

Stanozolol induces ventricular dysfunction by decreasing phospholamban phosphorylation in heart tissue of LDLr^{-/-} mice

O estanozolol induz disfunção ventricular pela redução da fosforilação do fosfolamban no tecido cardíaco de camundongos LDLr^{-/-}

El estanozolol induce disfunción ventricular al reducir la fosforilación de fosfolamban en el tejido cardíaco de ratones LDLr^{-/-}

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Abstract

Stanozolol is a steroid that causes lipid deposition in LDLr^{-/-} mice, although the mechanism by which this dyslipidemia results in cardiac dysfunction is little understood. The aim of this study was to evaluate the effect of stanozolol on cardiac contractility and the participation of myocardial phospholamban (pPLB) phosphorylation in an atherosclerosis mouse model. LDL receptor knockout mice (LDLr^{-/-}) were fed a standard chow diet and received weekly subcutaneous injections of either saline (control, C group) or 20 mg/kg stanozolol (S group). After 8 weeks, hemodynamic parameters were assessed in the left ventricle. The heart was collected, weighted for hypertrophy evaluation, and kept in formalin buffer for morphometric analysis (H&E) and collagen quantification (Picrosirius). Protein expression of PLB and its phosphorylated form (p-PLB) in the left ventricle was determined by western blot. We observed that stanozolol treatment favored cardiac hypertrophy and collagen deposition in heart tissue. Also, stanozolol induced left ventricle dysfunction, increasing PBL expression and decreasing the p-PLB/PLB ratio. Altogether, our data showed that stanozolol causes cardiac remodeling and ventricular dysfunction by decreasing PLB phosphorylation in the left ventricle of LDLr^{-/-} mice.

Keywords: Anabolic androgenic steroids; Hemodynamic parameters; Cardiac remodeling; Phospholamban phosphorylation.

Resumo

O stanozolol é um esteroide que promove deposição lipídica nas artérias de camundongos LDLr^{-/-}, entretanto, o mecanismo pelo qual a dislipidemia promove disfunção cardíaca nesses animais, ainda é pouco entendida. Deste modo, o objetivo do presente estudo é avaliar o efeito do stanozolol na contratilidade cardíaca e a participação da fosforilação da proteína fosfolambam (pPBL) miocárdica no modelo animal de aterosclerose. Os camundongos knock-out para receptor de LDL (LDLr^{-/-}) foram alimentados com dieta padrão para biotérios e receberam semanalmente injeções subcutâneas com salina (grupo controle, C) ou 20 mg/kg de stanozolol (grupo S). Depois de oito semanas de tratamento, os parâmetros hemodinâmicos foram avaliados no ventrículo esquerdo. O coração foi então coletado, pesado para determinação da hipertrofia e armazenado em tampão formalina para a determinação das análises morfométrica (H&E) e da quantificação de colágeno (picrossirius). A expressão da proteína fosfolambam (PBL) e da sua forma fosforilada (p-PBL) no ventrículo esquerdo foi determinado por western blot. Nós observamos que o tratamento com stanozolol favoreceu a hipertrofia e a deposição de colágeno no tecido cardíaco. Além disso, o stanozolol induziu a disfunção do ventrículo esquerdo, aumento da expressão do PBL e a redução da razão p-PBL/PBL. Em conjunto, nossos dados mostram que o stanozolol promove remodelamento cardíaco e disfunção ventricular pela redução da fosforilação do fosfolambam no ventrículo esquerdo em camundongos LDLr^{-/-}.

Palavras-chave: Esteróide anabólico androgênico; Parâmetros hemodinâmicos; Remodelamento cardíaco; Fosfolambam fosforilada.

Resumen

El estanozolol es un esteroide que promueve el depósito de lípidos en las arterias de ratones LDLr^{-/-}, sin embargo, el mecanismo por el cual la dislipidemia promueve la disfunción cardíaca en estos animales aún no se conoce bien. Por lo tanto, el objetivo del presente estudio es evaluar el efecto del estanozolol sobre la contractilidad cardíaca y la participación de la fosforilación de la proteína fosfolambano miocárdico (pPBL) en el modelo animal de aterosclerosis. Se alimentó a ratones sin receptor de LDL (LDLr^{-/-}) con una dieta estándar de casa de animales y recibieron inyecciones subcutáneas semanales de solución salina (grupo de control, C) o 20 mg/kg de estanozolol (grupo S). Después de ocho semanas de tratamiento, se evaluaron los parámetros hemodinámicos en el ventrículo izquierdo. Luego se recolectó el corazón, se pesó para determinar la hipertrofia y se almacenó en tampón de formalina para análisis morfométrico (H&E) y cuantificación de colágeno (picrossirius). La expresión de la proteína fosfolambano (PBL) y su forma fosforilada (p-PBL) en el ventrículo izquierdo se determinó por western blot. Observamos que el tratamiento con estanozolol favorecía la hipertrofia y el depósito de colágeno en el tejido cardíaco. Además, el estanozolol indujo disfunción ventricular izquierda, aumentó la expresión de PBL y redujo la relación p-PBL/PBL. En conjunto, nuestros datos muestran que el estanozolol promueve la remodelación cardíaca y la disfunción ventricular al reducir la fosforilación del fosfolambano del ventrículo izquierdo en ratones LDLr^{-/-}.

Palabras clave: Esteroide androgénico anabólico; Parámetros hemodinámicos; Remodelado cardíaco; Fosfolambano fosforilado.

1. Introduction

Anabolic androgenic steroids (AAS) are molecules derived from testosterone, the male sexual hormone (Kicman, 2008; Kuhn, 2002; Lippi et al., 2011). Among AAS, stanozolol is of greater concern because of its aesthetic use, as it has intense myogenic activity (Kicman, 2008; Lippi et al., 2011).

The abusive use of AAS leads to serious deleterious consequences, especially on the cardiovascular system, such as atherosclerosis (Fogelberg et al., 1990), hypertension (Franquni et al., 2013), cardiac remodeling (Brasil et al., 2015; Tadeu Uggere De Andrade et al., 2008; Nascimento et al., 2016), arrhythmias (Liu et al., 2003), and sudden death (Darke, Torok, & Duflo, 2014; Paolo et al., 2007). However, studies investigating the effects of stanozolol treatment on cardiac function, especially in subjects with predisposition to develop atherosclerosis, which is one of the most prevalent cardiovascular diseases (Herrington et al., 2016; Mozaffarian et al., 2016), are still lacking.

Atherosclerosis is a chronic and progressive inflammatory state that can obstruct blood flow and, consequently, cause tissue necrosis because of the absence of oxygen (Herrington et al., 2016). Obstruction of blood flow in the myocardium promotes cell death and inefficient cardiac pumping (Litwin et al., 1991; Pfeffer et al., 1979).

A previous study showed that the expression of sarcoendoplasmic reticulum Ca²⁺-ATPase (SERCA) in the aorta of ApoE^{-/-} mice is altered (Ewart et al., 2014). However, little is known about that of phospholamban (PLB) – a protein that plays a major role in the regulation of the cardiac cycle – in knockout mice (James et al., 1989; Simmerman & Jones, 2017).

There are many animal models to study atherosclerosis development (Lee et al., 2017; Veseli et al., 2017). One of the most used is the LDL receptor knockout mouse (LDLr^{-/-}) model, in which the animals develop a hypercholesterolemia more similar to that of humans (Veseli et al., 2017). Recently, our group demonstrated that an 8-week stanozolol treatment increased the vascular lipid deposition attributed, at least in part, to changes in the lipid profile and, probably, to systemic inflammation and oxidative stress in LDLr^{-/-} mice on a standard (non-atherogenic) diet (Tadeu Uggere de Andrade et al., 2019).

Based on those results, it is possible that the increased lipid deposition in aorta induced by stanozolol influences cardiac contractility by affecting the protein expression of PLB, which regulates the Ca²⁺ flow in cardiac muscle cells. Therefore, the aim of this study was to evaluate the effects of chronic stanozolol treatment on cardiac hemodynamic parameters of male LDLr^{-/-} mice on a standard diet, and the participation of phospholamban (PLB) and its phosphorylation in cardiac tissues.

2. Materials and Methods

2.1 Animals

All experimental procedures were performed in accordance with the guidelines for the care and handling of laboratory animals recommended by the National Institutes of Health (NIH) and were approved by the Institutional Animal Care Committee (Protocol n° 338/2014). Two-month-old male LDL receptor knockout (LDLr^{-/-}) mice weighing 25-30g were used in the experiments. The mice were kept in Alesco[®] miniinsulator IVC (Individually Ventilated Caging) racks at controlled temperature (~23 °C) and humidity and were exposed to a 12/12-h light-dark cycle with access to food (Standard rodent chow diet; Probiotério[®], Moinho Primor, S.A) and water *ad libitum*.

The animals were randomly separated into two groups: (a) Control group (C, n = 10), in which animals were treated with the vehicle for stanozolol (saline solution – NaCl 0.9%); and (b) Stanozolol treated-group (S, n = 10), treated with a high dose of stanozolol (20 mg/kg per week) (Tadeu Uggere de Andrade et al., 2019; Beutel, Bergamaschi, & Campos, 2005). Treatments were administered subcutaneously and maintained for eight weeks. The volume of saline injected into control animals was similar to that used for the S group. All experiments were performed one week after the last administration of the steroid or vehicle (Beutel et al., 2005).

2.2 Hemodynamic evaluation

After the experimental protocol, the animals were anesthetized with ketamine (100 mg/kg, i.p., Agener[®] União, Brazil) and xylazine (10 mg/kg, i.p., Bayer[®], Brazil). Left Ventricular (LV) function was assessed as previously described by Almeida et al., (Almeida et al., 2014). Briefly, the right common carotid artery was separated from connective tissue and catheterized with a fluid-filled polyethylene catheter (P50). The catheter was connected to a pressure transducer coupled to a MP-100 System Guide (model MP100-CE; Biopac Systems[®], Santa Barbara, CA, USA). After a 15-min stabilization, mean arterial pressure (MAP) and heart rate (HR) were recorded. The catheter was then advanced to the left ventricle. After an additional 15-min stabilization, the functional parameters +dP/dt_{max} and -dP/dt_{min} – which are the maximum and minimum rate of ventricular pressure increase, or the peak positive and negative values of the first derivative of the left ventricular pressure, expressed in mm Hg/s – and the time constant (Tau) of the LV isovolumic relaxation were measured. Following this procedure, the catheter was withdrawn from the LV and the arterial pressure measured again to determine whether the aortic valve was damaged (Nascimento et al., 2016).

2.3 Evaluation of cardiac hypertrophy

After hemodynamic evaluation the animals were euthanized. The heart was excised, cleaned, and weighted. Macroscopic cardiac hypertrophy was determined by the ratio between the organ weight and the final body weight (heart

weight/body weight ratio, mg/g). The left ventricle was split and one part frozen at -80°C for western blot analysis and the other kept in formalin buffer for morphometric analyses (Nascimento et al., 2016).

2.4 Morphometric analysis

The organs were kept in formalin buffer for 24 hours, dehydrated, diaphanized with xylol, and embedded in paraffin. Five micrometer-thick slices were stained with hematoxylin and eosin (H&E) or Picosirius red, to evaluate myocyte hypertrophy and collagen deposition, respectively (Junqueira, Bignolas, & Brentani, 1979; Lima et al., 2015). Ten photographs of the samples were obtained using an image acquisition system (Moticam Plus[®]; Motic Inc., Canada). Morphometric analysis was performed by determining the area of cardiac myocyte nuclei (μm^2) and collagen deposition under $400\times$ magnification. These analyses were performed by a blinded researcher using the free software Image J[®] (National Institutes of Health, Bethesda, MD, USA) (Nascimento et al., 2016).

2.5 Western blot analysis

Protein expression of phospholamban (PLB) and phosphorylated-phospholamban (p-Ser16-PLB) was assessed through western blot analysis. Samples of left ventricle tissue (80 mg) were homogenized in lysis buffer (100 mmol/L NaCl, 50 mmol/L Tris-base, 5 mmol/L EDTA, 2 Na, 50 mmol/L $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, 1 mmol/L MgCl_2 , 1% Nonidet P40, 0.3% Triton x-100, and 0.5% sodium deoxycholate; pH = 8), containing protease inhibitor (Sigma Fast; Sigma, USA) and phosphatase inhibitors (20 mmol/L NaF, 1 mmol/L Na_3VO_4). Total protein content was measured by the Bradford method (Bradford, 1976). Protein samples (50 mg) were diluted in buffer ($5\times$ 2 M Tris, pH = 6.8; 20% glycerol, 30% SDS, 25% mercaptoethanol, 0.1% Bromophenol Blue) and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Millipore, MA, USA). After incubation with blocking solution (20 mM Tris, 150 mM NaCl pH 7.6, 0.05% Tween and 4% albumin), the membranes were incubated overnight at 4°C with primary specific antibodies: anti-PLB ([1:1000], Abcam, Cambridge, MA, USA) and anti-p-Ser16-PLB ([1:1000], Santa Cruz Biotechnology, CA, USA). The membranes were then washed (5 min, three times) and incubated for two hours with secondary antibodies conjugated with peroxidase (HRP) [1:15,000]: anti-mouse IgG (Sigma Aldrich, St. Louis, MO, USA), anti-goat IgG (Millipore, Bedford, USA), and anti-rabbit IgG (Millipore, Bedford, USA). Immunoreactive bands were detected through chemiluminescence using peroxidase substrate (Luminata HRP Substrate-Millipore) and exposed to X-ray film. Densitometry was evaluated using ImageJ[®] (National Institutes of Health, Bethesda, MD, USA). GAPDH expression level was used to normalize protein expression (Nascimento et al., 2016).

2.6 Statistical analysis

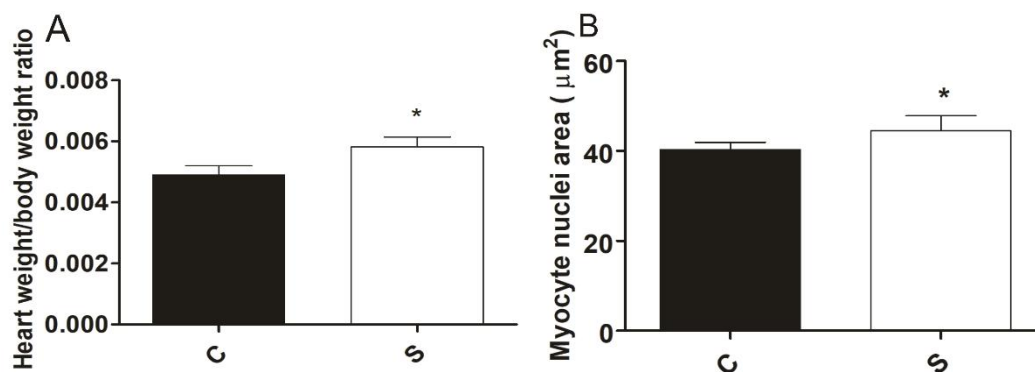
The data are expressed as mean \pm standard error of the mean (S.E.M.). The data were evaluated using the D'Agostino & Person's normality test. Statistical evaluation was performed through the unpaired Student's *t* test using the software Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA); differences were considered significant when $p < 0.05$.

3. Results

3.1 Stanozolol treatment induces cardiac hypertrophy

Chronic treatment with stanozolol increased the heart weight-to-body weight ratio (C: 0.004902 ± 0.0003053 ; S: 0.005819 ± 0.0003225 mg/g, Figure 1A) and myocyte nuclei area (C: 40.29 ± 0.7304 ; S: 44.49 ± 1.67 , Figure 1B) in $\text{LDLr}^{-/-}$ mice.

Figure 1: Macroscopic and morphometric analyses of cardiac hypertrophy. Macroscopic evaluation of cardiac hypertrophy, represented by heart weight/body weight (A). Graphical representation of the myocyte nuclei area (B). Values are shown as mean \pm standard error of mean (SEM). * $p < 0.05$ compared with C group.

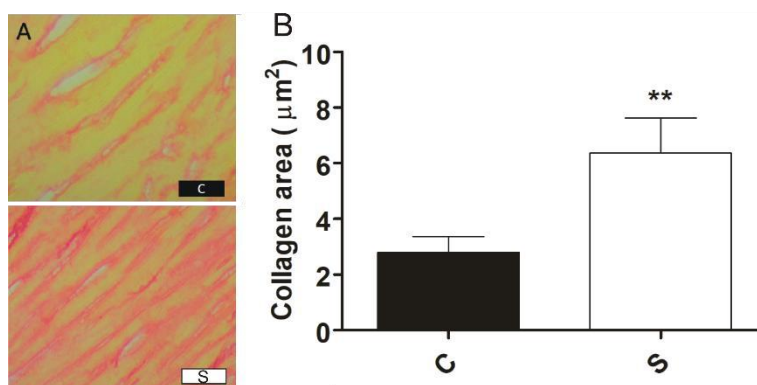


Fonte: Autores.

3.2 Stanozolol treatment determines cardiac remodeling

Cardiac hypertrophy was accompanied by a significant collagen deposition, indicating cardiac remodelling (C: 2.799 ± 0.56 , S: $6.360 \pm 1.27 \mu\text{m}^2$, Figure 2).

Figure 2: Histological analysis to evaluate collagen deposition. Representative image of blades stained with picosirius red (A). Collagen area deposited in cardiac tissue (B). Values are shown as mean \pm standard error of mean (SEM). * $p < 0.05$ compared with the C group.

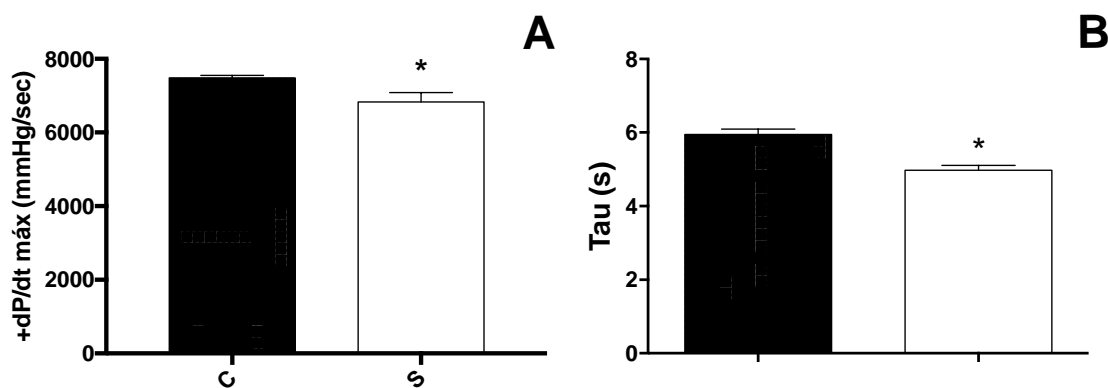


Fonte: Autores.

3.3 Stanozolol treatment induces left ventricle dysfunction in *LDLr^{-/-}* mice

The influence of the stanozolol treatment on left ventricular systolic and diastolic function was revealed by $+dP/dt_{\text{max}}$ (Table 1) and Tau analysis (Figure 3). $+dP/dt_{\text{max}}$ and Tau values were decreased in the stanozolol-treated group in comparison with the control group. No changes were observed in MAP, HR, or $-dP/dt_{\text{min}}$ (Table 1).

Figure 3: Evaluation of left ventricle function. Positive first derivatives +dP/dt maximum of the left ventricle (+dP/dt_{max}, mmHg/s) (A). Time constant of the isovolumetric relaxation of the left ventricle (Tau; seconds) (B). Values are shown as mean ± standard error of mean (SEM). *p < 0.01 compared with the C group.



Fonte: Autores.

Table 1: Hemodynamic evaluation in animals chronically treated with stanazolol.

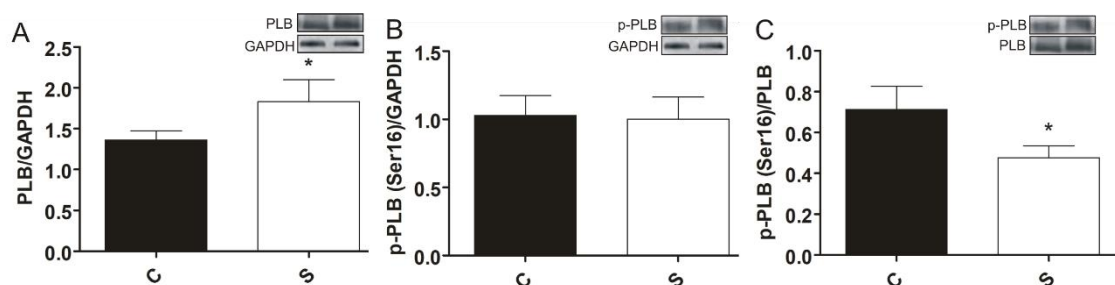
Parameter	Group	
	C	S
Mean Arterial Pressure (MAP) mmHg	50.81 ± 0.23	49.57 ± 0.20
Heart Rate (HR) bpm	499.5 ± 2.86	497.9 ± 2.89
Positive first derivative (+dP/dt) (mm Hg/s)	7477.0 ± 55.74	7269 ± 87.11*
Negative first derivative (-dP/dt) (mm Hg/s)	-7236 ± 105.2	-7087 ± 107.3
Tau (s)	5.929 ± 0.09	5.002 ± 0.13*

Values are shown as mean ± standard error or mean (SEM). *p<0.05 compared with the control group. Data were analyzed using unpaired Student's *t* test. Differences were considered significant when p < 0.05. Fonte: Autores.

3.4 Stanazolol treatment decreased the p-PLB/PLB ratio on heart tissue of LDLr^{-/-} mice

To evaluate the molecular mechanisms underlying the alterations observed in cardiac function, total phospholamban, phosphorylated phospholamban, and the ratio between them were determined (Figure 4). While total phospholamban expression levels increased in the stanazolol-treated group (C: 1.36 ± 0.11; S: 1.83 ± 0.27, Figure 4A), those of phosphorylated phospholamban remained unchanged (C: 1.03 ± 0.15; S: 1.00 ± 0.16, Figure 4B). As expected, the ratio between pPBL/PBL decreased in heart tissue (C: 0.71 ± 0.11; S: 0.48 ± 0.06, Figure 4C).

Figure 4: Protein expression of phospholamban in the left ventricle. Total phospholamban expression (A); Phosphorylated phospholamban (p-PLB) expression (B). Ratio between p-PLB (Ser16) and total phospholamban expression (C). Values are shown as mean ± standard error of mean (SEM). * p < 0.05 compared with the C group.



Fonte: Autores.

4. Discussion

In the present study, we detected left ventricle dysfunction after an 8-week treatment with high dose of stanozolol in LDLr^{-/-} mice under a standard, non-atherogenic diet. The change in cardiac physiology that manifested itself through left ventricular contractile and relaxation dysfunction (reduction of +dP/dt_{max} and Tau) was accompanied by a reduced p-PLB/PLB ratio and cardiac remodeling.

The treatment with stanozolol induced morphological changes in the heart, characterized by increases in heart weight/body weight ratio, area of cardiac myocyte nuclei, and collagen deposition in the heart, indicating cardiac hypertrophy and remodeling. This is the first time these cardiac effects are demonstrated in LDL^{-/-} mice treated with stanozolol on a standard chow diet, although similar results have been reported in other models of cardiovascular disease and using other AAS (Ammar, Said, & Hassan, 2004; Fontana, Oliveira, Leonardo, Mandarim-de-Lacerda, & Cruz-Hofling, 2008; Franquni et al., 2013).

Cardiac hypertrophy induced by AAS is caused by an increase in parallel sarcomere deposition, which causes a concentric hypertrophy, as previously demonstrated (Tadeu Uggere De Andrade et al., 2011; Fernandes, Soci, & Oliveira, 2011; Tanno et al., 2011). Beutel et al., (Beutel et al., 2005) evaluated the effect of stanozolol in rats and demonstrated cardiac hypertrophy, although only macroscopically. We observed that the increase in cardiac mass was followed by changes on myocyte morphology and collagen deposition.

In this context, the cardiac remodeling is characterized by the accumulation of collagen – especially type I and II – in cardiac tissue due to external stimuli, such as the use of AAS (Lima et al., 2015) or even a stroke (Almeida et al., 2018, 2014). These structural changes in the organization of the myocardium impair heart function (Sutton & Sharpe, 2000; Tanno et al., 2011). Therefore, cardiac remodeling becomes a risk factor for cardiac complications (3, 32–37).

Previous studies demonstrated that atherosclerosis can promote cardiac hypertrophy in both ApoE^{-/-} and LDLr^{-/-} atherosclerosis models (Mishra et al., 2015; Seto, Krishna et al., 2014; Silva et al., 2015; Viana Gonçalves et al., 2017). However, LDL-receptor gene deletion by itself does not induce cardiac hypertrophy in the absence of an atherogenic diet (Garcia et al., 2008). Interestingly, we observed cardiac hypertrophy and collagen deposition in LDLr^{-/-} mice on a standard, non-atherogenic diet. This effect could, therefore, be attributed to the action of stanozolol.

It has been reported that cardiac remodeling impairs the contractile capacity of the ventricle, what can result in heart failure (Almeida et al., 2018; Bozi et al., 2013; Gardner et al., 2002; Smith et al., 2000). Cardiac dysfunction is compatible with changes in ventricular function parameters such as +dP/dt_{max} and Tau (Almeida et al., 2018; Davis et al., 1999; Nascimento et al., 2016). The first derivative of +dP/dt_{max} is largely affected by cardiac contractility (Gleason & Braunwald, 1962; Mattiazzi et al., 1986; Melo Junior et al., 2018), and Tau is an isovolumetric relaxation time constant, being a strong indicator of heart failure (Leite-Moreira & Gillebert, 1994; Nascimento et al., 2016; Norton et al., 2000). In the present study, the values of both variables were decreased.

Some studies have shown ventricular contractile alteration after treatment with AAS. Norton et al., (Norton et al., 2000) suggested that chronic intake of AAS at high doses decreases the myocardial contractile reserve to beta-adrenoceptor stimulation. In Wistar rats treated with nandrolone for four weeks, both cardiac contractility and relaxation were enhanced, as revealed by increased +dP/dt_{max} values and reduced Tau values (Nascimento et al., 2016). On the other hand, in male spontaneously hypertensive rats (SHR) both +dP/dt_{max} and -dP/dt_{min} values were increased by the activation of renin angiotensin system components (ACE, AT1R), followed by an impairment of intracellular Ca²⁺-handling proteins (Melo Junior et al., 2018). Altogether, these data suggest AAS can induce cardiac dysfunction by multiple mechanisms, with cardiac function parameters being affected in different ways, depending on the animal model, type of AAS used, and evaluation period (Marques-neto et al., 2014; Mattiazzi et al., 1986; Nascimento et al., 2016). In fact, LDLr^{-/-} mice treated with high doses of stanozolol (20 mg/kg) for

eight weeks had increased lipid deposition and systemic inflammation (Tadeu Uggere de Andrade et al., 2019), which may have contributed to the onset of heart failure in these animals, differently from other studies.

The relation between steroids and the autonomic nervous system is well established in the literature (Beutel et al., 2005; Chaves et al., 2006; Marques-neto et al., 2014). Steroids can modulate baroreflex responsiveness by enhancing cardiac vagal efferent activity (Tadeu U Andrade et al., 2008; El-Mas, 2002; El-Mas, Afify, Mohy El-Din et al., 2001). Besides, it has been shown that vagal stimulation decreases $+dP/dt_{max}$ and $-dP/dt_{min}$, impairing ventricular contraction and relaxation (R. J. Henning, et al., 1989; Henning et al., 1990; Robert J Henning & Levy, 1991). Our results are in line with the aforementioned data, as we observed a reduction in both $+dP/dt_{max}$ and Tau consistent with and enhancement of parasympathetic activity. Taken together, these results suggest that stanozolol causes heart dysfunction probably by increasing the parasympathetic tonus and decreasing $+dP/dt_{max}$ and Tau. This outcome can lead to further heart diseases, mainly in subjects with predisposition to develop atherosclerosis. In this respect, the influence of stanozolol-induced cardiac remodeling and dysfunction in the metabolism of calcium is little known, reaffirming the importance of our effort to better understand the effects of this drug on the heart.

Changes in intracellular Ca^{2+} levels are crucial for normal heart muscle contraction and relaxation, and decreases in the sarcoplasmic reticulum (SR) Ca^{2+} content can lead to heart failure and contribute to the pathophysiology and progression of this disease (Kho et al., 2012). PLB is a key regulator of sarcolemmal Ca^{2+} uptake in cardiomyocytes, acting as a major inhibitor of SERCA activity. Phosphorylation of PBL (p-PLB) can decrease SERCA inhibition, consequently increasing calcium reuptake into the SR (Chagwon et al., 2013).

We showed that the treatment of $LDLr^{-/-}$ animals with stanozolol reduced the p-PLB/PLB ratio and altered hemodynamic parameters, what could be explained by the decreased phosphorylation of PLB. Previous studies have shown that hypo phosphorylation of PLB is a common molecular feature in failing hearts (Huang et al., 1999; Larsen et al., 2006), and that diastolic alteration in heart failure is most probably a result of reduced Ca^{2+} uptake or storage by the SR (Davies et al., 1996). Evidence shows that acetylcholine may inhibit the functional effects of beta-adrenergic stimulation in part by inhibiting PLB phosphorylation, by both inhibiting the activation of adenylate cyclase and stimulating dephosphorylation (Robert J Henning & Levy, 1991; Lindemann & Watanabe, 1985). Therefore, these data confirm our previous hypothesis that stanozolol-treated mice have increased cardiac parasympathetic activity and reduced ventricular contractile activity. To the best of our knowledge, this is the first study that investigates the molecular effects of stanozolol in ventricular contractility in hypercholesterolemic animals.

5. Conclusion

In conclusion, our data showed that chronic treatment with stanozolol (8 weeks) induced left ventricle dysfunction in $LDLr^{-/-}$ mice. That could be explained, at least in part, by a decrease in p-PLB/PLB ratio, which, combined with the increase in collagen deposition and cardiac hypertrophy, led to cardiac remodeling even in the absence of a western-type diet. Therefore, we suggest that individuals predisposed to the development of atherosclerosis who use stanozolol at high doses will be at increased risk of developing heart disease.

The deleterious effects observed in this study provided new data suggesting stanozolol have increased cardiac parasympathetic activity and reduced ventricular contractile activity. Certainly, more studies are necessary to clarify the cardiovascular other functional and molecular mechanisms of stanozolol, including the involvement of vagal tonus and the participation of other proteins regulating intracellular Ca^{2+} as isoforms of sarcoplasmic/endoplasmic reticulum calcium ATPase 2 expression (SERCA2a) and expression of Na^{+}/Ca^{2+} exchangers (NCX) in atherosclerosis.

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Conflicts of Interest

The authors of this manuscript declare no conflicts of interest.

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