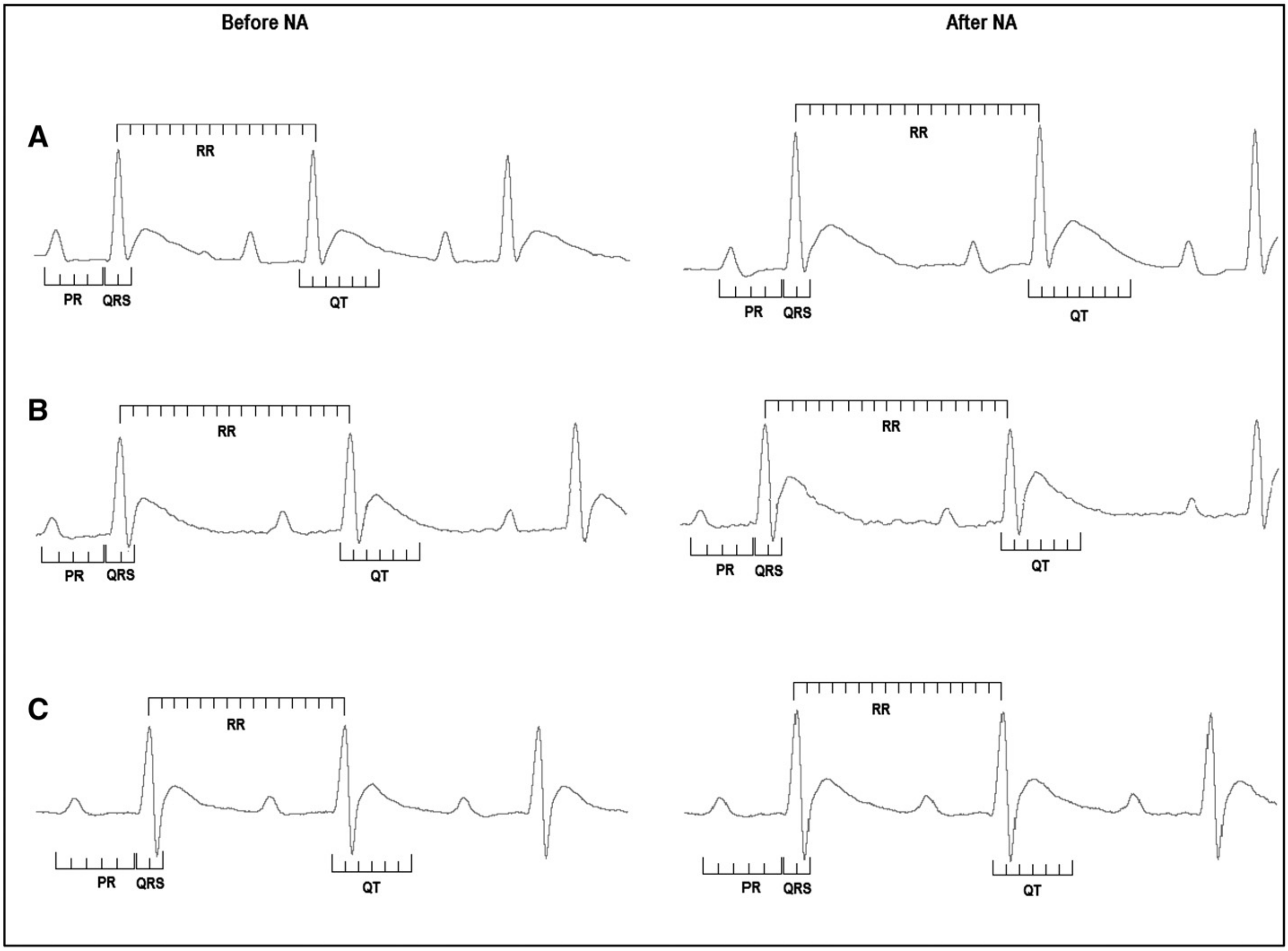

Fig. 2. Representative traces of ECG showing the effects of pre-treatment with pyridostigmine to prevent the alterations of the QT interval induced by 3 μg of NA, compared to the group that received saline.

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

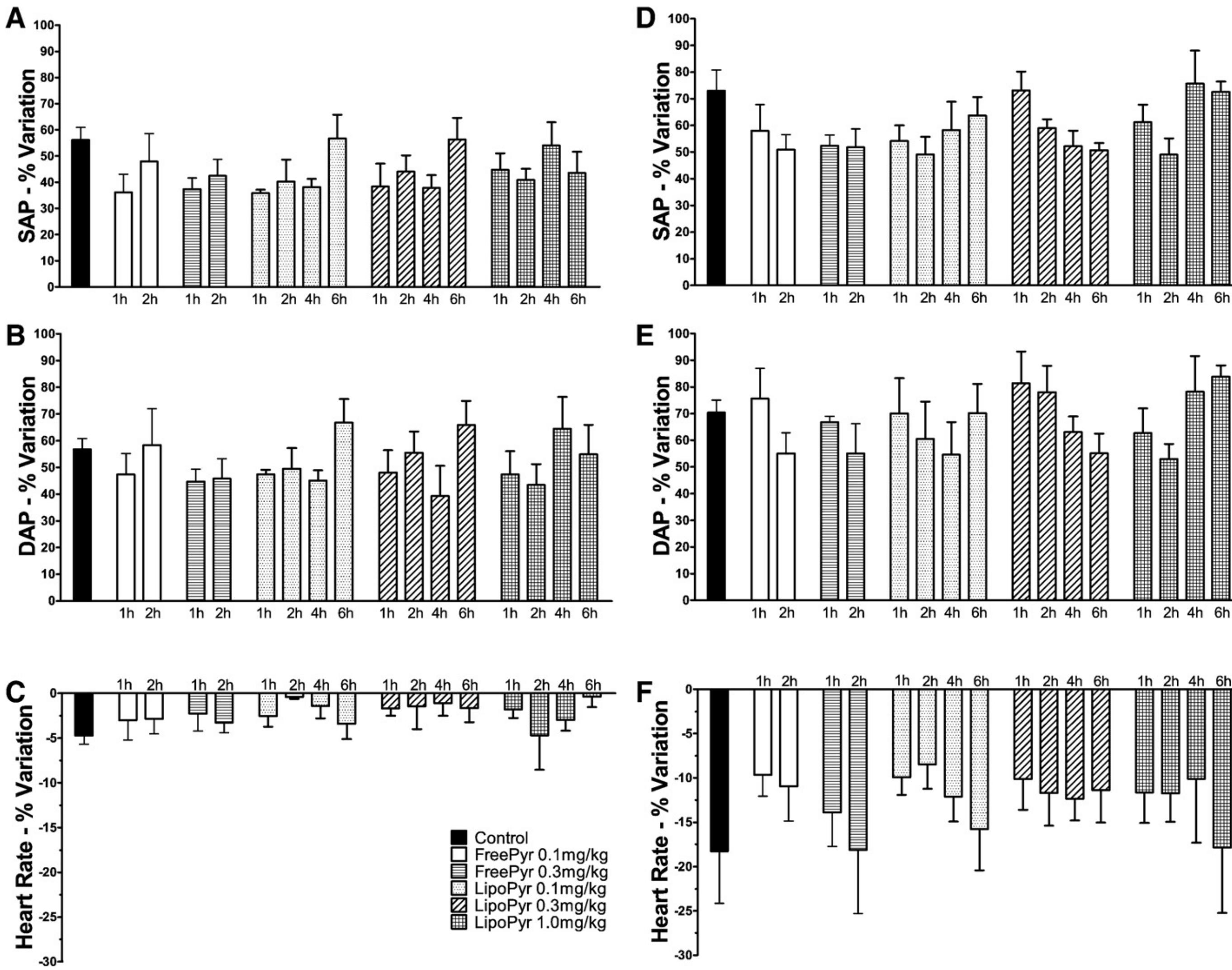

Fig. 3. Percentage variation of SAP, DAP and HR measured after the sympathetic stimulation with NA in animals pre-treated with saline, free or liposomal pyridostigmine at different times. (A), (B) and (C) after 1 µg of NA. (D), (E) and (F) after 3 µg of NA. FreePyr: free form of pyridostigmine; LipoPyr: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