

An Electrocardiographic and Morphological Study of Oral L-Arginine Administration Effects on a Model of Cardiotoxic β 2 Overstimulation

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ABSTRACT

Heart failure is one of the principal causes of death in the western world. Worldwide, it is estimated that cardiac disease caused approximately 22% of annual deaths by 2001. For example, Latin America reported a total incidence of 1%, with hospital readmission rates of 30% and a 1-year mortality rate of 24% representing that a heavy burden for health services and an important factor for disabilities and reducing life expectancy. Ischemic heart disease, hypertension, rheumatic/other valvular heart diseases, cardiopulmonary disease, and cardiomyopathy are considered the principal causes of heart failure, being, in consequence, the comprehension of the pathophysiology of these cardiac disease one of the most important aims in the biomedical research nowadays.

Keywords: Coronary artery disease; Speckle tracking echocardiography; Global longitudinal strain; Post systolic strain; Myocardial perfusion imaging; Coronary-angiography.

INTRODUCTION

Heart failure is one of the principal causes of death in the western world. Worldwide, it is estimated that cardiac disease caused approximately 22% of annual deaths by 2001 [1]. For example, Latin America reported a total incidence of 1%, with hospital readmission rates of 30% and a 1-year mortality rate of 24% [2], representing that a heavy burden for health services and an important factor for disabilities and reducing life expectancy. Ischemic heart disease, hypertension, rheumatic/other valvular heart diseases, cardiopulmonary disease, and cardiomyopathy are considered the principal causes of heart failure [3], being, in consequence, the comprehension of the pathophysiology of these cardiac disease one of the most important aims in the biomedical research nowadays.

As was stated above, cardiomyopathies are one of the principal causes of heart failure. Cardiomyopathies are defined as diseases of the myocardium with associated structural and/or functional abnormalities. Regarding the functional and structural criteria aforementioned, it may be classified into four principal

categories: dilated, hypertrophic, restrictive, and arrhythmogenic cardiomyopathy. Dilated cardiomyopathy comprising approximately 60% percent of all cardiomyopathy and is considered a “melting point” of pathological mechanisms of several cardiovascular pathologies [4].

Beta-adrenergic signaling is a key aspect of the pathophysiology of heart failure associated with cardiomyopathy. Beta-adrenergic receptors are divided among β 1, β 2, and β 3 receptors, with a differential tissular location and function. For cardiac tissue, the β 1 receptor is predominant in an approximate proportion of 3:1:0.5 regarding β 2 and β 3 respectively. β 1 adrenergic signaling is coupled to G's protein and generates AMPc and activates PKA with a subsequent rising of calcium concentration, heart rate and contraction force increasing (chronotropic and inotropic positive effect respectively) [5]. On the other hand, β 2 adrenergic receptors, generally associated with airways, are associated with Gs and Gi pathways [6], making more complex the understanding of receptor interplay in health and disease. For example, there are in vitro evidence that suggests that β 1 signaling may be cytotoxic and β 2 cardio-protective if β 2 receptors are signaled by Gi [7],

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through apoptosis inhibition by PI3K-AKT cell survival pathway [8]. However, β_2 receptors may also mediate cardio-toxic response. In this line of thought, low-dose administration of doxorubicin was not able to generate cardiomyopathy in a knockout β_2 mice regarding the wild type [9], evidencing the necessity of more clarity in the role of β_2 adrenergic signaling in heart failure.

On the other hand, the role of nitric oxide signaling (NO) associated pathways in the adrenergic response modulation has been highlighted in the last years. Neuronal nitric oxide synthase (NOS) dependent production of NO has been involved in the cardio protection mediated by β_3 receptors [10]. Inhibition of arginase II through β_2 signaling is associated with restoration of cardiac contractility in experimental models [11]. This evidence suggests that the interplay between beta-adrenergic signaling and nitric oxide may be involved in the hypertrophy genesis. Besides, the administration of L-arginine, the precursor of NO, has been linked to hypertrophy prevention in experimental models [12], but other works have failed to demonstrate beneficial effects [13], opening the field to the discussion. In this sense, this work aims to analyze the effect of orally given L-arginine on histological, morphological, serological, and electrocardiographic evidence of cardiac hypertrophy in a mouse model of β_2 adrenergic overstimulation.

MATERIALS AND METHODS

ETHICAL STATEMENT

Experiments were performed in strict accordance with "Bioethics and Biosafety Norms" (3rd edition) approved by Fondo Nacional de Ciencia y Tecnología de Venezuela (FONACIT), Ministerio de Ciencia y Tecnología of Venezuela (2011), the Asociación Venezolana para la Ciencia de los Animales de Laboratorio, and "International Ethical Standards for Research Biomedical in Animals of the WHO" (1982).

Adrenergic overstimulation

6-8 weeks old NMRI albino mice were divided into three groups (n=10): Salbutamol, Salbutamol plus L-arginine (henceforth LA), and Fenoterol. Salbutamol, a β_2 selective agonist, was administered via I.P. twice daily for seven days. Equally, the Salbutamol-LA group received treatment for 7 days and additionally were given orally with 3.75 mg/Kg of L-arginine diluted in the drinking water (water intake was estimated in 5 ml/Kg per mice). Finally, fenoterol, a β_2 agonist which acts only by Gs associated signaling pathway, was administered at 0.75 mg/Kg similarly than salbutamol.

Electrocardiogram recording

At the end of the β_2 adrenergic overstimulation protocol, mice were previously anesthetized with a single IP bolus of 25 mg/kg pentobarbital and 25 mg/kg ketamine. Electrocardiography (ECG) was performed using a bipolar system in which the electrodes were placed subcutaneously at the xiphoid cartilage (positive electrode), right shoulder (negative), and left shoulder as previously described [14]. Electrodes were connected to a Bioamp amplifier (AD Instruments, Bella Vista, Australia) and were digitalized through a PowerLab 8sp A/D converter (AD Instruments). Digital recordings were analyzed with Chart software for Windows v7.3.1 (AD Instruments), with events registered to 1 K/s and filtered to 60 Hz. Continuous ECG recordings were obtained for determining basal heart rate, defined as the point where there was no variation above 5%. At that point, electrocardiographic parameters were registered

and all groups were inoculated with 5 mg/Kg of salbutamol to assess β_2 dependent heart rate response by 5 minutes and variation of heart rate and T/S waves concerning pre-salbutamol values were determined, and wave morphology was recorded.

Tissue sampling and histological processing

After electrocardiographic analysis, hearts were collected from mice and placed in 10% neutral buffered formalin for 24 hr at room temperature, followed by incubation in 70% ethanol until processing. Hearts samples were then cut in a coronal shape and embedded in paraffin (Tissue Embedding Station Leica EG1150 H), and 3 μ m tissue sections were prepared (Microtome Leica 2125RTS), de waxed and rehydrated, stained with H&E staining and mounted permanently in Eukitt. As quick-hardening mounting medium (Biochemika, Fluka analytical). Sections were analyzed on a Leica microscope (Leica DM50) using the 20-40x magnification objectives.

For heart shape estimation, heart slices photo taken with a USB microscope with 10x magnification and a scale bar served to determine the pixel ratio with Image J software. The perimeter, estimated by tracing digitally a cardiac shape image and determining the pixel ration rate, was quantified to compare the mechanical response to adrenergic overstimulation. In the same way, inter ventricular septum and left and right ventricle thickness were estimated tracing a line from tendinous cord insertion area to the pericardium (left ventricle), the central portion (septum) and basal segment (right ventricle). Finally, the cardiac fiber perimeter and nuclei number were calculated in 100x magnification slides.

CK-MB activity measurements

The cardiac isoform of creatinine kinase (CK-MB) is an intracellular enzyme used as a marker of cardiac cell disruption in models of cardiac necrosis [15]. Enzymatic activity of CK-MB was determined following the instruction of the manufacturer (Elitech). Briefly, 10 μ L of serum taken by cardiac puncture and stored at -20 $^{\circ}$ C until use was plated on a 96 well plate and mixed with 250 μ L of work reagent provided by the manufacturer. Absorbance at 350 nm was determined at 37 $^{\circ}$ C by a Tecan absorbance reader (model), with measurements performed every 60 seconds. CK-MB activity was calculated with the mean of differential among the readings and multiplying the result by 8,254, a constant value estimated taking into account reference values of CK-MB activity. Interassay variation was estimated at 25%.

Statistical analysis

For in vivo experiments, data are shown as means \pm SEM. Significance was evaluated by Student's t-test when two groups were compared, by One-way ANOVA followed by the Tukey post-test for the analysis of three groups using Graph Pad Prism 5.00 software (La Jolla, CA, USA).

RESULTS

Salbutamol overstimulation and LA treatment

A protocol of cardiopathy induction with salbutamol was performed to evaluate the effects of L-Arginine treatment on β_2 overstimulation (Figure 1A). L-Arginine was able to induce a reduction of sinus tachycardia induced by salbutamol and produced a significant increase of QTc and R wave. Recovery of R voltage was even higher by the fenoterol group, a fact more sharply evident in

the comparison of QRS mean plots (Figure 1B).

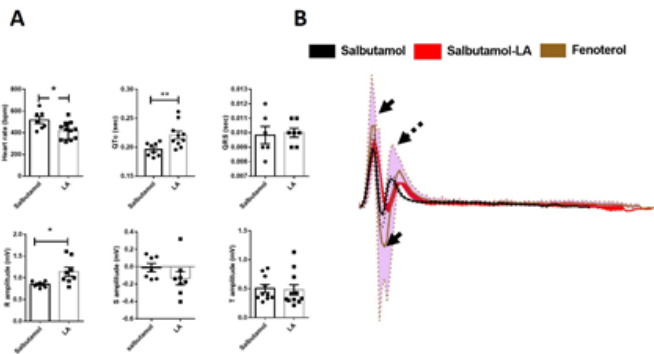


Figure 1: Salbutamol overstimulation and LA treatment. Panel “A” summarizes the main electrocardiographic parameters get in the analysis of Salbutamol and Salbutamol-LA (LA bars) treated groups. At Panel “B”, a mean electrocardiographic recording was plotted (n=4 different recordings for each group), including the fenoterol treated group. Standard error was represented as a dotted line and intervals were filled out (red, salbutamol-LA; magenta, fenoterol; colorless, salbutamol). The black arrows point R, S, and T waves where fenoterol showed higher voltage. Asterisks reflects P values: *, >0.05., **>0.01, ***>0.001.

Morphological analysis

Next, the effects of salbutamol overstimulation on cardiac morphology, cellularity, and integrity were assessed. LA treatment induced increased cellularity and a significant reduction of heart slices perimeter to expenses of septum width (Figure 2). Interestingly, LA treatment increased the area of individual cardiac fibers. fenoterol treatment reduced the perimeter of individual fibers and induced width increasing of the right ventricle and septum. Any effects were detected on the left ventricle. Representative histological micrograph showed fiber hypertrophy in salbutamol-LA group and nprotective effect of LA treatment in fenoterol overstimulated animals.

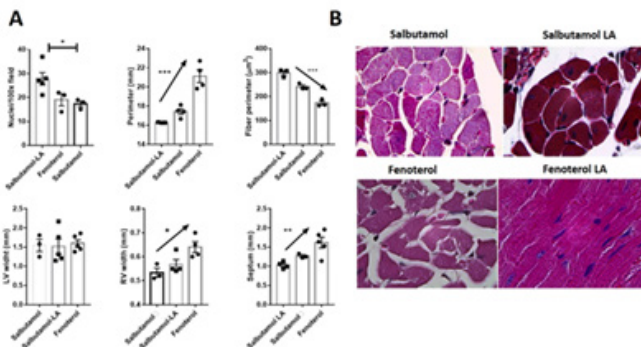


Figure 2: Morphological analysis. Nuclei number, heart perimeter, individual fiber perimeter, and Right Ventricle (RV), Left Ventricle (LV), and septum width were measured as stated at the the M&M section comparing salbutamol, salbutamol-LA, and fenoterol. (Left panel). Bars signal differences between individual groups and arrows indicate aa trend to right statistical differences, an ANOVA post-test. Representative micrographs, including in this case to Fenoterol LA is showed at the right side (100x). The black line-scale bar represents 50 μm. P values: *, >0.05., **>0.01, ***>0.001.

Fenoterol overstimulation and LA treatment

In this section, the interaction between LA treatment and fenoterol overstimulation was studied. L-arginine reversed the sinus tachycardia caused by fenoterol (Figure 3A). Additionally, LA treatment increased the electrocardiographic parameters associated with heart contractility (R voltage and QTc interval), as well as the S wave amplitude. LA treatment showed a dramatic decrease in the

serological marker of cardiac damage in the group overstimulated with fenoterol and treated with L-arginine (Figure 3B).

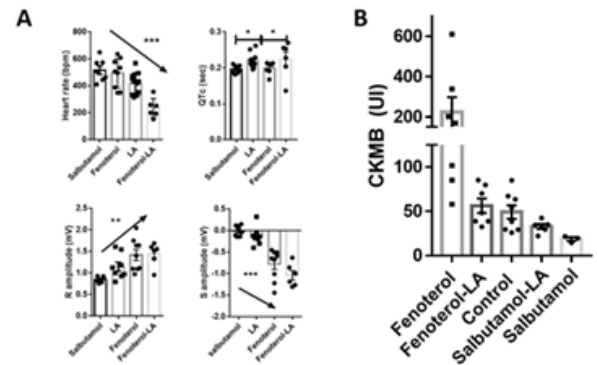


Figure 3: Fenoterol overstimulation and LA treatment. The electrocardiographical behavior of the treatment of LA in salbutamol stimulated animals was compared with fenoterol and fenoterol LA. The most significant parameters were plotted and statistical differences were assessed by a trend to right ANOVA post-test. P values: *, >0.05., **>0.01, ***>0.001. Panel B shows levels of Ck-Mb, a cardiac damage marker.

Heart rate kinetic study

Finally, we studied the effect of L-Arginine treatment on the dynamic of heart rate after injection of fenoterol and salbutamol. The group overstimulated with salbutamol showed and inverse-agonist effect, with a decreasing heart rate at the following five minutes post-stimulation probably due to the Gi protein effect. Fenoterol, an only Gs protein associated agonist, generated the expected mild sinus tachycardia. Notoriously, LA treatment was able to restore the positive chronotropic effect post-salbutamol stimulation in a similar fashion than fenoterol treated animals (Figure 4).

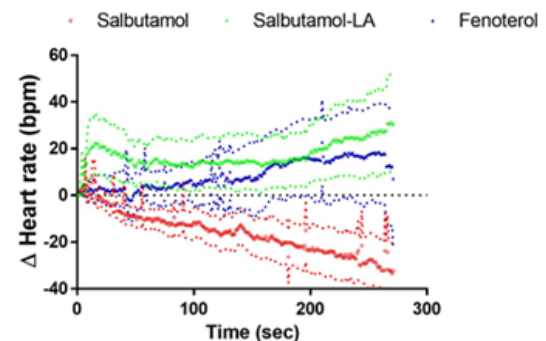


Figure 4: Heart rate kinetic study. Differential heart rate (Δ heart rate) was estimated by mean the differential between a given time point regarding injection of salbutamol/fenoterol. Standard error and confidence interval (95%) was plotted with small dots with the same color of the principal graph. N=9 for salbutamol groups and 8 for fenoterol.

DISCUSSION

Heart failure pathophysiology implies intricate mechanisms of intracellular signaling dysregulation in cardiac cells. β adrenergic signaling is composed of two principal receptors (β 1 and β 2) as regulators of heart rate, being β 1 potentially more cardiotoxic than β 2. Besides, β 3 has an emerging role in the comprehension of the heart failure process. Some review postulated that in conditions of heart failure, the hyperstimulated β 1-adrenoceptors induce a cardiotoxic effect, which could be counteracted by the cardioprotective effect of β 2-adrenoceptor-mediated Gi signaling. However, β 2-adrenoceptor-Gi signaling negates the stimulatory effect of the Gs signaling on cardiomyocyte contraction and further

exacerbates cardiodepression[16].

In our model, we overstimulated with salbutamol, a specific β_2 agonist, to simulate this condition in previously healthy mice. LA was able to revert sinus tachycardia and improve R voltage in overstimulated animals. One possibility is that low concentrations of NO, a product of the metabolism of L-arginine, inhibit phosphodiesterase III, thus preventing the hydrolysis of cAMP and causing the opening of sarcolemmal voltage-operated and sarcoplasmic ryanodine receptor $\text{Ca}(2+)$ channels [17]. In our case, our results are in contradiction with the reported increase of heart rate associated to NO signaling. However, in the absence of baroreflex buffering, inhibition of endogenous NO synthesis results in significant bradycardia, reflecting direct tonic modulation of heart rate by NO in healthy individuals [18]. As expected, Gs stimulation by fenoterol increases the voltage of the R wave and induced higher tachycardia.

Our team documented the ability of L-arginine orally given to improve the cardiac function in an experimental model of Chagas disease [19]. In this report, L-arginine was able to avoid cardiac ischemia-induced by isoproterenol in a similar fashion than observed in vasodilators agents, with a reduction of heart shape. In the present report, LA treatment reversed the hypertrophy induced for salbutamol. Reversion of tachycardia may be the most important pathway of cardioprotection, although is plausible that reduction of vascular tone associated with a higher NO-releasing in microvasculature may be involved. This hypothesis is reinforced by the reduction observed in the heart rate in the fenoterol overstimulated group supplemented with LA. Strikingly, LA treatment reduced substantially the levels of Ck-mb, a specific marker of cardiac damage, and is direct evidence of the cardioprotector effect of LA treatment. A further biochemical approach is necessary to complete this point.

Another possibility is associated with the stimulation of β_3 subtype adrenergic receptors through the NO pathway (GMPc). Some reports suggested that at least in some species, cardiac β_3 -mediated NO generation may act as a negative feedback system to regulate beta-adrenergic stimulated positive inotropy [20]. In such a sense, we may explain the reduction of heart rate in animals treated with fenoterol or salbutamol. However, is intriguing the mechanism associated with the ability of LA to reverse the reduction of heart rate in animals overstimulated with salbutamol in a similar fashion of fenoterol, suggesting the ability of NO to generate Gs signaling in animals overstimulated by β_2 agonist treatment. For example, fenoterol was able to stimulate NO production through Gs β_2 signaling [21]. It is plausible that NO, probably produced by iNOS or nNOS isoforms, can "rescue" β_2 Gs signaling in overstimulation conditions. The associated mechanisms may be a topic for future research.

CONCLUSION

In conclusion, this work was demonstrated the cardioprotection effects of L-arginine when is given orally in a model of β_2 adrenergic overstimulation. Differently than our previous report based on an inflammatory model of cardiac disease (Chagas disease), the protector effect of L-arginine supplementation seems to be centered on cardiac cell NO signaling, probably related to 1) heart rate reduction associated to GMPc pathway and 2) a probably shift from Gi signaling in a β_2 overstimulated cardiac cell to Gs signaling, which allows to heart exercising the pump function. Our goals were limited to describe the morphological

and electrocardiographic changes, but this work opens the window to explore the biochemistry mechanism involved and suggests possible beneficial effects of LA supplementation that need further corroborated.

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