Journal Pre-proof

Myo–inositol and D–chiro–inositol oral supplementation ameliorate cardiac dysfunction and remodeling in a mouse model of diet-induced obesity

Serena L'Abbate, Giuseppina Nicolini, Francesca Forini, Sabrina Marchetti, Nicole Di Lascio, Francesco Faita, Claudia Kusmic

PII: S1043-6618(20)31355-4

DOI: https://doi.org/10.1016/j.phrs.2020.105047

Reference: YPHRS 105047

To appear in: *Pharmacological Research*

Received Date: 30 April 2020

Revised Date: 13 June 2020

Accepted Date: 21 June 2020

Please cite this article as: L'Abbate S, Nicolini G, Forini F, Marchetti S, Di Lascio N, Faita F, Kusmic C, Myo–inositol and D–chiro–inositol oral supplementation ameliorate cardiac dysfunction and remodeling in a mouse model of diet-induced obesity, *Pharmacological Research* (2020), doi: https://doi.org/10.1016/j.phrs.2020.105047

Myo–inositol and D–chiro–inositol oral supplementation ameliorate cardiac dysfunction and remodeling in a mouse model of diet-induced obesity

Serena L'Abbate, Giuseppina Nicolini, Francesca Forini, Sabrina Marchetti, Nicole Di Lascio, Francesco Faita, Claudia Kusmic

Istituto di Fisiologia Clinica – Consiglio Nazionale delle Ricerche (CNR), Pisa, Italia

Corresponding author:

Claudia Kusmic

Istituto di Fisiologia Clinica – Consiglio Nazionale delle Ricerche (CNR)

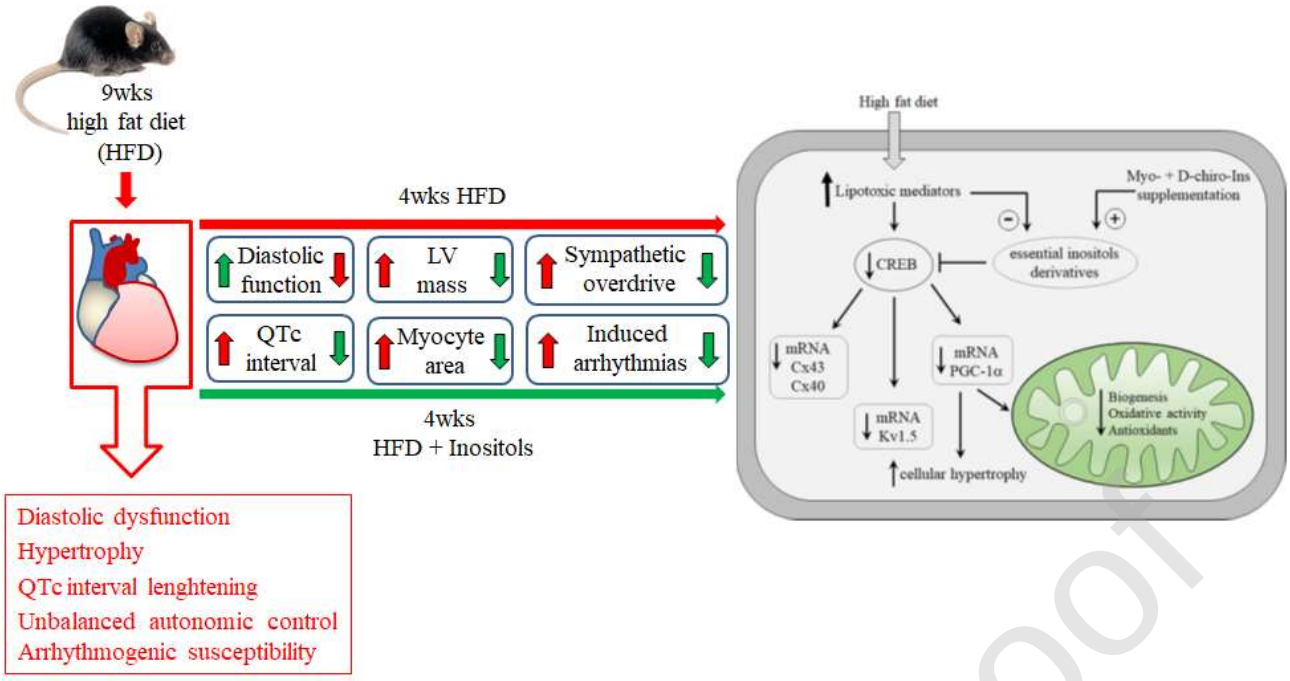
Via Moruzzi 1, 56124 Pisa, Italy

Phone: +39-050-3152669 Fax: +39-050-3152166

Email: kusmic@ifc.cnr.it

ORCID: 0000-0002-8064-8311

Graphical abstract



Abstract

Obesity is an independent risk factor to develop cardiac functional and structural impairments. Here, we investigated the effects of supplementation of inositols on the electrical, structural, and functional cardiac alterations in the mouse model of high fat diet (HFD) induced obesity. Three groups of C57BL6 mice (n=16 each) were studied: j) HFD feeding; jj) HFD feeding + inositols from week 9 to 13; jjj) standard diet feeding. Study observation period was 13 weeks. Inositols were administered as mixture of myo-inositol and D-chiro-inositol in the drinking water. Effects of inositols were evaluated based on electrical, structural, and functional cardiac features, autonomic sympatho–vagal balance and arrhythmogenic susceptibility to adrenergic challenge. Heart samples were collected for histological evaluations and transcriptional analyses of genes involved in defining the shape and propagation of the action potential, fatty acid metabolism and oxidative stress. Inositol supplementation significantly restored control values of heart rate and QTc interval on ECG and of sympatho–vagal balance. Moreover, it blunted the increase in left ventricular mass and cardiomyocyte hypertrophy, reversed diastolic dysfunction, reduced the susceptibility to arrhythmic events and restored the expression level of cardiac genes altered by HFD.

The present study shows, for the first time, how a short period of supplementation with inositols is able to ameliorate the HFD-induced electrical, structural and functional heart alterations including ventricular remodeling. Results provide a new insight into the cardioprotective effect of inositols, which could pave the way for a novel therapeutic approach to the treatment of HFD obesity–induced heart dysfunction.

Keywords: Ventricular remodeling, diastolic function, high fat diet, obesity mouse model.

Introduction

The negative effect of obesity on health has been documented by large epidemiological studies in terms of increased cardiovascular risk, even taking into account covariates such as diabetes, smoking and hypertension [1–2]. Over the last decades several studies on humans and animals focused on the characterization of the myocardial alterations in obesity that include hypertrophic remodeling, diastolic impairment and abnormal cardiac electrical activity [1, 3–8]. Despite several pathophysiological mechanisms may be involved in the development of these changes (e.g hypertension, endothelial dysfunction, ectopic fat accumulation), strong evidence suggests that lipid overload in cardiomyocytes plays a master role in causing cell dysfunction and structural derangement, a process known as lipotoxicity [9–10]. To date, the overall approach in preventing and controlling cardiovascular risk in obese subjects is mainly targeted to constrain body weight and obesity-associated metabolic disorders, including glucose intolerance, type 2 diabetes and dyslipidemia. To this purpose, behavioral risk factors and dietary interventions represent the first line strategies for the primary prevention of cardiac diseases in obese subjects [11]. Despite the development of anti-obesity effective pharmacologic agents [12], at present several studies reported serious adverse effects and conflicting evidence on their putative impact on cardiovascular risk factors that could limit their use [13–16]. For this reason, in both clinical and experimental studies, increasing attention has been paid to the beneficial effects deriving from supplementation with bioactive compounds and phytochemicals in the reduction of cardiometabolic risk, including metabolic syndrome and obesity. Recently, a few studies suggested the therapeutic potential in the treatment of cognitive, metabolic and endocrine disorders of naturally occurring compounds within the inositol family [17–19]. Inositols, also known as vitamin B8, are carbocyclic polyols with six–carbon ring structure naturally occurring in a number of biologically active stereoisomers, of which myo–inositol (myo–Ins) is the most common [20]. It is endogenously synthetized by human body (4 gr/die) and in small amounts provided by diets rich in vegetables, whole grains, nuts and legumes (1 gr/die) [21]. Myo–Ins is a component of membrane phospholipids and its phosphorylated derivatives act as second messengers in signal transduction pathways [22], mediate protein phosphorylation [23], participate in chromatin remodeling and gene expression [24–25], and facilitate mRNA export from the nucleus [26]. Two isomers, namely myo–Ins and D-chiro- inositol (D–chiro–Ins), have been shown to possess insulin-mimetic properties and to be efficient in lowering post-prandial blood glucose, supporting a role of inositol or its derivatives in glucose metabolism [27]. As myo-Ins can be obtained either through endogenous biosynthesis and from diet, an inositol deficiency is unlikely to happen. However, alterations in myo-Ins biosynthesis or extreme dietary conditions may lead to inositol shortage. Abnormalities in inositol metabolism have been implicated in the development of several diseases, including diabetic complications

[17, 19]. Moreover, a dietary myo–Ins (or its derivatives) deficiency causes high accumulation of triacylglycerol, cholesterol, and non-esterified lipids in the mammalian liver [28–30]. It is also noteworthy that mice fed with high fat diet show depletion of intracellular myo-inositol in some tissues, in particular those susceptible of developing diabetes complications [31]. By combining these evidences, it emerges the interest towards the therapeutic potential of myo–Ins and its derivatives also in many lifestyle diseases like obesity and diabetes. However, to the best of our knowledge, a study to investigate whether inositol supplementation is able to ameliorate obesity-associated cardiac alterations and the putative cellular pathways involved in such activity is still lacking.

In light of these considerations, the aim of the present study was to evaluate the effect of inositol administration in a mouse model of diet-induced obesity previously shown to reproduce significant structural and electro-mechanical cardiac changes [8]. In particular, C56BL6 mice fed with HFD, in the absence or presence of inositol supplementation, were characterized in terms of cardiac morphology and function, electrical activity, arrhythmogenic susceptibility, and autonomic control of heart rate, in the metabolic context of glucose tolerance (glucose test) and liver fat content (liver ultrasounds). In addition, at sacrifice, ventricular samples were collected for morphometric assessment of cardiomyocyte cross sectional area and analysis of transcriptional level of master key genes potentially involved in the HFD induced cardiac alterations.

Material and Methods

Animals and study design

The study was approved by the Local Ethical Panel (Prot. n° 397/2018-PR) and conforms to principles of laboratory animal care demanded by European Directive (2010/63/UE) and Italian laws (D.Lgs. 26/2014) on the protection of animals used for scientific purposes. Details on sample size calculation are provided in Supplementary Information S1. Forty–eight C57BL/6 mice (sex ratio 1:1, 7–9 week–old) were purchased from Charles Rivers Laboratories (Milano, Italy). See Supplementary Information S2 for full details on animal housing conditions and timeline of the study design. Briefly, 32 animals were fed a high-fat diet (HFD, PF4051/D, 60% of energy from animal fat; Mucedola srl, Settimo Milanese, Italy) for 9 weeks as previously described [8]. After confirming diastolic cardiac dysfunction by echocardiography, mice were divided into 2 groups and fed the HFD for additional 4 weeks in the absence (HFD group) or presence of supplementation with inositols (HFD+Ins group) for a total of 13 weeks. Allocation into the groups were performed using stratified randomization by sex. A third group was composed by age- and sex–matched control mice fed with normal chow for 13 weeks (C group). Mice were examined for cardiac and metabolic characterization at

baseline, after 9 weeks and again at 13 weeks of diet (endpoint) with non–invasive techniques: high-frequency ultrasound imaging, surface ECG recordings, glucose tolerance test. Body weight was assessed prior to each investigation. At the endpoint, mice were challenged with i.p. isoprenaline, a β_1-adrenoreceptor agonist, to test the susceptibility to arrhythmias.

At the end of the study, mice were anesthetized with isoflurane (1.5% in pure oxygen) and blood was immediately collected by cardiac puncture. After an overdose of isoflurane, hearts were harvested and washed in ice-cold saline solution. Then, each heart was dissected to obtain two cups of the ventricles through a short–axis midplane cut. Half ventricular tissue containing the base was fixed in formalin for histopathological analyses. The remaining cardiac sample was frozen and stored at -80 °C.

Inositol administration

Myo-inositol (lot # 171372) and D – Chiro-inositol (lot # 1946140) were supplied by Lo.Li Pharma Srl (Rome, Italy). A mixture of myo-inositol and D-Chiro-inositol, ratio 40:1, was freshly dissolved in distilled water and delivered to mice at the daily average dose of 0.8 and 0.02 mg/g of body weight, respectively. Doses were chosen to mimic the effect of the maximal dose tested and tolerated in human clinical trials [32] and converted to doses to mice using the Regan–Shaw calculation. The solution was administered as drinking water and changed 3 times a week. The actual estimate of ingested inositols was assessed by careful monitoring of the ingested water.

High frequency–ultrasound examinations

All the animals underwent examination with a high-resolution US imaging system (Vevo 3100, FUJIFILM VisualSonics Inc, Toronto, Canada). See paragraph S3 of Supplementary Information for more details on examination conditions and parameters analysed. Briefly, mice were anaesthetized with isoflurane by inhalation (1.5% v/v, after initial induction at 3% v/v in pure oxygen, at 1 L/min flow rate), and imaged in the supine position while placed on a temperature-controlled board. Heart rate was monitored via ECG electrodes built into the platform and temperature via a rectal probe using the Physiological Monitoring Unit provided with the imaging station. Regarding cardiac assessment, images were acquired using B-mode modality with the 40 MHz probe (FUJIFILM VisualSonics Inc, Toronto, Canada) in parasternal long axis and short axis views, and transmitral left ventricle (LV) inflow velocities by means of pulse–waved (PW) Doppler. All images were analyzed offline using the LV Trace software (MX 550S, FUJIFILM VisualSonics Inc, Toronto, Canada).

Concerning the evaluation of liver fat content, images from a sagittal projection showing the liver and the right kidney simultaneously were acquired using B-mode modality to calculate the sonographic hepato-renal ratio (steatoscore) as index of hepatic steatosis as previously described [8, 33].

Electrocardiographic recordings and analysis

Surface heart's electrical activity was recorded through standard lead configuration (i.e. type I, II, III, aVR, aVL, aVF) using needle electrodes inserted subcutaneously into the limbs of sedated mice (1% isoflurane) as previously described [8]. Measurements included heart rate (HR), P and QRS wave duration, PR interval, QRS duration, rate corrected QT interval (QTc) and the rate corrected JT interval (JTc), index of ventricular repolarization. For QTc and JTc calculation the correction equation recommended by Mitchell [34] was used, which is based on the Bazett formula and adapted for mice: $QTc = QT/(RR/100)^{1/2}$

To investigate the sympathovagal balance we assessed heart rate variability (HRV) as previously described [31]: the beat-to-beat interval variation in 2–min segment of ECG recording was analyzed by the HRV LabChart module (ADInstruments Ltd.). See paragraph S4 of Supplementary Information for more details.

Arrhythmogenic response to β-adrenergic challenge

The arrhythmic response to β-adrenergic stimulation was tested in sedated mice (1% isoflurane in pure oxygen) by isoproterenol challenge (isoprenaline hydrochloride, Monico S.p.A., Venezia, Italy) through intraperitoneal bolus administration at a dose of 2 mg/kg of body weight.

ECG was continuously recorded in a time frame of 15 min, with baseline ECG recordings initiated at 5 min before the isoproterenol injection. See Supplementary Information S5 for more details on arrhythmias' classification.

Glucose tolerance test and plasma lipid measurements

To assess systemic responsiveness to glucose loading, intraperitoneal glucose tolerance test (GTT) were performed on awake mice fasted for 6 h. See Supplementary Information S6 for more details.

Total cholesterol and triglycerides determinations were obtained on whole blood collected by tail vein puncture (reflectometric suction strips, MultiCare IN, BSI Diagnostic, Arezzo, Italy) on awake mice fasted for 6 h.

Histological analysis studies

Mouse hearts were fixed in buffered 10% formalin and paraffin-embedded. All sections (5 μm thick) were taken from the longitudinal midpoint of the LV, at the level of the papillary muscles, and stained with hematoxylin-eosin for blinded determination of cardiomyocyte cross-sectional area (CSA) and myocardial collagen deposition. Further details are reported in Supplementary Information S7.

Quantitative Real-time Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was extracted from homogenized ventricles using the miRNeasy Mini Kit Reagent (Qiagen s.r.l, Milano, Italy) according to the manufacturer's instructions. Total RNA integrity was detected by standardized gel electrophoresis in 1.5% Agarose (Bio-Rad, Milan, Italy). Aliquot of 1 μg RNA was run with reverse transcriptase by using the QuantiTect Reverse Transcription Kit (Qiagen). The amplification reaction was performed in triplicate in a Rotor-Gene Q real-time machine (Qiagen) using 10 ng cDNA and the Quantifast SYBR Green Mix (Qiagen). Expression quantification was performed for the following genes: Nav1.5, Kv1.5, Kv1.4, Cx40, Cx43, Creb1, Cpt2, Pgc–1α, Sod2. Genes were selected on the basis of their pivotal role in defining the shape and propagation of the action potential, regulation of cellular metabolism and mitochondrial function. PCR conditions and primers' sequence are reported in paragraph S8 of Supplementary Information (Table S1). Gene transcript values were normalized with those obtained from the amplification of hypoxanthine phosphoribosyltransferase (Hprt) and hydroxymethylbilane synthase (Hmbs).

Statistical analysis

Data were analyzed with SPSS Version 23 (IBM, New York, NY, USA). All data were tested for normality and presented as mean ± standard deviation (SD). Inter-group differences were examined by one-way ANOVA, followed by Bonferroni's post hoc test. The effect of time per treatment was evaluated by ANOVA for repeated measurements and intra-group longitudinal variations by means of Student's t-test for paired samples. Chi–square test was run to compare the incidence of global arrhythmic events under isoproterenol challenge among groups. Tests were considered statistically significant when $p < 0.05$.

Results

Effect of 9 weeks of HFD regimen

The outcome of 9–week HFD feeding on body weight, metabolic features and cardiac performance of mice is reported in the paragraph S9 of Supplemental Information (Table S2). Briefly, 9 weeks of HFD increased body weigh as

compared with the standard diet (30 ± 5.8 g vs 27 ± 3.4 g); increase, however, did not reach the significance level (p=0.07). Tolerance to intraperitoneal glucose load, as expressed by the area under the glucose tolerance curve (28 ± 6.2 (mg/dl*min)*10^3 in HFD vs 20 ± 2.9 (mg/dl*min)*10^3 in C mice), but not fasting glycemia, (146 ± 29 mg/dl in HFD vs 141 ± 26 mg/dl in C mice) was significantly impaired ($p \leq 0.001$ vs standard diet). Likewise, mice fed with HFD showed a significant increase in the hepatic fat content compared with C group (0.72 ± 0.1 a.u. vs 0.64 ± 0.1 a.u., $p<0.03$ vs standard diet).

As cardiac function is concerned, 9–week period of HFD produced a diastolic impairment, expressed as an increase of both E/A ratio and time of early ventricular filling (Dt), without any sign of systolic dysfunction. Conversely, the time of LV repolarization on ECG traces was significantly lengthened as marked by the increase in the QTc interval (~ 12% compared to the standard diet) and JTc (~ 30% compared to the standard diet). Moreover, the FFT spectral analysis of heart rate revealed the HFD consumption decreased HRV by increasing normalized LFr power and reducing normalized HFr with respect to standard diet ($p<0.001$), thus reflecting unbalanced autonomic control with sympathetic overdrive in the HFD–Group as expressed by the elevated LFr/HFr ratio ($p<0.001$ vs standard diet).

Effect of inositol on body weight, plasma lipids, glucose metabolism and liver steatosis

All the parameters featuring the general and metabolic characteristics of the three groups at the endpoint of 13 weeks are reported in Table 1. Compared with controls, mice on HFD, both in the absence or presence of inositol supplementation, gained more body weight. At the endpoint, mice fed HFD showed a weight increase of 26% and 21% compared to the standard diet group, in the absence or presence of supplementation with inositols (p=0.039 of both vs standard diet). There was no difference in the weight gained over the 4–week inositol intake between HFD and HFD+Ins groups (11.9 ± 6.4 g and 11.3 ± 6.5 g, respectively; p= ns). In 6–hour fasting animals, the blood glucose was similar in the three groups (138 ±19 mg/dl in C–Group, 149 ± 39 mg/dl in HFD group and 148 ± 37 mg/dl in HFD+Ins Group, p=ns) and compatible with values reported as the physiological range for mice matched for strain (100-160 mg/dl). Alike, the circulating levels of total cholesterol did not differ among the three groups. In contrast, the hyperlipidic diet caused an increase in the levels of free triglycerides ($p <0.005$ HFD vs C group). The administration of inositols, although not significantly, reduced the HFD–induced rise of circulating levels of triglycerides by about 15% ($p <0.05$ vs standard diet, p = ns vs HFD).

On the other hand, the response to an intraperitoneal glucose load showed impaired glucose homeostasis in mice under fat diet regimen compared to mice fed with the standard diet (p=0.005 HFD group vs C), with a trend to a mild recover with inositol administration (~ 6.5% vs HFD, p=ns, and $p<0.02$ vs C group).

As far as the accumulation of liver fat is concerned, 13 weeks of HFD further worsened steatoscore index with respect to 9 weeks, with an increase of about 30% compared to the control group ($p<0.001$). The administration of inositols in the last 4 weeks effectively counteracted further accumulation of hepatic fat ($p=0.001$ vs HFD, and p=ns vs C group). The longitudinal analysis, in fact, indicates that while the steatoscore increased significantly between 9 and 13 weeks in the HFD group (0.72 ± 0.1 and 0.87 ± 0.1, respectively; $p=0.002$), its value remained constant in the HFD+Ins group (0.72 ± 0.1 and 0.72 ± 0.2, respectively: p=ns).

Inositols substantially prevented cardiac alterations induced by high fat diet

Ultrasound imaging analysis. As previously reported [8], 13 weeks of HFD regimen have no obvious effect on systolic function (Table 2). On the contrary, diastolic function was significantly impaired compared to C group, as expressed by the alteration of almost all the distinctive parameters, some of which resulted affected already at 9 weeks of HFD (cfr. with Table S1 in the Supplementary). At the endpoint, compared to control mice, the time of early ventricular filling (Dt, $p=0.005$), the total time of mitral flow (MVt, $p=0.005$), and the IVRT ($p<0.05$) were longer and the E/A ratio ($p<0.005$) was higher in HFD mice than in C group (Table 2 and Figure 1a). The administration of inositols in the last 4 weeks of HFD was effective in preventing the worsening of diastolic dysfunction. The values measured were significantly lower in all the parameters with respect to HFD mice, except for IVRT. Moreover, all the assessments were not different from those in the control group (Table 2).

The comparison of the morphometric measurements obtained from B–mode images of HFD group highlighted a significant increase in the LV mass ($p<0.05$) and the diastolic thickness of both the interventricular septum (IVSTd) and the LV posterior wall (LVPWTd) ($p<0.05$ for both) with respect to control mice (Figure 1 b–d). Even in this case, the administration of inositols significantly prevented the increase of the above parameters ($p\leq0.001$ and $p< 0.05$ vs HFD group for LV mass and both wall thicknesses; p=ns vs C group for all).

ECG analysis. The main data measured at the endpoint are reported in Table 3. The ANOVA analysis revealed that 13 weeks of HFD induced a significant decrease of the R–R interval compared to standard diet ($p<0.05$ vs C group), whose values corresponded to HR of 543 ± 74 bpm and 481 ± 48 bpm, respectively. Moreover, the marked lengthening of total electrical ventricular activity (QTc) and time of repolarization (JTc), already overt at 9 weeks of HF diet, was further worsened at 13 weeks in HFD–group, with QTc and JTc interval values higher of about 20% and 39% with respect of C group ($p<0.001$ for both). Inositol supplementation in the last four weeks was able to prevent HR increase (494 ± 86 bpm, p=ns vs C group) as well as to maintain interval values within the control range (57.2 ± 3.0 ms for QTc and 32.8 ± 5.0 ms for JTc; $p<0.001$ vs HFD group and p= ns vs C group for both).

The longitudinal analysis within the HFD group pointed out a significant increase, according to the time of diet exposure, of HR, QTc and JTc ($p=0.023$, $p=0.027$ and $p=0.037$, respectively for 9 vs 13 weeks). Conversely, the same longitudinal analysis within the HFD+Ins group revealed a significant reduction of the interval values associated with the 4–week period of inositol administration of both QTc and JTc ($p<0.001$ and $p=0.002$, respectively for 9 vs 13 weeks).

The heart rate variability analysis indicated that HFD regimen shifted the sympathovagal balance in favor of the sympathetic system, as indexed by both the altered values of normalized LFr and HFr, and the increased LFr/HFr ratio compared to C group (Table 3).

The intake of inositols had a significant effect in counteracting the activation of the sympathetic branch of the autonomic nervous system induced by HFD and in restoring a balanced autonomic control of the heart rate.

Inositols dampened the increased susceptibility to arrhythmias under adrenergic stimulation

In all groups the administration of i.p. isoproterenol caused a similar sudden increase in HR that reached values above 700 bpm in the first minutes while progressively lowered to values around 650–660 bpm over 10 min. A few minutes following the administration of isopreterenol, various types of arrhythmic events were recorded in some mice within each group (Table 3 and Figure S1 in paragraph S5 of Supplementary Information). Sinus arrhythmias and sporadic AV block events (type Mobitz 2) were recorded in all experimental groups. However, atrio-ventricular dissociation events were recorded exclusively in the HFD group, and a higher number of subjects (50%) showed atrial fibrillation and/or flutter compared to C group (6%) and HFD+Ins group (6%). Chi–square test run to compare the incidence of global arrhythmic events showed a significant difference between HFD and C groups ($\chi 2$ 14.6, df=1, $p<0.001$) and between HFD and HFD+Ins groups ($\chi 2$ 4.6, df=1, $p<0.03$). No difference was found between C and HFD+Ins groups ($\chi 2$ 3.5, df=1, p=ns).

Inositols prevented HFD induced myocardial cellular hypertrophy

To relate the increased LV mass and wall thickness to myocardial hypertrophy under HFD regimen, the measure of the LV cardiomyocyte's cross sectional area (CSA) was performed in the three groups (Figure 2). The average value of CSA of mice fed with a standard diet was 248 ± 53 μm^2 and it was significantly increased by about 30% in HFD group (326 ± 57 μm^2, $p<0.001$). The myocyte area of HFD+Ins group was significantly reduced compared to the HFD mice (258 ± 46 μm^2, $p<0.001$ vs HFD) and did not differ from that of standard diet animals.

Expression of connexins and selected ion channels was differentially regulated by HFD and restored by inositol administration

The transcriptional levels of ion channels relevant to cardiomyocyte action potential (AP) and gap junction proteins were investigated in the LV tissue of the three groups to verify their putative role in the HFD–induced changes in electrical activity and diastolic function. Namely, we evaluated the mRNA expression levels of subunits Kv1.4 and Kv1.5 of potassium channels (responsible for the repolarizing currents involved both in the early and late phases of AP), Nav1.5 of sodium channel (responsible for the depolarizing current of AP) and of the connexin 40 (Cx40) and 43 (Cx43). Figure 3a shows the expression levels of genes expressed as fold change with respect to C group. After 13 weeks of HFD mice showed a significant increase in the mRNA levels of Nav1.5, approximately 40% ($p<0.001$ vs C group), paralleled by 10% decrease in the transcriptional level of Kv1.5 which approached but did not reach the significance level ($p=0.07$) compared to C group. The mRNA levels of Kv1.4 were not significantly affected by HFD. On the contrary, a marked reduction in both Cx40 and Cx43 expression levels ($p<0.001$ vs C group) was observed compared to controls. Overall, these results point in favor of the lengthening of myocyte AP and of a less effective conduction of the cellular excitation, in line with the alterations noted on the ECG recordings. The administration of inositols in the last 4 weeks of HFD was effective in dampening the increase of Nav1.5 mRNA ($p<0.05$ vs HFD diet) keeping values close to those of control mice (p=ns). In addition, inositol supplementation increased the expression of Kv1.5 with respect to HFD group but not C ($p<0.05$ vs HFD group; p=ns vs C group), and blunted the reduction in the expression of Cx40 and Cx43 ($p<0.001$ for both vs HFD group; p=ns vs C group). These results are consistent with the positive effect of inositols shown on the HFD–induced alterations of ECG trace.

Expression of key regulatory genes for cellular and mitochondrial function were negatively controlled in HFD regimen and restored by inositol administration

To address the hypothesis that cardiac hypertrophy and diastolic dysfunction of HFD mice were secondary to increased cardiomyocyte uptake of circulating fatty acids (FAs), we assessed the transcriptional levels of some key genes involved in the regulation of mitochondrial function, long chain FA handling and antioxidant defence. We tested the transcriptional levels of the master regulators of mitochondrial metabolism and function as peroxisome proliferator-activated receptor gamma coactivator-1α (Pgc–1α), carnitine palmitoyltransferase 2 (Cpt2), and superoxide dismutase 2 (Sod2). Moreover, to investigate the putative underpinning mechanism we tested whether high fat diet affected the expression level of cAMP response element-binding protein 1 (Creb1), a cellular key transcription factor that regulates, among the others, the transcription of Pgc-1α gene.

Figure 3b summarizes the main evidences in mRNA expression levels expressed as fold change with respect to control mice. HFD group showed a significant increase in Cpt2 expression ($p=0.001$ vs C group), possibly induced by the excess of substrate (dietary FAs). This was accompanied by a significant reduction in Pgc–1α and its transcriptional factor Creb1 ($p<0.05$ vs C group for both) and a light, but not significant, decrease in Sod2 expression ($p=0.08$). In addition, inositol administration, on the one hand, had no effect on the HFD–induced increase of Cpt2 mRNA levels ($p<0.001$ vs C group, p=ns vs HFD group), on the other hand preserved the physiological transcriptional profile of Creb1, Pgc–1α and Sod2 ($p< 0.001$ vs HFD group for Pgc–1α, and $p<0.05$ vs HFD group for the other genes, p=ns vs C group for all).

Discussion

In the present study, the supplementation of myo-Ins and D–chiro–Ins was evaluated for its putative effects on cardiac dysfunction developed by high-fat-diet-induced obesity in mice. To this end, a model of C57BL6 mouse fed with HFD diet for 13 weeks was used in which we previously reported cardiac structural and electro–mechanical alterations even in the absence of overt diabetes mellitus, but with impaired glucose tolerance and insulin resistance [8]. The cardiac dysfunction developed by such a diet-induced obesity model recapitulates the main features of the metabolic heart disease observed in patients with obesity and diabetes [35–36]. It is characterized by diastolic electro–mechanical impairment, cellular hypertrophy and greater susceptibility to develop arrhythmias under adrenergic stimulation in a framework of disturbed glucose and lipid homeostasis [8]. In the present study, we report for the first time that a mixture of myo–Ins and D–chiro–Ins orally administered for 4 weeks during hyperlipidic dietary regimen can prevent or dampen all the main observed myocardial alterations in HFD fed mice, despite only a moderate effect on their systemic metabolic ailments (plasma lipids and glucose tolerance). Hence, the emerging picture suggests that such an overall positive effect of inositol supplementation on cardiac alterations may result mainly from a direct action on the myocardial pathways affected by HFD. Nevertheless, it is plausible that inositol supplementation can also have beneficial effects on other insulin sensitive tissue and organs. Despite the exact mechanisms of action of myo–Ins and other inositol isomers or derivatives still remain uncertain, the great majority of reports proposes that an intake of extra inositols sustains the formation of putative insulin–mimetic mediators that contain inositols as constituents [17, 37–38]. On the other hand, according to Michell [18], it seems more likely that the extra dietary supplement of inositols can correct a tissue deficiency induced by pathophysiological conditions that restricts the production of an abundant and crucial inositol-containing cell component. According to the author, such pivotal cell component is phosphatidylinositol, which is essential to maintain endoplasmic reticulum membrane homeostasis, whose unbalance

plays key role in the pathogenesis of many diseases [39]. In our study, we are not able to ascribe the observed effect to the compounds *per se* or to their derivatives resulting from cellular metabolism.

Myo–Ins and D–chiro–Ins supplementation decreased hepatic fat accumulation

As expected, high fat diet, both in the absence or presence of inositol supplementation, had a negative impact on circulating lipids (especially on triglycerides) and on tolerance to glucose load, but not on fasting glycemia with respect to chow diet. This result is in line with data reported by Croze [31] in a similar animal model fed with HFD or HFD plus myo–Ins supplementation (0.58 mg/g daily). However, conflicting data are present in the literature on this issue in animal models of disturbed glucose homeostasis [40–44]. These heterogeneous results may be ascribed to the different genetic background of the mice strain and/or the experimental conditions used.

Concerning the effect of a lipid enriched diet on the liver, in accordance with our previous observation [8] there was a progressive hepatic fat accumulation in HFD mice, whose steatoscore values became significantly higher with respect to control group. Supplementation with a mixture of myo–Ins and D–chiro–Ins during the HFD feeding was able to protect liver tissue from steatosis. In agreement with our data, Hu et al [45] showed that the intake of a D–chiro–Ins enriched herbal extract alleviated liver damage and steatosis in a mouse model of high fructose–induced metabolic disorder, suggesting that inositols may blunt tissue lipotoxiticy by promoting liver fat metabolism. In addition, to substantiate a key role of myo–Ins and its derivatives in the hepatic triglyceride homeostasis, several studies reported that their deprivation in the diet determined a fatty liver condition in rats [28–30].

Beneficial effect of Myo-Ins and D-chiro-Ins on the HFD-induced cardiac impairment: evidence of a pleiotropic action

In the present study, we confirm the previously reported evidences of cardiac impairment induced by HFD [6–8, 46–47]. Briefly, 13–week period of HFD altered all the outstanding parameters of diastolic function (E/A, Dt, MVt and IVRT) with respect to standard chow. Besides, HFD mice showed an increased heart rate, a marked lengthening of ventricular repolarization (QTc/JTc) and an increased susceptibility to develop supraventricular arrhythmias (sinus arrhythmia, atrial flutter and fibrillation) under adrenergic challenge compared to the control group. Moreover, under HFD regimen, an impaired autonomic modulation of sinoatrial activity with a prevalence of the sympathetic arm was evident at HRV analysis. Possibly allied with the diastolic functional alteration, the fat diet regimen resulted even in an increased mass of the left ventricle with significant thickening of the ventricular walls. As previously observed in this model [8], the structural remodeling was mainly ascribable to cell hypertrophy, in the absence of significant interstitial fibrosis (Figure S2 in paragraph S7 of Supplementary information). Since we previously showed significant

relationships between the variables analysed by a correlation analysis [8], it is conceivable to feature a complex frame of cross–talking outcomes with probably one or a few common underlying mechanisms. The most obvious candidate is cardiac lipotoxicity secondary to a diet induced excess supply of fatty acids, which has been associated with a shift in energy balance, low grade of inflammation and endoplasmic reticulum stress [10, 48]. The administration of myo–Ins and D-chiro–Ins in the last 4 weeks of fat diet, rather surprisingly, resulted effective in restoring within normal intervals all the altered parameters of electrical activity and diastolic function. Even the sympathetic overdrive was reversed leading to a balanced autonomic control of the heart rate. This latter effect, however, is not surprising as we have previously observed on the one hand a linear relationship between autonomic alterations and disturbances of ventricular repolarization (QTc/JTc) [8] and, on the other hand, that inositol supplementation was able to counteract the lengthening of QTc/JTc. Moreover, HFD–induced cardiomyocyte hypertrophy was blunted by inositol supplementation. The pleiotropic nature of the positive effects associated with supplementation of inositols strongly suggests that one essential, but still unidentified, Ins-dependent pathway of regulation, acting on the multiple cardiac functions, operates less than optimally in HFD mice and that an extra supply of inositols is needed to support this function.

Myo–Ins and D–chiro–Ins supplementation mediated regulatory network for cardioprotection

In our study, the HFD-driven transcriptional alterations in the LV are consistent with an imbalance between fatty acid (FA) uptake and oxidation that leads to accumulation of long-chain FAs. The excess of FAs can be incorporated into a multitude of other lipid subspecies, known to be associated with progression of ventricular dysfunction. One of the main finding of our study is that HFD negatively affects the expression levels of Creb1. Pgc-1α gene possesses a binding site for Creb, spanning the region 2146 to 2129 in Pgc-1α promoter in mice, suggesting that its reduced transcription induced by HFD may be secondary to the downregulation of the expression level of Creb1. In the presence of elevated levels of circulating diet–derived FAs, myocardial lipid uptake increases dramatically [49]. Although the increase in transcription levels of Cpt2, observed in HFD mice by us and others [50], may indicate an increase in mitochondrial substrate useful for β oxidation, the concomitant down regulation of Pgc–1α suggests a reduced metabolic turnover of lipid derivatives, an indication of mismatch between substrate uptake and metabolism. Indeed, Pgc–1α coactivator plays a key role in the maintenance of lipid balance via engagement in numerous mitochondrial metabolic processes (i.e., Krebs cycle, β oxidation, oxidative phosphorylation and electron transport chain) [51–54]. Reduced expression level of Pgc–1α was also shown in mouse model of cardiac lipotoxicity due to cardiomyocyte-specific overexpression of the FA transport protein, with development of cardiac hypertrophy and diastolic dysfunction [9] and in transgenic mice with cardiac specific overexpression of the FA-activated nuclear receptor Pparα [55]. Moreover, the reduction of Pgc-1α

expression has been associated with development of cardiac hypertrophy and failure [56–57]. In our HFD mice supplemented with myo–Ins and D–chiro–Ins the transcriptional levels of Creb1 and Pgc–1α were returned to the values of control animals. The optimal levels of expression of Pgc–1α could, on the one hand guarantee an adequate oxidative metabolism of fatty acids and, on the other hand, burst mitochondrial function, as expressed by the higher levels of the antioxidant enzyme Sod2 transcript with respect to HFD group. Indeed, Pgc–1α interacts with a plethora of transcription factors involved in a wide range of biological responses, including mitochondrial biogenesis and function, and cellular hypertrophy [54, 36, 58]. Alike, myo–Ins and D–chiro–Ins supplementation entirely reversed the HFD–induced alteration of expression levels of ion channel subunits (Nav 1.5 and Kv1.5) and gap junction proteins (Cx43 and Cx40), returning the values to the physiological range.

An interesting question to be addressed is whether the HFD–induced transcriptional alterations associated with electrical remodeling have a common mechanism with metabolic ones. Some information in this regard has been provided in a recent study on mice fed with HFD for 18–20 weeks [6], in which the authors demonstrated, as in our study, that the lengthening of QT was accompanied by reduced ventricular expression of channel Kv1.5. In addition, the authors demonstrated that the decreased mRNA level of the channel was caused by the reduction of the transcription factor Creb1 that directly regulates a broad range of genes, and is known to have a role also in the regulation of cellular metabolism [59–60]. Moreover, some studies have confirmed, on the one hand, the presence of Creb-binding sites in the Cx43 gene, and on the other hand, that Creb1 downregulation by siRNA reduced Cx43 expression [61]. Combining this information with our findings, we envision a regulatory pathway mediated by inositol derivatives for cardioprotection from obesity–driven alterations. As illustrated in Figure 4, in cardiomyocyte the HFD–induced lipotoxic stimuli affect inositol metabolism leading to a tissue reduction of myo–Ins and derivatives, and favor the downregulation of Creb1 and the subsequent reduction of transcriptional levels of connexins, Kv1.5 subunit of potassium channel and Pgc–1α. In this scenario, at the mitochondrial level, dowregulation of Pgc–1α reduces organelle biogenesis and function and impairs oxidative activity, exacerbating the production of ROS and noxious lipid metabolites. Myo–Ins and D–chiro–Ins supplementation, by restoring cardiac homeostasis of essential inositols through replacement, may counteract the pernicious cascade of events triggered by lipotoxic mediators, thus exerting a beneficial effect on related cardiac alterations.

One limitation of the present study is that we have tested only some of the paths proposed in Figure 4 and therefore further investigations on some nodal points of whole pathway and further validations are necessary to confirm the validity of such a regulatory network. Furthermore, although supported by data of the literature, in our study gene expression analysis was performed only at mRNA level but not validated at level of protein expression or activity; this

implies that some genes modulated at translational level might be overlooked. Moreover, the hypothesis of the relationship between gene expression of channel subunits and the effect on the ECG tracing needs to be verified by electrophysiological recording of specific cell currents and action potential profiles in cardiomyocytes. Nevertheless, the ideas put forward here pave the way to further exploration of inositol–dependent regulatory mechanisms, previously underestimated, as the basis for the beneficial role of inositols in obesity-induced cardiac disease.

Conclusions

The present study has shown, for the first time, how a short period of supplementation with a mixture of myo–Ins and D–chiro–Ins can reverse the complex pathophysiologic picture of obesity-induced cardiac impairments in mice, with a central role for mitochondrial oxidative function. The data presented here provide a novel insight into the cardioprotective effect of inositols. In this perspective, our results support further research to completely unreveal the inositol–dependent regulatory mechanisms and be helpful in translating inositol supplementation into a therapeutic approach to the treatment of obesity-induced cardiac disease.

Authors' contribution

CK and SLA designed the study and conducted the in vivo experiments. SLA and SM performed the histological analysis. NDL,FFa and CK, performed imaging analysis. GN and FFo performed gene expression experiments. All the authors analyzed data. CK and SLA wrote the draft manuscript. All the authors discussed, read and approved the final version of the manuscript.

Funding. This study was partially supported by LoLi Pharma s.p.a (Rome, Italy) through the research agreement [AA123456] with Consiglio Nazionale delle Ricerche (CNR).

Ethics approval. The study was approved by the Local Ethical Panel (Prot. n° 397/2018-PR), conforms to principles of laboratory animal care demanded by European Directive (2010/63/UE) and Italian laws (D.Lgs. 26/2014) on the protection of animals used for scientific purposes, and comply with the ARRIVE guidelines.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors take complete responsibility for the integrity of the data and the accuracy of the data analysis. Role of the funding source: none.

Declaration of interests

None

Acknowledgements. The authors wish to express their deep appreciation to Prof. Antonio L'Abbate for his helpful criticism and valuable suggestions, and thank Mrs. Cecilia Ciampi and Mrs. Sara Ciampi for their assistance in animal care.

References

1. Wang TJ, Parise H, Levy D, D'Agostino RB Sr, Wolf PA, Vasan RS, Benjamin EJ (2004) Obesity and the risk of new-onset atrial fibrillation. JAMA 292:2471-2477 doi:10.1001/jama.292.20.2471
2. Aune D, Sen A, Norat T, Janszky I, Romundstad P, Tonstad S, Vatten LJ (2016) Body mass index, abdominal fatness, and heart failure incidence and mortality: a systematic review and dose-response meta-analysis of prospective studies. Circulation 133: 639–649 doi:10.1161/CIRCULATIONAHA.115.016801
3. López-Jiménez F, Cortés-Bergoderi M (2011) Obesity and the heart. Rev Esp Cardiol 64:140-149 doi:10.1016/j.recesp.2010.10.010
4. Calligaris SD, Lecanda M, Solis F, Ezquer M, Gutierrez J, Brandan E Leiva A, Sobrevia L, Conget P (2013) Mice long-term high-fat diet feeding recapitulates human cardiovascular alterations: an animal model to study the early phases of diabetic cardiomyopathy. PLoS One 8: e60931 doi:10.1371/journal.pone.0060931
5. Alpert MA, Karthikeyan K, Abdullah O, Ghadban R (2018) Obesity and cardiac remodeling in adults: mechanisms and clinical implications. Prog Cardiovasc Dis 61:114-123 doi:10.1016/j.pcad.2018.07.012
6. Huang H, Amin V, Gurin M, Wan E, Thorp E, Homma S, Morrow JP (2013) Diet-induced obesity causes long QT and reduces transcription of voltage-gated potassium channels. J Mol Cell Cardiol 59:151-158 doi:10.1016/j.yjmcc.2013.03.007
7. Zhang F, Hartnett S, Sample A, Schnack S, Li Y (2016) High fat diet induced alterations of atrial electrical activities in mice. Am J Cardiovasc Dis 6:1-9.
8. L'Abbate S, Di Lascio N, Nicolini G, Forini F, Faita F, Kusmic C (2019) Murine model of left ventricular diastolic dysfunction and electro-mechanical uncoupling following high-fat diet. Int J Obesity doi:10.1038/s41366-019-0500-3
9. Elezaby A, Sverdlov AL, Tu VH, Soni K, Luptak I, Qin F, Liesa M, Shirihai OS, Rimer J, Schaffer JE, Colucci WS, Miller EJ (2015) Mitochondrial remodeling in mice with cardiomyocyte-specific lipid overload. J Mol Cell Cardiol 79:275-283 doi:10.1016/j.yjmcc.2014.12.001
10. Sletten AC, Peterson LR, Schaffer JE (2018) Manifestations and mechanisms of myocardial lipotoxicity in obesity. J Intern Med 284:478-491 doi:10.1111/joim.12728
11. Korakas E, Dimitriadis G, Raptis A, Lambadiari V (2018) Dietary Composition and Cardiovascular Risk: A Mediator or a Bystander? Nutrients 10: E1912 doi:10.3390/nu10121912

12. Andrew CA, Saunders KH, Shukla AP, Aronne LJ (2019) Treating obesity in patients with cardiovascular disease: the pharmacotherapeutic options. Expert Opin Pharmacother 20:585–593 doi:10.1080/14656566.2018.1561867
13. Valsamakis G, Konstantakou P, Mastorakos G (2017) New targets for drug treatment of obesity. Ann Rev Pharmacol Toxicol 57:585-605 doi:10.1146/annurev-pharmtox-010716-104735
14. Crawford AR, Alamuddin N, Amaro A (2018) Cardiometabolic effects of anti-obesity pharmacotherapy. Curr Atheroscler Rep 20:18 doi:10.1007/s11883-018-0719-9
15. Patel DK, Stanford FC (2018) Safety and tolerability of new-generation anti-obesity medications: a narrative review. Postgrad Med 130:173-182 doi:10.1080/00325481.2018.1435129
16. Bramante CT, Raatz S, Bomberg EM, Oberle MM, Ryder JR (2020) Cardiovascular risks and benefits of medications used for weight loss. Front Endocrinol (Lausanne) 10:883 doi:10.3389/fendo.2019.00883
17. Croze ML, Soulage CO (2013) Potential role and therapeutic interests of myo-inositol in metabolic diseases. Biochimie 95:1811–1827 doi:10.1016/j.biochi.2013.05.011
18. Michell RH (2018) Do inositol supplements enhance phosphatidylinositol supply and thus support endoplasmic reticulum function? Br J Nutr 3:1-16 doi:10.1017/S0007114518000946
19. Chhetri DR (2019) Myo-Inositol and its derivatives: their emerging role in the treatment of human diseases. Front Pharmacol 10:1172 doi:10.3389/fphar.2019.01172
20. Majumdar AL, Johnson MD, Henry SA (1997). 1L–myo-Inositol–1- phosphate synthase. Biochim. Biophys. Acta 1348: 245–256 doi:10.1016/ S0005-2760(97)00122-7
21. Goodhart RS (1973) Bioflavonoids. In Goodhart RS, Shils ME (eds) Modern Nutrition in Health and Disease; Dietotherapy, 5th edn. Lea & Febiger: Philadelphia USA, pp 259–267
22. Berridge MJ (2009) Inositol trisphosphate and calcium signalling mechanisms. Biochim Biophys Acta 1793: 933-940 doi:10.1016/j.bbamcr.2008.10.005
23. Saiardi A, Bhandari R, Resnick AC, Snowman AM, Snyder SH (2004) Phosphorylation of proteins by inositol pyrophosphates. Science 306:2101-2105 doi:10.1126/science.1103344
24. Odom AR, Stahlberg A, Wente SR, York JD (2000) A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. Science 287:2026-2029 doi:10.1126/science.287.5460.2026
25. Shen X, Xiao H, Ranallo R, Wu WH, Wu C (2003) Modulation of ATP-dependent chromatin-remodeling complexes by inositol polyphosphates. Science 299:112-114 doi:10.1126/science.1078068

26. York JD, Odom AR, Murphy R, Ives EB, Wente SR (1999) A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science 285:96-100 doi:10.1126/science.285.5424.96

27. Croze ML, Vella RE, Pillon NJ, Soula HA, Hadji L, Guichardant M, Soulage CO (2013) Chronic treatment with myo-inositol reduces white adipose tissue accretion and improves insulin sensitivity in female mice. J Nutr Biochem 24:457-466 doi:10.1016/j.jnutbio.2012.01.008

28. Burton LE, Wells WW (1974) Studies on the developmental pattern of the enzymes converting glucose 6-phosphate to myo-inositol in the rat. Dev Biol 37:35-42 doi:10.1016/0012-1606(74)90167-5

29. Hayashi E, Maeda T, Tomita T (1974a) The effect of myo-inositol deficiency on lipid metabolism in rats. I. The alteration of lipid metabolism in myo-inositol deficient rats. Biochim Biophys Acta 360:134-145 doi:10.1016/0005-2760(74)90163-5

30. Hayashi E, Maeda T, Tomita T (1974b) The effect of myo-inositol deficiency on lipid metabolism in rats. II. The mechanism of triacylglycerol accumulation in the liver of myo-inositol-deficient rats. Biochim Biophys Acta 360:146-155 doi:10.1016/0005-2760(74)90164-7

31. Croze ML, Géloën A, Soulage CO (2015) Abnormalities in myo-inositol metabolism associated with type 2 diabetes in mice fed a high-fat diet: benefits of a dietary myo-inositol supplementation. Br J Nutr 113:1862-1875 doi:10.1017/S000711451500121X

32. Carlomagno G, Unfer V (2011) Inositol safety: clinicalevidences. Eur Rev Med Pharmacol Sci 15: 931 –936

33. Di Lascio N, Kusmic C, Stea F, Lenzarini F, Barsanti C, Leloup A, Faita F (2018) Longitudinal micro-ultrasound assessment of the ob/ob mouse model: evaluation of cardiovascular, renal and hepatic parameters. Int J Obes (Lond) 42: 518-524 doi:10.1038/ijo.2017.219

34. Mitchell GF, Jeron A, Koren G (1998) Measurement of heart rate and Q-T interval in the conscious mouse. Am J Physiol 274:H747-H751 doi:10.1152/ajpheart.1998.274.3.H747

35. Li W, Bai Y, Sun K, Xue H, Wang Y, Song X Fan X, Song H, Han Y, Hui R (2009) Patients with metabolic syndrome have prolonged corrected QT interval (QTc). Clin Cardiol 32: E93-E99 doi:10.1002/clc.20416

36. Ortega FB, Lavie CJ, Blair SN (2016) Obesity and cardiovascular disease. Circ Res 118:1752-1770 doi:10.1161/CIRCRESAHA.115.306883

37. Noventa M, Vitagliano A, Quaranta M, Borgato S, Abdulrahim B, Gizzo S (2016) Preventive and therapeutic role of dietary Inositol supplementation in periconceptional period and during pregnancy: a summary of evidences and future applications. Reprod Sci 23:278-288 doi:10.1177/1933719115594018

38. Monastra G, Unfer V, Harrath AH, Bizzarri M (2017) Combining treatment with myo-inositol and D-chiro-inositol (40:1) is effective in restoring ovary function and metabolic balance in PCOS patients. Gynecol Endocrinol 33:1-9 doi:10.1080/09513590.2016.1247797

39. Lin JH, Walter P, Yen TS (2008) Endoplasmic reticulum stress in disease pathogenesis. Annu Rev Pathol 3:399-425 doi:10.1146/annurev.pathmechdis.3.121806.151434

40. Ortmeyer HK (1996) Dietary myoinositol results in lower urine glucose and in lower postprandial plasma glucose in obese insulin resistant rhesus monkeys. Obes Res 4:569-575 doi:10.1002/j.1550-8528.1996.tb00271.x

41. Dang NT, Mukai R, Yoshida K, Ashida H (2010) D-pinitol and myo-inositol stimulate translocation of glucose transporter 4 in skeletal muscle of C57BL/6 mice. Biosci Biotechnol Biochem 74:1062-1067 doi:10.1271/bbb.90963

42. Yap A, Nishiumi S, Yoshida K, Ashida H (2007) Rat L6 myotubes as an in vitro model system to study GLUT4-dependent glucose uptake stimulated by inositol derivatives. Cytotechnology 55:103–108 doi:10.1007/s10616-007-9107-y

43. Ortmeyer HK, Huang LC, Zhang L, Hansen BC, Larner J (1993) Chiroinositol deficiency and insulin resistance. II. Acute effects of D-chiroinositol administration in streptozotocin-diabetic rats, normal rats given a glucose load, and spontaneously insulin-resistant rhesus monkeys. Endocrinology 132:646-651 doi:10.1210/endo.132.2.8425484

44. Pak Y, Huang LC, Lilley KJ, Larner J (1992) In vivo conversion of [3H]myoinositol to [3H]chiroinositol in rat tissues. J Biol Chem 267:16904–16910

45. Hu Y, Zhao Y, Ren D, Guo J, Luo Y, Yang X (2015) Hypoglycemic and hepatoprotective effects of D-chiro-inositol-enriched tartary buckwheat extract in high fructose-fed mice. Food Funct 6:3760–3769 doi:10.1039/c5fo00612k

46. Bruder-Nascimento T, Ekeledo OJ, Anderson R, Le HB, Belin de Chantemèle EJ (2017) Long term high fat diet treatment: an appropriate approach to study the sex-specificity of the autonomic and cardiovascular responses to obesity in mice. Front Physiol 8:32 doi:10.3389/fphys.2017.00032

47. Nguyen S, Shao D, Tomasi LC, Braun A, de Mattos ABM, Choi YS, Villet O, Roe N, Halterman CR, Tian R, Kolwicz SC, Jr (2017) The effects of fatty acid composition on cardiac hypertrophy and function in mouse models of diet-induced obesity. J Nutr Biochem 46:137-142 doi:10.1016/j.jnutbio.2017.05.009

48. Han J, Kaufman RJ (2016) The role of ER stress in lipid metabolism and lipotoxicity. J Lipid Res 57:1329–1338 doi:10.1194/jlr.R067595

49. Goldberg IJ, Trent CM, Schulze PC (2012) Lipid metabolism and toxicity in the heart. Cell Metab 15:805–812 doi:10.1016/j.cmet.2012.04.006

50. Fujita M, Momose A, Ohtomo T, Nishinosono A, Tanonaka K, Toyoda H, Morikawa M, Yamada J (2011) Upregulation of fatty acyl-CoA thioesterases in the heart and skeletal muscle of rats fed a high-fat diet. Biol Pharm Bull 34:87–91 doi:10.1248/bpb.34.87

51. Ventura-Clapier R, Garnier A, Veksler V (2008) Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. Cardiovasc Res 79:208-217 doi:10.1093/cvr/cvn098

52. Supruniuk E, Mikłosz A, Chabowski A (2017) The implication of PGC-1α on fatty acid transport across plasma and mitochondrial membranes in the insulin sensitive tissues. Front Physiol 8:923 doi:10.3389/fphys.2017.00923

53. Lehman JJ, Boudina S, Banke NH, Sambandam N, Han X, Young DM, Leone TC, Gross RW, Lewandowski ED, Abel ED, Kelly DP (2008) The transcriptional coactivator PGC-1alpha is essential for maximal and efficient cardiac mitochondrial fatty acid oxidation and lipid homeostasis. Am J Physiol Heart Circ Physiol 295:H185-H196 doi:10.1152/ajpheart.00081.2008

54. Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, Kelly DP (2000) Peroxisome proliferator-activated receptor γ coactivator-1 promotes cardiac mitochondrial biogenesis. J Clin Invest 106:847–856 doi:10.1172/JCI10268 141

55. Duncan JG, Bharadwaj KG, Fong JL, Mitra R, Sambandam N, Courtois MR, Lavine KJ, Goldberg IJ, Kelly DP (2010) Rescue of cardiomyopathy in peroxisome proliferator-activated receptor-alpha transgenic mice by deletion of lipoprotein lipase identifies sources of cardiac lipids and peroxisome proliferator-activated receptor-alpha activators. Circulation 121:426-35 doi:10.1161/CIRCULATIONAHA.109.888735

56. Lehman JJ, Kelly DP. (2002). Transcriptional activation of energy metabolic switches in the developing and hypertrophied heart. Clin Exp Pharmacol Physiol 29**:**339-345 doi:10.1046/j.1440-1681.2002.03655.x

57. Garnier A, Fortin D, Delomenie C, Momken I, Veksler V, Ventura-Clapier R. (2003). Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. J Physiol 551**:**491-501 doi:10.1113/jphysiol.2003.045104

58. Russell LK, Mansfield CM, Lehman JJ, Kovacs A, Courtois M, Saffitz JE, Medeiros DM, Valencik ML, McDonald JA, Kelly DP (2004) Cardiac-specific induction of the transcriptional coactivator peroxisome

proliferator-activated receptor γ coactivator-1α promotes mitochondrial biogenesis and reversible cardiomyopathy in a developmental stage-dependent manner. Circ Res 94: 525–533 doi:10.1161/01.RES.0000117088.36577.EB

59. Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M (2001) CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature 413:179–183 doi:10.1038/35093131
60. Altarejos JY, Montminy M (2011) CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol 12:141–151 doi:10.1038/nrm3072
61. Zhang L, Keung W, Samokhvalov V, Wang W, Lopaschuk GD (2010) Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle. Biochim. Biophys. Acta 1801:1–22 doi:10.1016/j.bbalip.2009.09.014

Figure legends

Figure 1. (a) Representative PW-Doppler profile of transmitral flow velocity obtained at endpoint (13 weeks) in parasternal 4-chamber view in the C group (left), HFD group (middle) and HFD+Ins group (right). E: peak of early LV filling wave; A: peak of late filling wave; Dt: deceleration time of early LV filling: HR: heart rate (b–d) Histograms show LV mass (b), diastolic interventricular septum thickness (IVSTd) (c) and diastolic left ventricular posterior wall thickness (LVPWTd) (d) in the control group (open bars), HFD group (black bars) and HFD+Ins group (gray bars) at 13 weeks of diet. Data are presented as mean ± SD (n=16 for each group). *p<0.05 vs C group; §p<0.05 and §§p<0.001 vs HFD+Ins group.

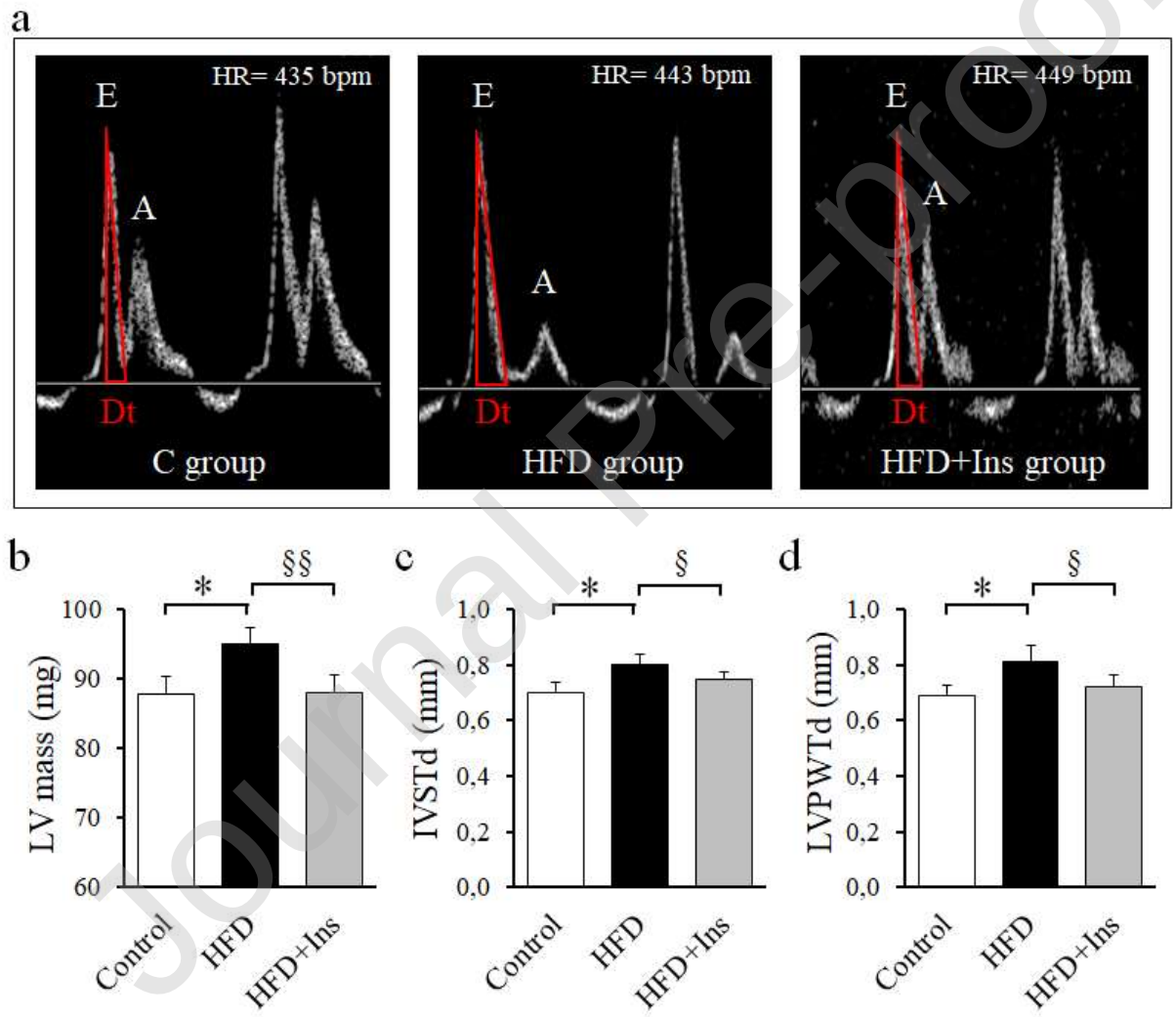


Figure 2. Micrographs of cross sectioned cardiac tissue stained with hematoxylin–eosin from C group (a), HFD group (b) and HFD+Ins group (c). All panels: bar 40µm. (d) Quantitative analysis of cardiomyocyte cross sectional area

(CSA). Data are expressed as mean ± SD (n=120 cells for each group). **p<0.001 vs C group and §§p<0.001 vs HFD+Ins group.

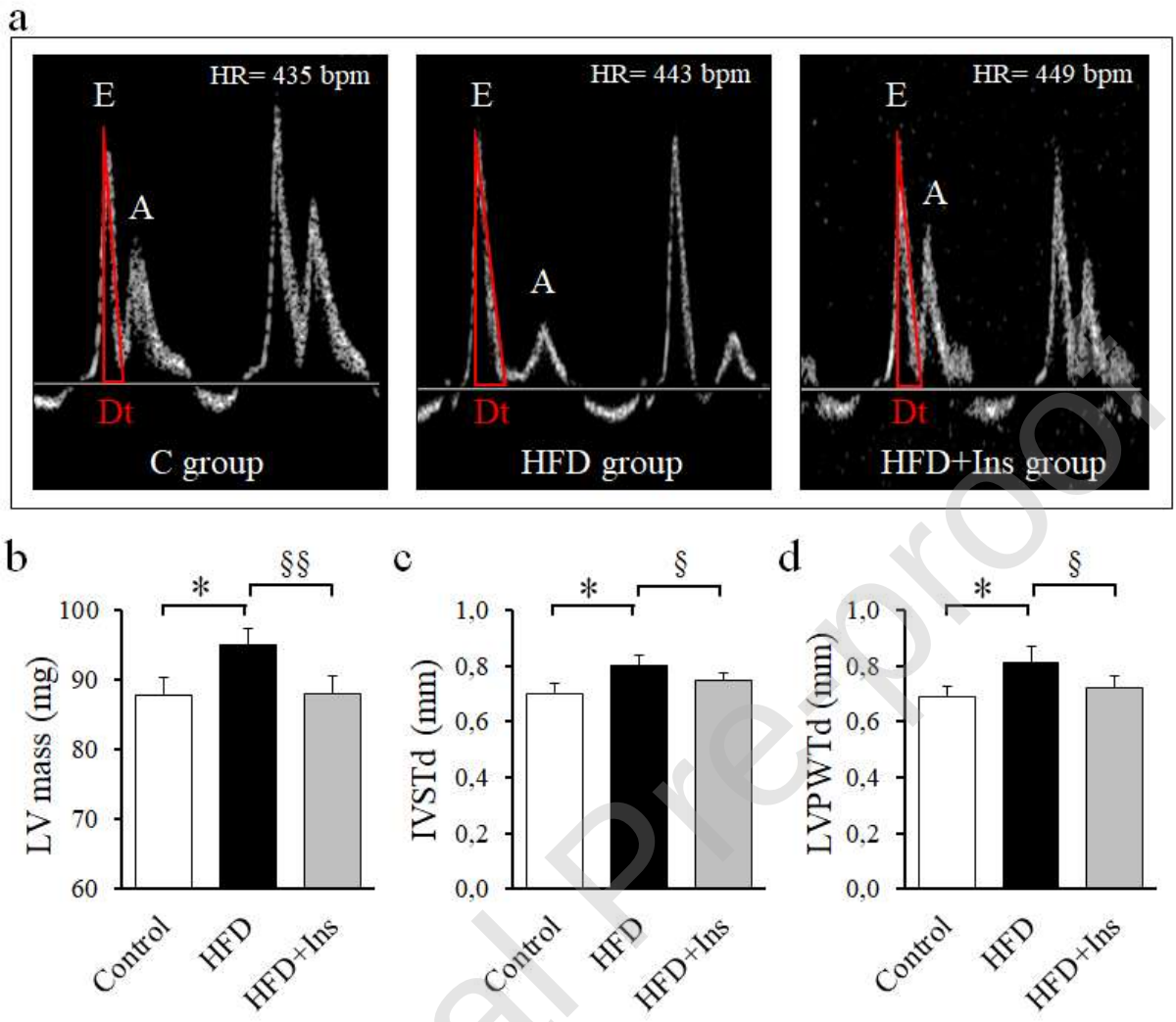


Figure 3. Relative transcriptional expression of genes encoding for ion channels relevant to cardiomyocyte action potential and gap junction proteins (a) and genes involved in the regulation of metabolism and mitochondrial function (b). Open bars: C group; black bars: HFD; gray bars: HFD+Ins. Data on the vertical axes are normalized and expressed as fold change vs C group. Data are expressed as mean ± SD (n=10 for each group). *p<0.05 and **p<0.001 vs C group; §p<0.05 and §§p<0.001 vs HFD+Ins.

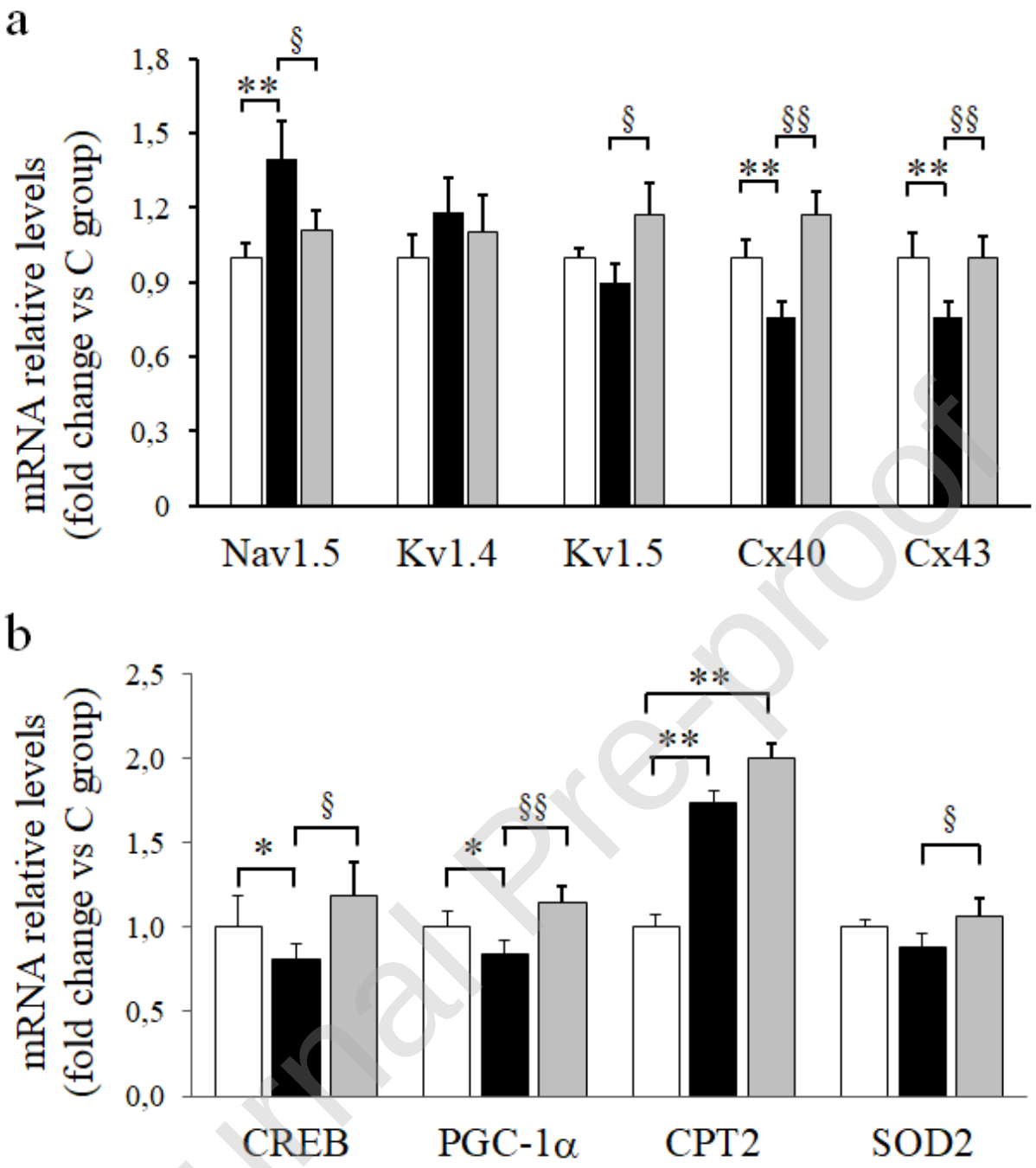


Figure 4. Hypothetical inositol-based mechanisms for cardioprotection from obesity–driven alterations. The scheme postulates the pleiotropic operation of Creb1 and its modulated pathways in the cardiomyocyte, being negatively regulated by HFD–induced lipotoxic stimuli and positively regulated by supplementation with Myo–inositol and D–chiro–inositol.

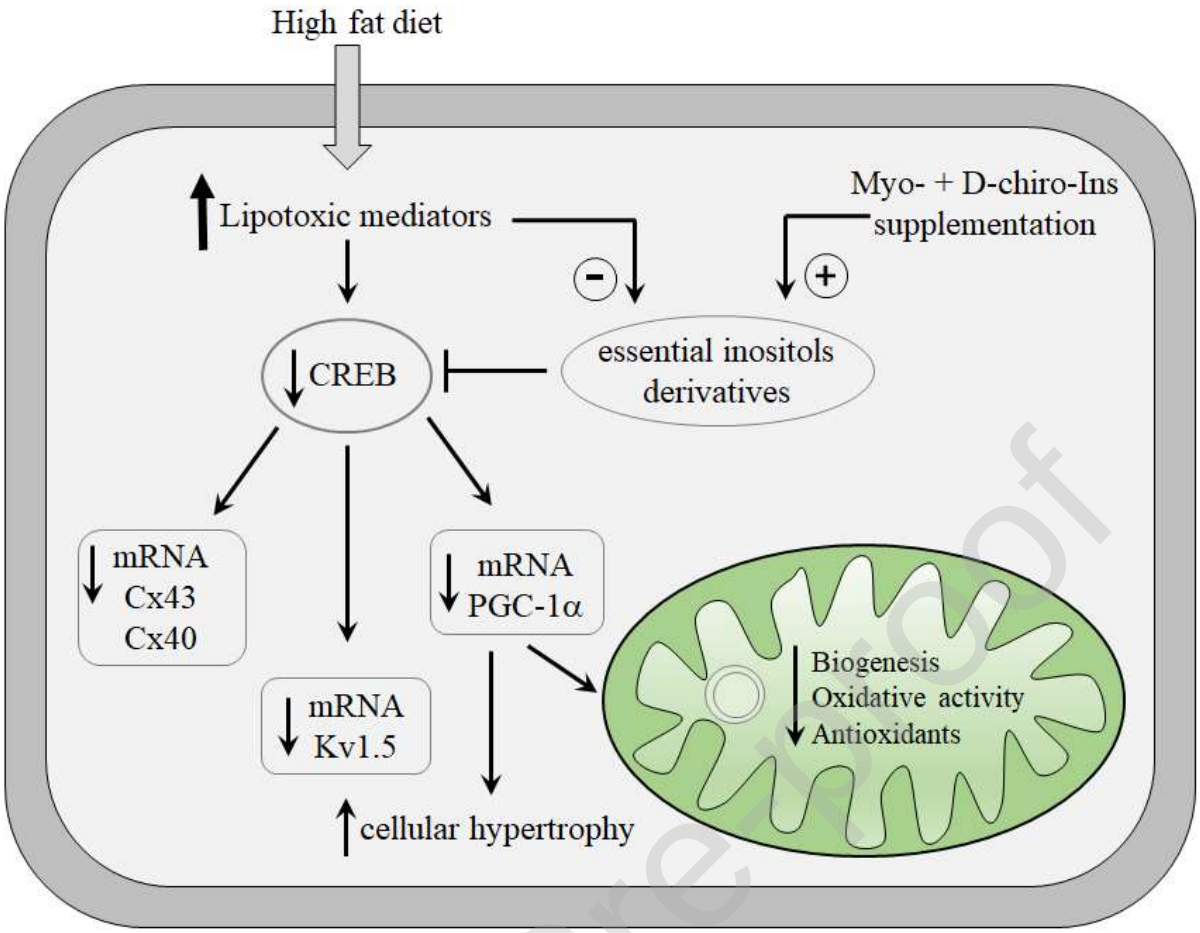


Table 1. Metabolic and plasma parameters at the end of the study

	C group	**HFD group**	**HFD+Ins group**
Body weight (g)	28 ± 3.5	35 ± 8.3*	34 ± 8.0*
GTT–AUC (mg/dl*min)*10^3	22 ± 1.2	31 ± 11**	29 ± 6.7*
Steatoscore (a.u.)	0.67 ± 0.1	0.87 ± 0.1†	0.72 ± 0.1
Glycemia (mg/dl)	138 ± 19	149 ± 39	148 ± 37
Cholesterol (mg/dl)	157 ± 15	170 ± 17	167 ± 23
Triglycerides (mg/dl)	129 ± 23	202 ± 30**	172 ± 35*

GTT–AUC: glucose tolerance test–area under the curve; HFD: high fat diet; Ins: inositols (myo–Ins + D–chiro–Ins). Data are expressed as mean ± SD, n= 16 in each group. *p<0.05 and **p≤0.005 vs C group; †p≤0.001 vs both C group and HFD+Ins group

Table 2. Ultrasonographic parameters of LV function and morphometry at 9 and 13 weeks of HFD

	9 weeks of HF diet		**13 weeks of HF diet**		
	C n=16	**HFD** n=32	**C** n=16	**HFD** n=16	**HFD+Ins** n=16
Systolic function					
Vol_s (µl)	42 ± 7.4	39 ± 6.8	43 ± 10	37 ± 6.2	37 ± 6.1
Vol_d (µl)	67 ± 9.9	62 ± 7.9	66 ± 7.7	63 ± 7.9	62 ± 8.81
SV (µl)	25 ± 5.1	24 ± 3.0	24 ± 4.8	26 ± 5.7	24 ± 2.9
EF%	46 ± 6	48 ± 7	45 ± 4.6	48 ± 7.2	48 ± 4.6
FS%	22 ± 4	25 ± 5	22 ± 5.3	25 ± 4.0	23 ± 6.0
IVCT (ms)	16 ± 3.0	16 ± 2.3	14 ± 3.6	14 ± 3.1	16 ± 5.3
Aet (ms)	51 ± 6.7	49 ± 2.9	49 ± 7	48 ± 7	50 ± 11
Diastolic function					
E/A (a.u.)	1.7 ± 0.4	2.4 ± 0.6**	1.6 ± 0.3	2.3 ± 0.5††	1.8 ± 0.4
Dt (ms)	17.3 ± 5.7	24.5 ± 8.0*	18 ± 6.0	24 ± 7.9**§	20 ± 6.2
MVt (ms)	61 ± 9.3	63 ± 10.3	60 ± 6.0	79 ± 11**§	67 ± 13
IVRT (ms)	15 ± 4.9	17 ± 1.8	16 ± 2.1	18 ± 3.0*	17 ± 2.8
Morphometry					
LV mass (mg)	86.7 ± 7.0	88.5 ± 7.6	87.8 ± 2.5	95.1 ± 2.2*§§	87.9 ± 2.7
IVSTd (mm)	0.69 ± 0.07	0.73 ± 0.08	0.70 ± 0.03	0.80 ± 0.04†	0.75 ± 0.02
LVPWTd (mm)	0.72 ± 0.08	0.72 ± 0.11	0.69 ± 0.04	0.81 ± 0.06†	0.72 ± 0.04

Values are mean ± SD. Vol_s: systolic volume; Vol_d: diastolic volume; SV: stroke volume; EF%: ejection fraction; FS%: fractional shortening; IVCT: isovolumic contraction time; Aet: aortic ejection time; E/A: E/A ratio. Dt: deceleration time of early filling; MVt: mitral valve flow time; IVRT: isovolumic relaxation time. LV: left ventricle; IVSTd: diastolic inter–ventricular septum thickness; LVPWTd: left ventricular posterior wall thickness. a.u.: arbitrary units. *$p<0.05$ and **$p\leq0.005$ vs C group; §$p<0.05$ vs HFD+Ins; †$p<0.05$ and ††$p<0.001$ vs C and HFD+Ins groups.

Table 3. Electrocardiographic characteristics of groups at 9 weeks and 13 weeks

	9 weeks of HF diet		**13 weeks of HF diet**		
	C n=16	**HFD** n=32	**C** n=16	**HFD** n=16	**HFD+Ins** n=16
ECG analysis					
R–R (ms)	130.6 ± 6.0	122.0 ± 7.0*	126 ± 11	112 ± 15†	124 ± 19
HR (bpm)	469 ± 43	489± 41	481 ± 48	543 ± 74*	494 ± 86
P duration (ms)	10.5 ± 1.6	10.1 ± 1.0	10.5 ± 1.8	10.4 ± 1.4	10.2 ± 1.3
PR (ms)	41.1 ± 3.3	39.2 ± 2.0	39 ± 2	40 ± 2	39 ± 2
QRS (ms)	9.0 ± 0.5	8.6 ± 0.1	9.2 ± 1	8.6 ± 1	8.8 ± 1
QTc (ms)	57.3 ± 4.0	64.6 ± 4.0**	56.3 ± 5	67.5 ± 4††	57.2 ± 3
JTc (ms)	31.2 ± 5.5	39.3 ± 4.9**	30.0 ± 3	41.8 ± 4††	32.8 ± 5
HRV analysis					
LFr (n.u.)	7.5 ± 3.4	14.2 ± 5.8**	7.2 ± 4.1	17.9± 8.0††	7.1 ± 3.2
HFr (n.u.)	92.2 ± 3.5	84.8 ± 6.7**	92.7 ± 4.1	78.8 ± 7.2††	91.0 ± 3.8
LFr/HFr	0.08. ± 0.04	0.18 ± 0.06**	0.078 ± 0.05	0.250 ± 0.19††	0.090 ± 0.05

Values are mean ± SD. R–R: time between consecutive QRS complexes; HR: heart rate; P: wave of atrial depolarization; PR: interval from the beginning of the P wave to the beginning of the QRS complex; QRS: complex of ventricle depolarization; QTc: QT interval corrected for heart rate; JTc: JT interval corrected for heart rate. LFr: low frequency; HFr: high frequency: LFr/HFr: ratio between LFr and HFr; n.u.: normalized units. *p<0.05 and **p<0.001 vs C group; †p<0.05 and ††p<0.001 vs both C and HFD+Ins groups.

Table 4. Arrhythmic events following isoproterenol challenge

	C group	**HFD group**	**HFD+Ins group**
Isoproterenol challenge			
Atrial flutter/fibrillation	1/16	8/16	1/16
Atrial sinus arrhythmias	3/16	6/16	6/16
AV dissociation	—	2/16	—
AV block (Mobitz 2)	1/16	3/16	2/16
PVB	—	3/16	2/16

AV: atrio–ventricular: PVB: premature ventricular beat