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A new experimental model of chemical ablation of the Intrinsic Cardiac Nervous System reduces heart contractility and causes a type of dilated cardiopathy in rats

Um modelo experimental de ablação do Sistema Nervoso Intrínseco Cardíaco reduz a contratilidade do coração de ratos

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Abstract

Introduction: **The function of the Intrinsic Cardiac Nervous System is largely unknown, as is its role in heart disease. In the digestive system, a topic application of Benzalkonium chloride leads to intrinsic denervation of the viscera. Thus, our aim was to obtain an experimental model of cardiac intrinsic denervation by the application of Benzalkonium chloride on the heart.**

Method: **Thirty male Wistar rats received intrapericardial injections of 0.3% Benzalkonium chloride (BC animals) and thirty similar animals received saline (C animals). After 15 days the animals were divided in three groups, with 10 CB-treated and 10 saline-treated animals in each. Group I was submitted to radiological and morphologic studies. The cardiac shadow area (CSA) and cardiothoracic index were calculated in roentgenograms with a semi-automatic image analysis system (MINI-MOP). The day after the animals were weighed and sacrificed with heart, liver and lung collected for histopathologic analysis. The animals of Group II were submitted to a hemodynamic study. Measurements of blood pressure, heart rate and cardiac output were performed using the Cardiomax II thermodilution system and a Thermistor sensor. With the animals of the Group III, the integrity of the extrinsic parasympathetic cardiac innervation was examined by measuring heart rate response to electrical stimulation of the right vagus. Electrical activity was assessed by ECG.**

Results: **CB animals presented increases in cardiothoracic index, CSA, body and liver weight. In these animals the histopathologic analysis showed passive chronic congestion and reduction of the number of atrial neurons. In the hemodynamic study, total peripheral resistance and heart rate were similar in both groups, but blood pressure and cardiac index were reduced in the CB group. The vagal stimulation and ECG were similar in both groups.**

Conclusion: **Intrinsic Cardiac Nervous System denervation caused dilated cardiopathy in rats with left and right heart failure. The etiology of some dilated cardiopathies in human is largely unknown. This unpublished experimental model should provide future studies with the objective of elucidating the relationship between neuronal injures and heart disease.**

Descriptors: **Myocardial contraction. Heart conduction system. Autonomic nervous system. Ganglia, parasympathetic. Autonomic denervation. Benzalkonium compounds. Cardiomyopathy, congestive. Heart failure, congestive.**

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Resumo

Objetivo: **A função do Sistema Nervoso Intrínseco Cardíaco e o seu papel na doença cardíaca permanecem pobremente compreendidos. Sabe-se que o cloreto de benzalcônio (CB) induz a desnervação intrínseca do tubo digestivo. O objetivo deste estudo foi tentar produzir um modelo experimental de desnervação intrínseca do coração utilizando o CB.**

Método: **Trinta ratos Wistar foram submetidos à aplicação intrapericárdica de CB (0,3%) e trinta animais controle receberam a solução salina. Após 15 dias, os animais foram divididos em três grupos, com 10 animais tratados e 10 controles em cada. Os animais do grupo I foram submetidos a estudo radiológico e histopatológico. A área cardíaca e o índice cardiotorácico (ICT) foram medidos nas radiografias. Os animais do grupo II foram submetidos a estudo hemodinâmico com registro da pressão arterial, freqüência cardíaca e débito cardíaco. No grupo III, a integridade da inervação parassimpática extrínseca do coração foi avaliada por estimulação vagal direita. O sistema de condução foi avaliado pelo ECG basal.**

Resultados: **A aplicação de CB acarretou aumento do ICT, da área cardíaca, pressão arterial e débito cardíaco, bem como do peso ponderal e do fígado. Nestes animais, a análise histopatológica mostrou redução do número de neurônios atriais e congestão passiva crônica do fígado. A estimulação vagal não mostrou diferenças entre os grupos experimentais.**

Conclusão: **A ablação do sistema nervoso intrínseco propiciou o aparecimento de cardiopatia dilatada com insuficiência cardíaca direita e esquerda. Esse modelo experimental inédito deverá nortear futuros estudos na tentativa da elucidação da relação entre lesão neuronal e miocardiopatia.**

Descritores: **Contração miocárdica. Sistema de condução cardíaco. Sistema nervoso autônomo. Gânglios parassimpáticos. Denervação autônoma. Compostos de benzalcônio. Miocardiopatia congestiva. Insuficiência cardíaca congestiva.**

INTRODUCTION

Intrinsic cardiac neurons have been classically considered as being only efferent parasympathetic post-ganglionic neurons that receive impulses from efferent parasympathetic pre-ganglionic neurons, with the intrinsic ganglions working as cardiac transmission stations of central nervous system impulses. Later, efferent neurons involved in a local network of interconnections were identified, that contained both efferent and afferent sympathetic and parasympathetic post-ganglionic neurons [1], with this entire complex being called the Cardiac Intrinsic Nervous System (CINS). However, the function of the CINS and its role in the cardiac physiopathology are poorly understood. The numerous published experimental models of extrinsic denervation of the heart [2-4], have partially collaborated to the comprehension of cardioregulation.

All these models demonstrated cardiac denervation, but the authors did not take into account an important fact of denervation in heart transplantation, which does not include intrinsic denervation, because intracardiac ganglions are not affected in this kind of procedure. In fact, whilst human heart grafts continue to work extrinsically denervated, they present viability of the intrinsic nerves [5] and the activities of the intrinsic cardiac ganglionic plexus return [6]. Thus, there are no descriptions in the literature of a specific experimental model of denervation of the CINS, which preserves the extrinsic system.

Benzalkonium chlorate (BC) is a cationic surfactant that, when applied topically to the digestive tract, causes denervation of intrinsic neurons, causing significant alterations to the physiology and morphology of this organ [7,8]. As the cardiac neurons are located in the epicardium [9], by analogy, the intra-pericardial administration of BC should destroy them, producing an experimental model of denervation. Thus, the proposal of this study is to determine the effects of the ablation of the CINS on the morphology, contractility and heart rate of rats, to better understand its role in the cardiac physiopathology.

METHOD

Animals

Sixty male Wistar rats, with body weights of 200g at the beginning of the experiment, were utilized. All animals received care according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and to the "Guide for the Care and Use of Laboratory Animals", published by the National Institute of Health (NIH publication 86-23, reviewed 1985).

Groups and experimental design

Thirty Wistar rats were submitted to intra-pericardial application of BC (0.3%) and 30 control animals received a saline solution. After 15 days, the animals were allocated to three groups, with 10 treated and 10 control animals in each. The animals of Group I were submitted to radiologic and histopathologic studies. The cardiac area and cardiothoracic index were measured in radiographs. The animals of Group II were submitted to a hemodynamic study measuring the

arterial blood pressure, heart rate and cardiac outflow. In Group III the integrity of extrinsic parasympathetic innervation of the heart was evaluated by right vagal stimulation. The conduction system was evaluated by ECG.

Intrinsic heart denervation

After anesthesia by inhalation of sulfuric ether in a closed chamber, transverse laparotomy of approximately 4 cm in the upper abdomen was performed and using a syringe and 25G needle the pericardial sac was punctured at the central tendon of the diaphragm and 0.2 mL of 0.3% BC was injected. Closure of the cutaneous and muscular layers was performed using continuous 2-0 cotton sutures. The other group (control animals) received 0.9% sodium chloride solution in the pericardial sack.

Radiologic study

The rats were submitted to simple radiologic chest examinations in an anterior-posterior projection using a dental radiograph apparatus placed on the animal dorsum. The Cardiac area, and thoracic and cardiac side-to-side diameters were evaluated from measurements on the radiography using a semi-automatic system of image analysis (MINI-POP, Kontron®). The cardiothoracic index (CTI) was also calculated.

Histopathologic study

On the day following the radiologic examination, the animals, that presented a good general state, were weighed and were sacrificed with an intravenous infusion of potassium chloride (1 meq/mL), aiming at inducing cardiac arrest at diastole. Subsequently, the hearts and livers were excised and weighed and fragments of right inferior lobe of the lung were collected and sent for routine histological examinations. A series of 15 cross-sectional sections of the atria of each animal were assessed to determine the number of heart intrinsic neurons, according to the method described by OLIVEIRA et al. [8].

Aiming at verifying the specificity of BC for denervation, the same procedure using an intrapericardial injection of carrageenin was performed in a group of five animals. This substance is known to induce an inflammatory reaction. These animals' hearts were submitted to the same aforementioned histopathologic study.

Electrocardiographic and vagal electrical stimulation studies

The integrity of the extrinsic parasympathetic innervation of the heart was examined by measuring of response of the heart rate to electrical stimulation of the right vagus nerve. The heart rate and the integrity of the cardiac conduction system were evaluated by electrocardiography (ECG).

Three electrodes were subcutaneously implanted in the dorsum of each rat and connected to a multiple channel recorder (Physiograph Four-A, E & M. Instrument Company, New York, NY). The morphology of the ECG and the drop in the heart rate produced by electrical stimulation of the right vagus nerve were studied in rats anesthetized with endovenous Chloralose. The interval between stimuli was determined by the time required for the heart rate to return to the pre-stimulation level.

Hemodynamic study

This experiment consisted of hemodynamically measuring the responses obtained after intrinsic denervation of the heart and, thus, studying its contractility. Polyethylene cannulae were inserted in the right femoral artery and the right jugular vein, with the animal under intraperitoneal anesthesia using nembutal and a Thermistor temperature sensor (Columbus Instruments) was inserted in the right common carotid artery as far as the aortic crest. A Cardiomax II 85 thermodilution flowmeter (Columbus Instruments) was used to perform the measurements. These measurements were taken 15 minutes after the surgical procedure or when the hemodynamic parameters were stabilized.

The mean arterial blood pressure (MAP) and the heart rate (HR) were measured by flowmetry using the femoral artery cannula. The systolic volume (SV) was calculated using thermodilution curves. A solution of 0.9% NaCl at a low temperature was injected through the jugular vein cannula using a Micro Injector 400 (Columbus Instruments) and the Thermistor measured variations in temperature.

SV was calculated by the formula: $SV = \frac{(Tb-Ti)xVi}{AUC}$; where:
SV - Systolic Volume,
Tb - blood temperature, Ti- Injection Temperature, Vi- Injected Volume, AUC - Area under the curve
The following parameters were calculated:

- COM (Cardiac Outflow Minute): COM = HR x SV
- CI (Cardiac index): $CI = \frac{COM}{100g}$
- SI (Systolic Index): $SI = \frac{SV}{100g}$
- TPVR
  (Total Peripheral Vascular Resistance): $TPVR = \frac{MAP}{COM}$

Statistical analysis

The results were presented as means ± standard deviation. The Student t-test was utilized for comparative analysis, with level of significance established for alpha errors of 5%.

RESULTS

The radiologic study demonstrated an increase in the cardiac area and cardiothoracic index in the BC animals when compared to the control animals (Table 1). An increases in the mean body and liver weights of the BC animals compared with the control animals was observed, but no alterations in heart weights were seen (Table 2).

Table 1. Results of radiological analysis of images obtained from the posterior-anterior plane

	Control animals	CB animals
cardiothoracic index	0.34 ± 0.02	0.42 ± 0.08*
Cardiac area (mm2)	204.61 ± 15.05	283.37 ± 33.63*

* CB > C (p<0,05)

Table 2. Body, heart and liver weights.

	Control animals	CB animals
liver weight (g)	9.78 ± 1.03	11.64 ± 1.28*
heart weight (g)	1.01 ± 0.09	1.04 ± 0.11
Body weight (g)	242.20 ± 15.73	274.09 ± 23.98*

* CB > C (p<0,05)

The histopathologic study showed that the hearts of the control animals were normal and in the BC animals the hearts presented necrosis in the ganglionic plexus, and alternate myocardial hypertrophic and hypotrophic areas. Discrete epicardium thickening was observed in the BC animals, but no significant adhesions or acute inflammatory activity were noted. In the hearts of the rats treated with carrageenin, discrete pericardial thickening and absence of other histopathologic alterations were observed, as preservation of the intrinsic cardiac neurons was maintained.

A chronic passive congestion characterized by dilatation of the centrilobular vein together with sinusoidal dilatation was observed in the livers of the BC animals. Histopathologic alterations were not observed in the livers of the control and carrageenin animals. The lungs were histologically normal in all animals. The neuron count demonstrated a reduction in the number of cardiac neurons in the BC animals when compared to the control animals (Table 3).

The hemodynamic study (Table 4) demonstrated that the BC rats presented decreases in the PAM, COM, SV and CI, when compared to the control animals, however, the heart rate and the TPVR did not present significant alterations.

The ECG showed a decrease in the variability of the RR interval in the BC animals, with all the other parameters similar in all animals and within normal limits. The drops in heart rates, produced by electrical stimulation in the right vagus nerve, were similar in all animals (Figure 1).

Table 3. Neuron count results

	Control animals	CB animals
Number of atrial neurons (per 100µm of myocardium)	380.90 ± 44.75	112.51 ± 21.28*

* CB < C (p<0,05)

Table 4. Results of the hemodynamic study in animals in the CB and control animals

	Control animals	CB animals
Heart rate (beats per min)	405 ± 30	429 ± 39
Mean arterial blood pressure (mmHg)	117 ± 5	96 ± 3*
Cardiac outflow (mcL/min)	137 ± 9	101 ± 6*
Systolic volume (µL/100g)	342 ± 21	241 ± 18*
Cardiac index (mcl/min/g)	0.47 ± 0.05	0.32 ± 0.04*
Peripheral vascular resistance (mmHg/mcl/min)	0.87 ± 0.40	1.00 ± 0.14

* CB < C (p<0,05)

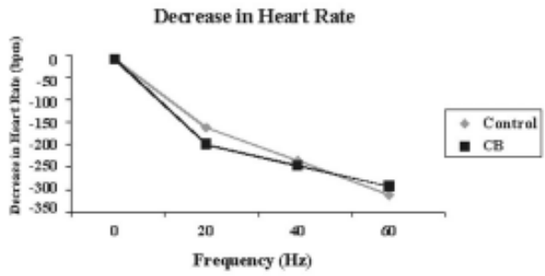

Fig. 1 – Resting heart rate and bradycardia response to electrical stimulation of the right vagus nerve (5V, 2 ms, 1-128 Hz) (n=10, NS).

COMMENTS

Studies have demonstrated that the ICNS supplies neuro-humoral stimuli to the heart, modulating the heart rate and the cardiac contractility [10]. The ICNS acts as a distribution processor, integrating parasympathetic and sympathetic centrifugal efferent information to the heart, as well as centripetal information originating from the sensitive nerve fibers [1].

The role of ICNS in the cardiac pathogenesis is unknown. Autonomic dysfunction is a serious complication of diabetes mellitus and can cause ventricular dysfunction [11], as well as in chagasic heart disease [12]. A common useful

instrument to study functional aspects of the nervous system and visceral innervation is to verify the effects of removing parts of the nervous system. The development of a new ablation model of the ICNS using benzalkonium chloride (BC) has been investigated over some time [7]. The destruction of intrinsic myoenteric neurons caused by the topical administration of BC on the digestive tract serosa in rats and the fact that this procedure causes great alterations in the physiology and morphology of the organ have already been confirmed. Thus, this type of denervation leads to significant distension of these viscera affecting their motility has been described [12,l3]. The cardiac neurons are located on the epicardium of the heart [10] and by analogy using the digestive tract model, the intrapericardial administration of BC could destroy these ganglia, producing an experimental model of intrinsic denervation of the heart.

This was the fundamental idea of this investigation, in which the principal findings are:

1) The radiologic study showed that the CTI and the cardiac area of the BC animals were significantly greater than the control animals;

2) The BC animals presented increases in the body weight and liver weight;

3) Histopathologic analysis of the liver was normal in the control animals and showed chronic passive congestion in the BC animals. In the heart, alternating hypertrophic and hypotrophic areas and a reduction in the neuron count in the atria were observed in the BC animals;

4) Vagal stimulation did not demonstrate significant alterations in the heart rate of the animals, and the ECG only demonstrated a reduction of the R-R interval in the BC animals;

5) The systemic vascular resistance and heart rate were similar in all animals as seen in the hemodynamic study, but the arterial blood pressure and the cardiac index were significantly lower in the BC animals.

The histopathologic study demonstrated a discrete pericardial thickening, but no active inflammatory process could explain the other morphologic and functional findings. Injuries to the cardiac intrinsic neurons were not evidenced in the animals submitted in the application of carrageenin, which shows the specificity of BC to produce these injuries.

Destruction of the ICNS was partial in the developed experimental model, as was confirmed by the neuron count. Animals after denervation put on weight which may be caused by hydric retention, which is usually seen with congestive heart insufficiency.

In conclusion, denervation of the ICNS causes a type of dilated heart disease as was demonstrated by the radiologic study but without muscle mass increases as seen by comparing the weights of the denervated and control hearts. The hemodynamic study indicated that the heart disease evolves with a reduction in the heart contractility.

The conduction system and the extrinsic parasympathetic innervation of the heart were preserved, which is confirmed as the ECG at rest and the response to electrical stimulation of the vagus nerve were identical in all animals. However, the decrease in the R-R interval in the ECG of BC animals is an indirect sign of severe heart disease.

The analysis of hemodynamic parameters, mainly the drop in cardiac index and the ejection fraction evaluated by the systolic volume, associated with chronic passive hepatic congestion in the BC animals, evidenced left and right congestive heart insufficiency. The reduction of the mean arterial blood pressure (MAP) in the BC animals suggests cardiocirculatory failure which is present in uncompensated cardiac insufficiency. There were no statistical differences in the heart rate and total peripheral vascular resistance between the two groups corroborating the hypothesis that alterations are related to the myocardial contractility.

Only hypotheses in respect to mechanisms that reduce the cardiac contractility after denervation of ICNS can be raised. It is consensus that cardiac insufficiency is accompanied by a significant disturbance in the autonomic nervous system. This disautonomy in cardiac insufficiency is generally characterized by increases in the activity of the sympathetic nervous system and a reduction in the activity of the parasympathetic nervous system [14]. A physiopathologic mechanism of heart failure common to laboratory models and the population is the neuroendocrine activation [15], with elevations in the levels of noradrenalin [16], vasopressin, natriuretic atrial factor, endothelin and increase of aldosterone-angiotensin-renin system [17].

One manner to study the function of the cardiac intrinsic innervation would be to evaluate the alterations which occur from denervation in chagasic heart disease [14]. However, the disadvantage that the experimental model of Chagas Disease presents is due to the difficulty of standardized denervation and by the fact that in this heart disease there are coexistent immunologic phenomena and alterations in the microvasculature [18] that make the identification of the role of denervation in the functional and morphological alterations of the heart impossible.

In models of denervation published in the literature, such as plexectomy [19], transplantation [20], the Randall/ vagotomy technique [21] and autotransplantation [22] only extrinsic innervation was involved both in animals and human beings. These models do not lead to an alteration in the cardiac physiology or morphology. The studied model fundamentally differs because it causes an exclusively intrinsic denervation and causes a significant alteration in the cardiac contractility. In this sense, the cardiac intrinsic denervation is similar to non-ischemic dilated heart disease, similar to chagasic and diabetic cardiopathies. In these

diseases, there is an imbalance in the autonomic nervous system and intrinsic denervation has, certainly, a great importance. This is an experimental detail that makes denervation with BC a novel model. These results introduce many perspectives for future studies in respect to the role of intracardiac neurons in the physiopathology of the heart.

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