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HIGH FAT DIET AGGRAVATES CARDIOMYOPATHY IN MURINE CHRONIC CHAGAS DISEASE

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Abstract

Infection with *Trypanosoma cruzi*, the etiologic agent in Chagas disease, may result in heart disease. Over the last decades, Chagas disease endemic areas in Latin America have seen a dietary transition from the traditional regional diet to a western style, fat rich diet. Previously, we demonstrated that during acute infection high fat diet (HFD) protects mice from the consequences of infection-induced myocardial damage through effects on adipogenesis in adipose tissue and reduced cardiac lipidopathy. However, the effect of HFD on the subsequent stages of infection – the indeterminate and chronic stages – has not been investigated. To address this gap in knowledge, we studied the effect HFD during indeterminate and chronic stages of Chagas disease in the mouse model. We report, for the first time, the effect of HFD on myocardial inflammation, vasculopathy, and other types of dysfunction observed during chronic *T. cruzi* infection. Our results show that HFD perturbs lipid metabolism and induces oxidative stress to exacerbate late chronic Chagas disease cardiac pathology.

Keywords

Chagas disease; chagasic cardiomyopathy; High fat diet; lipid metabolism; mitochondrial dysfunction; RAGE; inflammation

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⁵.CONFLICT OF INTEREST STATEMENT

None of the authors have conflict of interest.

1. INTRODUCTION

Chagas disease, caused by the parasite *Trypanosoma cruzi*, is a potentially deadly disease endemic in Latin America [1, 2]. Approximately 30% of the infected patients develop cardiac problems, including Chronic Chagasic Cardiomyopathy (CCM), which is associated with high mortality [3,4]. The pathogenesis of CCM is not completely understood. Many factors, including parasite-induced myocardial damage, immune-mediated myocardial injury, mitochondrial dysfunction and microvascular and neurogenic disturbances, have been suggested as causes for the development of CCM [5–9]. Furthermore, no effective drugs or vaccines are available to counteract or prevent CCM [3].

Chagas disease in human patients characteristically presents as two phases, acute and chronic, each with distinct clinical features [10, 17]. The chronic phase can take one of two forms: (i) the indeterminate form, which represents about 60–70% of the cases and does not present any clinical symptoms, and (ii) the determinate form, which is characterized by cardiac and digestive pathologies [10]. Chronic chagasic cardiomyopathy (CCM) is the most clinically important chronic form of Chagas' disease due to its high rate of morbidity and mortality in the endemic regions of Chagas disease, with a significant medical and social impact [11].

In the endemic regions of Chagas disease, as elsewhere, traditional food patterns rich in complex carbohydrates, micronutrients, fiber, and phytochemicals are being replaced with diets high in refined sugars, animal products, and highly processed foods [18, 19]. However, how such a diet transition may influence the outcome of chronic Chagas disease has not been studied. Various murine experimental Chagas models have been used to understand the molecular mechanism(s) involved in the pathogenesis of acute and chronic Chagas disease [12–16]. *T. cruzi* infected CD1 mice usually present with three distinct phases of the disease (acute, indeterminate and cardiac chronic) based on the serum parasitemia and inflammatory signaling, and are therefore a suitable model to investigate the factors responsible for the pathogenesis of the disease and the transition from the indeterminate stage to determinate cardiac stage (CCM) [12–15]. It has been demonstrated that acute infection in a murine model of Chagas disease is associated with alterations in cardiac lipid metabolism and lipiodopathy, which in turn causes myocardial injury, cardiac oxidative stress and inflammation [13–16]. We also demonstrated that during acute *T. cruzi* infection, a high fat diet (HFD, 60% fat calories) significantly protected the infected mice from mortality by regulating cardiac immuno-metabolic signaling via increased adiposity in the adipose tissue [15,16].

In this study we investigated the effect of HFD on regulating cardiac pathology during the indeterminate and chronic cardiac stages in the murine model of Chagas disease. In particular, we examined the effect of HFD on cardiac lipid metabolism, oxidative stress, and inflammatory signaling to analyze their roles in causing myocardial inflammation, vasculopathy, and other dysfunctions observed during chronic *T. cruzi* infection using an established murine Chagas disease model. The results shed light on how metabolic regulation influences the development of cardiomyopathy and heart failure during chronic Chagas disease.

2. MATERIALS AND METHODS:

2.1 Ethical approval:

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Albert Einstein College of Medicine (No. 20130202) and the Rutgers Biomedical and Health Sciences (No. 15107), adhere to the National Research Council guidelines (Guide for the Care and Use of Laboratory Animals: Eighth Edition, Washington, DC: The National Academies Press, 2011).

2.2 Experimental animal model:

The Brazil strain of *T. cruzi* was maintained by passage in C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME). Male CD-1 mice (Jackson Laboratories) were infected intraperitoneally (i.p.) at 6–8 weeks of age with 10^3 trypomastigotes of the Brazil strain [20]. Mice were maintained on a 12-hour light/dark cycle. Acute infection results in increased parasitemia and serum inflammatory cytokines [14]. The parasitemia and the levels of pro-inflammatory signaling significantly reduces after 30dpi in *T. cruzi* infected CD1 mice [14, 17]. In this Experiment (replicated), starting at the day of 35 post infection (after acute infection), mice were randomly divided into two groups (n = 20 per group) and fed on either high fat diet (HFD; 60% fat D12492 Research Diets, Inc., New Brunswick, NJ) or regular diet (RD, 10% fat D12450 Research Diets, Inc., New Brunswick, NJ) [14]. Uninfected were fed on either HFD (n=20) or RD (n=20) and used as respective controls in all the experiments (Experimental design Supplemental Fig.1A).

Mice were euthanized and hearts were harvested for histological and molecular analysis at early chronic stage (d120pi) and late chronic stage (d160pi) [14, 20]. Plasma samples were obtained from 75 μ l of blood collected from the orbital venous sinus (using isoflurane anesthesia) 160 days post infection (dpi).

2.3 Magnetic resonance imaging (MRI) analysis:

Cardiac gated MRI was performed on uninfected and infected mice at early and late chronic stages of infection (100 and 150 dpi, before sacrificing the mice at d120pi and 160pi respectively) were imaged using a 9.4 T Varian Direct Drive animal magnetic resonance imaging and spectroscopic system (Agilent Technologies, Inc. Santa Clara, CA) as previously published [14]. Briefly, anesthesia was induced with 2% isoflurane in air, mice were positioned supine inside an MR compatible holder and positioned within a 35-mm ID quadrature ^1H volume coil (Molecules2Man Imaging Co., Cleveland, OH). Body temperature was maintained at 34 ~35 °C using warm air with feedback from a body surface thermocouple. A respiratory sensor balloon was taped onto the abdomen. Cardiac (ECG electrodes inserted subcutaneously in front left paw and rear right paw) and respiratory signal (from sensor balloon taped to the abdomen) were continuously monitored and used for MR gating/triggering by an SA Monitoring and Gating System (Small Animal Instruments, Inc., Stony Brook, NY). Ten to fourteen 1-mm-thick slices without gap was acquired in short-axis orientation covering the entire heart using an ECG-triggered and respiratory gated multi-frame tagged cine sequence. The imaging parameters used were field of view (FOV) of 40 $\times$ 40 mm², matrix size of 256 $\times$ 256, TE of 2.6 ms, TR of 5.5 ms, flip

angle of 25°, number of averages of 2. The number of frames was twelve to eighteen. Data were transferred to a PC and analyzed using MATLAB-based software. Left ventricle (LV) and right ventricle (RV) dimensions in millimeters were determined from the images representing end-diastole. The left ventricular wall is the average of the anterior, posterior, lateral, and septal walls. The right ventricular internal dimension is the widest point of the right ventricular cavity.

2.4 Immunoblot analysis:

Technique was performed as described [15,20] using the following antibodies: fatty acid synthase (ab22759) from Abcam Inc. (Cambridge, MA); β -actin (A2066) from Sigma-Aldrich; phospho-Cav1.2 a1C (AB9022) from Chemicon (Temecula, CA); LDLr specific rabbit monoclonal antibody (1:1000 dilution, AB52818 Abcam, Cambridge, MA), lipoprotein lipase (LPL) specific mouse monoclonal antibody (1:1000 dilution, AB21356, Abcam), adipose triglyceride lipase (ATGL) specific rabbit monoclonal antibody (1:1000 dilution, AB 109251), hormone sensitive lipase (HSL) specific rabbit monoclonal antibody (1:1000 dilution, AB 45422), lipoprotein lipase (LPL) specific mouse monoclonal antibody (1:1000 dilution, AB 21356) or TNF- α specific rabbit polyclonal antibody (1:1000 dilution, AB6671, Abcam) were used as primary antisera. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (1:2000 dilution, Amersham Biosciences, Piscataway, NJ) or horseradish peroxidase- conjugated goat anti-rabbit immunoglobulin (1:5000 dilution, Amersham Biosciences) were used to detect specific protein bands (explained in Figure Legends) using a chemiluminescence system [14,17]. GDI (1: 10000 dilution, 71-0300, and rabbit polyclonal, Invitrogen, CA) and a secondary antibody horseradish peroxidase conjugated goat anti-rabbit (1:2000 dilution, Amersham Biosciences) was used to normalize protein loading.

2.5 Polymerase Chain Reaction Array:

An RT² Profiler (SA Biosciences, Valencia, CA) custom designed PCR array for mouse genes involved in lipid metabolism, oxidative stress and inflammatory signaling was used to analyze gene expression. Data analysis was performed normalized to the expression of 18sRNA using the CT method according to the manufacturer's protocol (SABiosciences) and as previously mentioned [14, 21].

2.6 Immuno-histochemical analysis (IHC):

Hearts were isolated, fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 5-mm sections with a microtome, and stained with hematoxylin and eosin (H&E) as previously published [14]. Four to six sections of each heart were scored blindly. For each myocardial sample, histologic evidence of myocarditis, cellular hypertrophy and inflammation was graded on a six point scale ranging from 0 to 5+ as previously published. A zero score indicated lowest or negligible changes and 4 the most damaged state. The levels of ApoB, UCP3, and CETP were quantitated in the sections of right and left ventricles using anti-ApoB, anti-UCP3, and anti-CETP antibodies (1:250 dilution) followed by Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (1:1000 dilution) by IHC analysis as previously mentioned [17]. The stain intensities of the images were quantified using NIH-Image J program for four to six images of each heart.

2.7 Cholesterol measurement:

Cholesterol levels were quantified in the hearts and livers [22] of mice at day 160 pi using a colorimetric assay kit, and samples were prepared and assayed following the manufacturer's protocol (Total cholesterol colorimetric assay kit, Cell Biolabs Inc., CA).

2.8 Soluble-RAGE (S-RAGE) measurement:

sRAGE levels were quantified in the serum of mice at day 160 pi using a ELISA kit, and samples were prepared and assayed following the manufacturer's protocol (Rat/mouse soluble RAGE ELISA, Aviscera Bioscience, CA).

2.9 Statistical Analysis:

Data represent means $\pm$ S.E. Data were pooled and statistical analysis was performed using a Student's t-test (Microsoft Excel) as appropriate and significance differences were determined as p values between < 0.05 and < 0.001 as appropriate. Gene array analyses were done in duplicates as described earlier [14, 21].

3. RESULTS

3.1 Diet has no effect on the survival of mice during chronic Chagas disease:

Earlier we demonstrated that HFD increases the survival rate of *T. cruzi* infected mice during the acute stage of Chagas disease [14, 15]. To study the effect of HFD on the mortality and pathogenesis of subsequent, chronic *T. cruzi* infection, at d35 post infection we began feeding the infected mice with either a high fat diet (HFD, 60% fat calories) or a control regular diet (RD, 10% fat calories) for approximately 18 weeks (till d160pi). We observed no significant difference in the survival rate between infected, uninfected, HFD-fed and RD-fed mice between d35pi and d160pi (two mice died in each group after d120pi because of bite wounds due to fighting). As expected, HFD fed mice showed greater body weight compared to RD fed mice (Supplementary Fig.1B). Also as expected, infected mice displayed lower body weights compared to uninfected mice on the same diet (Supplementary Fig. 1B).

3.2 Diet regulates ventricular dilation during chronic Chagas disease:

Earlier we demonstrated significant alterations in cardiac morphology in *T. cruzi* infected mice during the acute phase infection, including a reduction in the left ventricle internal diameter (LVID), an increase in the right ventricle internal diameter (RVID), and an increase in the cardiac wall thickening (at both diastole and systole) [14]. We also reported that these alterations in heart morphology were less pronounced in the acutely infected mice fed on a HFD compared to the acutely infected mice fed on a RD [14]. Chronically infected CD1 mice develop cardiomyopathy between d90 and d150pi [12, 17]. To investigate the effect of a switch to HFD on the cardiac morphology in mice during the early and late stages of chronic *T. cruzi* infection, we performed cardiac MRI analysis at d100 pi and d150 pi., respectively. Compared to RD fed uninfected mice, RD fed infected mice demonstrated no significant difference in LVID and a significant decrease in RVID (both diastole and systole) during the early chronic stage (d100pi) of infection and an increased LVID (and no

significant difference in the RVID) at the late chronic stage (d150 pi) of infection (Fig. 1). Compared to RD fed uninfected mice, infected mice fed on a HFD showed no significant difference in the LVID or RVID (except a significant decrease in LVID diastole) at d100 pi and displayed a significantly increased RVID at d150 pi (Fig.1). The wall average (diastole) significantly increased in the infected RD mice and decreased in the infected HFD mice compared to RD fed uninfected mice (Fig.1). These data demonstrated significant alterations in the cardiac morphology between the early and late chronic stages of infection and showed that prolonged HFD intake causes RV dilation.

3.3 Histopathological changes in the heart:

There were significant histological differences in the hearts of infected (RD and HFD fed) mice compared to RD fed uninfected mice at both early (d120pi) and late chronic stages (d160pi) of infection (Fig. 2A and 2B). Cellular hypertrophy and inflammation were prominent in the infected hearts sections. The pattern of pathology (hypertrophy, inflammation and accumulation of lipid droplets) differed between RV and LV (additional images are presented as Supplementary Fig. 2): infected RD fed mice showed significantly higher levels of inflammation in the RV compared to infected HFD mice, whereas there was no significant difference between the infected RD and HFD mice in the levels of inflammation observed in the LVs at d120pi (Fig. 2A). At the later stage of chronic infection (d160pi), the hearts obtained from infected RD fed mice displayed no significant pathological changes in the RV compared to uninfected RD mice, but did display significant pathological changes at the LV (Fig. 2B). Interestingly, HFD fed mice showed significantly increased pathology at both RV and LV irrespective of infection status compared to uninfected RD fed mice (Fig. 2B). We performed histological scoring of the hearts, with scores ranging from 0 to 5+ in the categories of inflammation, cellular hypertrophy, lipid droplet accumulation and increased capillary size. Infected (RD and HFD) animals had higher scores than uninfected RD fed animals at d120pi (Fig. 3). Infected HFD animals had greater scores than infected RD fed animals at 160pi. These data demonstrate that infected mice fed on a HFD are more susceptible to developing cardiac pathology involving both RV and LV compared to RD fed mice during the late chronic stages of infection.

3.4 HFD increases cholesterol levels in the livers but not the hearts of the chronically infected mice:

Earlier we showed that cholesterol accumulation in the myocardium is associated with CCM in the murine Chagas model and in a human CCM biopsy samples [14,23]. To evaluate the effect of HFD on cardiac cholesterol levels during late chronic infection, we measured the levels of cholesterol in the hearts, as well as in the livers, of the mice fed on either a HFD or RD at 160dpi. The mice showed no significant differences in the levels of cholesterol in the hearts between infected HFD fed and RD fed mice (Supplementary Fig. 3). However, the livers of HFD fed mice (both infected and uninfected) showed greater cholesterol levels than the RD fed mice (Supplementary Fig. 3). Also, the weights of hearts and livers correlated with the cardiac levels of cholesterol in these experimental groups (Supplementary Figs 1(C&D) and 3).

To test whether LDL was a major source of cholesterol in the infected mice [14,23], we quantified ApoB levels in the LV and RV separately at d120 and 160pi by IHC analysis (Fig. 4 and Supplementary Fig. 3). We found significant differences in ApoB levels between the RV and LV sections, between the infected RD and HFD groups, and also between the early and late chronic time points (Supplementary Fig. 3). During the early chronic phase (d120pi), ApoB levels of the LV significantly decreased in the infected mice compared to uninfected mice irrespective of the diets. However, only the RV of the infected HFD mice showed significantly reduced ApoB levels at d120pi. During the late chronic stage (d160pi), ApoB levels significantly increased in both RV and LV of the infected RD mice compared to the infected HFD mice (Supplementary Fig. 3).

3.5 HFD enhances disruption of cardiac lipid metabolism during chronic Chagas disease:

Earlier we demonstrated that *T. cruzi* infection affects cholesterol trafficking and efflux mechanisms and lipid metabolism during acute infection [15]. To evaluate the effect of diet on regulating lipid metabolism during the early and late stages of chronic infection, we measured the protein levels of cholesterol efflux protein ATP binding cassette transporter 1 (Abca1), cholesterylester transfer protein (CETP), and lipid oxidation regulator PPAR α , in the hearts of the infected mice at d120pi and 160pi (Fig. 5). The protein levels of Abca1, CETP and PPAR α significantly increased in the infected HFD mice compared to uninfected HFD mice at d120pi. The infected RD mice also demonstrated greater levels of CETP and PPAR α compared to uninfected RD (no significant difference in Abca1 levels) at d120pi. During the late chronic stage (d160pi), the infected HFD mice showed either no significant change or a decrease in the cardiac levels of Abca1, CETP and PPAR α compared to uninfected HFD mice. In the infected RD mice, the levels of these proteins significantly increased compared to uninfected RD mice (Fig. 5A and Fig. 5a). Overall, the levels of Abca1, CETP and PPAR α were lower in the infected HFD mice compared to the infected RD mice and also significantly reduced (except CETP) during the late chronic stage relative to RD fed uninfected mice (Fig. 5A and 5a). IHC analysis also demonstrated significantly reduced CETP levels in both the RV and LV of the infected HFD mice compared to the infected RD mice (Supplemental Fig. 4).

We also measured the cardiac levels of some of the following lipid metabolic proteins to investigate the effect of HFD on the late chronic stage of infection. We measured the levels of lecithin-cholesterol acyltransferase (LCAT), an enzyme that converts free cholesterol into cholesteryl ester, in the hearts of mice at 160pi. Immunoblot analysis demonstrated a significant decrease in the levels of LCAT in the infected HFD fed mice compared to all the other groups (Fig. 5B and 5b). These data suggest that HFD significantly affects cardiac cholesterol metabolism at the later stages of chronic *T. cruzi* infection. We also measured the cardiac levels of key proteins involved in fatty acid metabolism, such as fatty acid synthase (FAS), acyl-coA synthase (ACSL1), ATP citrate lyase, and UCP3 by immunoblot analysis in the hearts of mice at d160pi (Fig. 5B and 5b). The levels of proteins involved in the fatty acid metabolism were significantly decreased in the infected HFD mice compared to the infected RD mice. We also found a significant decrease in UCP3 in the LVs of the infected HFD mice compared to all other experimental groups – the quantitated intensities of the IHC

images showed significant differences between RV and LV, between the infected RD and infected HFD groups, and between the early and late chronic phases (Supplementary Fig. 4).

3.6 HFD aggravates mitochondrial oxidative stress during chronic CD:

It has been demonstrated that mitochondrial dysfunction and oxidative stress are involved in the pathogenesis of Chagasic cardiomyopathy [8,13]. To evaluate the effect of prolonged HFD treatment on the cardiac mitochondrial oxidative capacity in Chagas disease, we measured the protein levels of succinate dehydrogenase complex flavoprotein subunit A (SDHA) and heat shock protein 60 (HSP-60) (Fig. 6A). SDHA is a major catalytic subunit of succinate-ubiquinone oxidoreductase, a complex of the mitochondrial respiratory chain which is vulnerable to reactive oxygen species (ROS) [24]. HSP60 is a chaperone originally identified in the mitochondria, which is responsible for refolding and transportation of proteins between the mitochondrial matrix and the cytoplasm of the cell [25]. HSP60 associates with a number of cytosolic proteins involved in apoptosis and its level correlates with oxidative stress [25]. We found that SDHA1 and HSP60 levels significantly increased in the hearts of infected RD mice and that only HSP60 levels were elevated in the hearts of infected HFD mice compared to uninfected RD mice at d 120 pi (Fig.6A). However, in the late chronic phase mice (d160 pi), infected HFD mice showed a significant decrease in SDHA1 levels (no significant change was observed in the infected RD mice) compared to uninfected RD mice (Fig.6A). HSP60 levels were significantly down regulated in both infected groups (i.e. RD and HFD). Additionally, qPCR analysis showed that the mRNA levels of the genes involved in the mitochondrial oxidative functions were significantly altered in the RV of the infected HFD mice compared to the infected RD mice (Supplemental Table 1A). We also analyzed the levels of anti-oxidant genes, such as glutathione peroxidases, peroxiredoxine1, catalase and super oxide dismutase in the RV and LV of the late chronic mice and found that they were significantly reduced in both RV and LV of the infected HFD mice compared to infected RD mice (Supplemental Table 1B).

3.7 HFD increases cardiac levels of RAGE and cleaved RAGE:

The receptor for advanced glycation end products (RAGE) mediates responses to cell danger and stress [26]. When bound by its many ligands (which include advanced glycation end products, certain members of the S100/calgranulin family, extracellular high-mobility group box 1, the integrin Mac-1, amyloid beta-peptide and fibrils), RAGE activates programs responsible for acute and chronic inflammation [27]. We found that during early chronic stages of infection (d120pi), the cardiac levels of RAGE significantly increased in the infected HFD mice compared to uninfected RD fed mice (Fig. 6B and 6b). Furthermore, immunoblot analysis demonstrated significantly increased levels of the proteolytically cleaved RAGE (a.k.a. soluble RAGE) in both uninfected and infected HFD fed mice compared to uninfected mice. During the late chronic stages of infection (d160pi), the cardiac levels of RAGE significantly increased in the infected RD mice compared to uninfected RD mice. However, unlike at d120pi, the levels of RAGE and cleaved RAGE significantly decreased in the infected HFD mice compared to the infected RD mice at d160pi (Fig. 6B and 6b). We also analyzed the serum levels of soluble RAGE (sRAGE) at d160pi using a RAGE ELISA kit. The levels of sRAGE were significantly reduced

($p = 0.0002$) in the infected HFD mice compared to RD fed uninfected mice (Supplementary Fig. 5).

3.8 HFD modulates cardiac inflammation during chronic Chagas disease:

It has been shown that inflammatory signaling plays a major role in determining cardiac pathogenesis in chronic Chagas disease [6]. For instance, inflammation induced cardiac remodeling is observed both in the animal models of Chagas disease and in Chagasic cardiac patients [28]. Thus, we analyzed the effect of HFD on cardiac inflammatory cytokine levels. Immunoblot analysis demonstrated significantly decreased levels of pro-inflammatory cytokines TNF α and IFN γ in the infected RD fed mice during the early chronic stage (d120pi), but both of these cytokines significantly increased during the late chronic stage (d160pi) compared to uninfected RD mice (Fig. 6C and 6c). The levels of TNF α in the infected HFD mice showed no significant difference relative to uninfected HFD mice (d160pi). The levels of IFN γ were significantly reduced in both the infected RD fed and HFD fed mice ($p = 0.051$) during the early stages of chronic infection relative to RD fed uninfected mice (Fig. 6C and 6c). However, the levels of IFN γ significantly increased in the infected RD mice compared to the infected HFD mice during later stages of chronic infection. Interestingly, immunoblot analysis showed a significant increase in the levels of both TNF α and IFN γ in the infected HFD mice compared to the infected RD mice at the early chronic stage, which reversed to significantly reduced levels by d160pi (Fig. 6C and 6c). The levels of pro-inflammatory cytokines correlated with the levels of cardiac RAGE levels (Fig. 6A and 6a) in all experimental groups.

4. Discussion

In the present study we validated the role of HFD introduced after the acute stage and during the indeterminate stage of Chagas disease as a regulator of the pathogenesis of CCM. In particular we measured the effect of HFD on cardiac lipid metabolism and inflammation, and morphology during early and late chronic stages of infection. We found that HFD significantly reduced cardiac pro-inflammatory markers and increased cardiac dilation and dysfunction during later stages of chronic infection compared RD in our murine Chagas model. Together, these data show that HFD intake during indeterminate/early chronic stages increases the risk of developing cardiomyopathy and cardiac dysfunction.

The various techniques we employed to investigate cardiac structure and function clearly demonstrated that during the late chronic stages (d150pi) of infection in HFD-fed mice there was a significant cardiac dysfunction associated with right ventricular enlargement compared to RD-fed mice. Even though there were no significant alterations at the LVID and RVID observed in the infected HFD fed mice compared to uninfected RD-fed mice at an early time point of the chronic infection stage (d100pi), infected HFDfed mice developed increased RVID over an extended period of HFD feeding as shown by the MRI data at d150pi (Fig. 1). Interestingly, infected RD fed mice showed significantly increased LVID ($p = 0.05 - 0.01$) and no significant change in RVID compared to uninfected RD mice at d150pi (Fig. 1). These observations suggest that HFD and RD, which are composed of 60% and 70% (k cal) of fat and carbohydrates respectively, differently regulate cardiac

enlargement in infected mice. These MRI data suggest that diet plays a major role in determining cardiac and ventricular enlargement during chronic stages of infection.

Previously we demonstrated that altered lipid metabolism of the heart contributes to myocarditis in acute *T. cruzi* infection [13, 14, 23]. We showed that cardiac lipid metabolism is modulated by HFD, which alters the cardiac pathogenesis of acute *T. cruzi* infection, resulting in reduced myocardial fibrosis, inflammation and mortality of CD1 mice [13, 14]. We and others have reported cardiac lipid accumulation that may regulate cardiac pathogenesis in chronic Chagas disease [13,14]. These accumulated lipids can either be effluxed or utilized via mitochondrial oxidation to prevent cardiac lipotoxicity. Our results here showed that HFD decreases the efficiency of clearing cardiac cholesterol accumulation, as indicated by the reduced levels of the cholesterol efflux protein ABCA1 and free cholesterol converting enzyme LCAT (Fig. 5). HFD also reduced fatty acid metabolism and oxidation as demonstrated by reduced levels of cardiac PPAR α and UCP3 (Fig. 5) in the infected mice. Increased intracellular lipid accumulation causes mitochondrial stress and exacerbates mitochondrial oxidative capacity, resulting in mitochondrial dysfunction [29]. While we observed significantly increased mitochondrial oxidative function in the infected HFD mice at early stages of chronic infection, as indicated by the increased cardiac levels of SDHA and UCP3, their levels significantly decreased at the later stages of chronic infection (Figs. 5 and 6A). These information suggest that HFD treatment during the early chronic stage overloads cardiac lipid levels and exceeds mitochondrial oxidative capacity (Fig. 6A), and increases oxidative stress (Table. 1), and prolonged HFD treatment results in the exhaustion of mitochondrial function in the infected HFD mice. These data indicate that cholesterol efflux mechanisms (Fig. 5), lipid oxidation (Fig. 5), and mitochondrial dysfunction (Fig. 6A) are all affected by HFD during chronic stages of infection, resulting in aggravated cardiac lipotoxicity, vascular and cardiac accumulation of lipid droplets, and vascular dilation (Fig. 6 and Table 1).

HFD also caused significant changes in the levels of cardiac inflammatory cytokines. While HFD increased cardiac levels of the pro-inflammatory cytokines such as TNF α and IFN γ in the early chronic phase (d120pi) compared to RD in infected groups, their levels significantly decreased in the late chronic phase (d160pi), suggesting that increased cardiac lipid droplets (Fig. 3) may play a role in decreased TNF α levels. Indeed, the inverse regulation mechanisms between lipid droplets/361 adipocytes and TNF α have been reported [30]. Our data also showed significantly decreased levels of RAGE/proteolytically cleaved RAGE in the heart, and reduced serum soluble RAGE in the infected HFD mice (Fig. 6B and Sup Fig. 5). This result correlates well with the cardiac pro-inflammatory cytokine levels because increased TNF α levels have been shown to positively regulate RAGE levels through nuclear factor-kappa B in human vascular endothelial cells [26,27]. Increased soluble RAGE was protective against inflammation during early chronic stage which diminished in the hearts of infected HFD mice at late chronic stage suggesting a possible protective role of soluble RAGE in preventing CCM.

HFD is known to alter the metabolic state of the host, which in turn can regulate host immune signaling [15, 31]. In recent decades, a western style diet high in fat has become more accessible in the endemic regions of Chagas disease, which may adversely impact the

outcome of chronic Chagas disease [18, 19]. Indeed, our data suggest that HFD aggravates the pathogenesis of cardiomyopathy by causing mitochondrial dysfunction due to elevated cardiac lipidopathy. Fat and carbohydrate rich diets differentially altered ventricular enlargements (i.e. RV vs LV) during the late chronic stage. In light of our studies, CCM may be considered not just a chronic inflammatory disease, but also one with an associated metabolic pathology. Further dietary studies need to be conducted to completely understand the mechanisms of the pathogenesis of CCM and their metabolic regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference

1. Montgomery SP, Starr MC, Cantey PT, Edwards MS, Meymandi SK. Neglected parasitic infections in the United States: Chagas disease. *Am J Trop Med Hyg* 2014;90 :814–8. [PubMed: 24808250]
2. Steverding D The history of Chagas disease. *Parasit Vectors* 2014; 7:317. [PubMed: 25011546]
3. Rassi A, Jr, Neto M, Antonio J, Rassi A. Chronic Chagas cardiomyopathy: a review of the main pathogenic mechanisms and the efficacy of aetiological treatment following the BENznidazole Evaluation for Interrupting Trypanosomiasis (BENEFIT) trial. *Mem Inst Oswaldo Cruz* 2017; 12:224–235
4. Mensah GA, Burns KM, Peprah EK, Sampson UK, Engelgau MM. Opportunities and challenges in chronic chagas cardiomyopathy. *Glob Heart* 2015 ;3:203–7
5. Tanowitz HB, Machado FS, Jelicks LA, Shirani J, de Carvalho AC, Spray DC et al. Perspectives on *Trypanosoma cruzi*-induced heart disease (Chagas disease). *Prog Cardiovasc Dis* 2009; 51:524–39. [PubMed: 19410685]
6. Machado FS, Tanowitz HB, Ribeiro AL. Pathogenesis of Chagas cardiomyopathy: role of inflammation and oxidative stress. *J Am Heart Assoc* 2013; 2: e000539. [PubMed: 24152984]
7. Machado FS, Jelicks LA, Kirchhoff LV, Shirani J, Nagajyothi F, Mukherjee S et al. Chagas heart disease: report on recent developments. *Cardiol Rev* 2012 ;2:53–65
8. Zacks MA, Wen JJ, Vyatkina G, Bhatia V, Garg N. An overview of chagasic cardiomyopathy: pathogenic importance of oxidative stress. *An Acad Bras Cienc* 2005 ;77 :695–15. [PubMed: 16341444]
9. Teixeira AR, Hecht MM, Guimaro MC, Sousa AO, Nitz N. Pathogenesis of chagas' disease: parasite persistence and autoimmunity. *Clin Microbiol Rev* 2011;24:592–30. [PubMed: 21734249]
10. https://www.cdc.gov/parasites/chagas/gen_info/detailed.html
11. Botoni FA, Ribeiro AL, Marinho CC, Lima MM, Nunes MD, Rocha MO. Treatment of Chagas cardiomyopathy. *Biomed Res Int* 2013; 24:2013 :849504
12. De Souza AP, Tanowitz HB, Chandra M, Shtutin V, Weiss LM, Morris SA, Factor SM, Huang H, Wittner M, Shirani J, Jelicks LA. Effects of early and late verapamil administration on the development of cardiomyopathy in experimental chronic *Trypanosoma cruzi* (Brazil strain) infection. *Parasitol Res* 2004; 92:496–501. [PubMed: 14999469]
13. Johndrow C, Nelson R, Tanowitz H, Weiss LM, Nagajyothi F. *Trypanosoma cruzi* infection results in an increase in intracellular cholesterol. *Microbes Infect* 2014; 4 :337–44
14. Nagajyothi F, Weiss LM, Zhao D, Koba W, Jelicks LA, Cui M, et al. High fat diet modulates *Trypanosoma cruzi* infection associated myocarditis. *PLoS Negl Trop Dis* 2014; 8:e3118. [PubMed: 25275627]

15. Brima W, Eden DJ, Mehdi SF, Bravo M, Wiese MM, Stein J, et al. The brighter (and evolutionarily older) face of the metabolic syndrome: evidence from *Trypanosoma cruzi* infection in CD-1 mice. *Diabetes Metab Res Rev* 2015; 31:346–59. [PubMed: 25613819]
16. Dhiman M, Garg NJ. NADPH oxidase inhibition ameliorates *Trypanosoma cruzi*-induced myocarditis during Chagas disease. *J Pathol* 2011;225:583–96. [PubMed: 21952987]
17. Andrade DV, Gollob KJ, Dutra WO. Acute chagas disease: new global challenges for an old neglected disease. *PLoS Negl Trop Dis* 2014;8:e3010. [PubMed: 25077613]
18. Bermudez OI, Tucker KL. Trends in dietary patterns of Latin American populations. *Cad Saude Publica* 2003; 19:S87–99. [PubMed: 12886439]
19. Popkin BM, Adair LS, Ng SW. Global nutrition transition and the pandemic 426 of obesity in developing countries. *Nutr Rev* 2012;70 :3–21. [PubMed: 22221213]
20. Combs TP, Nagajyothi F, Mukherjee S, de Almeida CJ, Jelicks LA, Schubert W, et al. The adipocyte as an important target cell for *Trypanosoma cruzi* infection. *J Biol Chem* 2005; 280: 24085–94. [PubMed: 15843370]
21. Zhao D, Lizardo K, Cui MH, Ambadipudi K, Lora J, Jelicks LA, et al. Antagonistic effect of atorvastatin on high fat diet induced survival during acute Chagas disease. *Microbes Infect* 2016 ; 18 :675–86. [PubMed: 27416748]
22. Lizardo K, Almonte V, Law C, Aiyyappan JP, Cui MH, Nagajyothi JF. Diet regulates liver autophagy differentially in murine acute *Trypanosoma cruzi* infection. *Parasitol Res* 2017;116 : 711–23. [PubMed: 27987056]
23. Nagajyothi F, Weiss LM, Silver DL, Desruisseaux MS, Scherer PE, Herz J, et al. *Trypanosoma cruzi* utilizes the host low density lipoprotein receptor in invasion. *PLoS Negl Trop Dis* 2011; 5: e953. [PubMed: 21408103]
24. Nojiri H, Shimizu T, Funakoshi M, Yamaguchi O, Zhou H, Kawakami S, et al. Oxidative stress causes heart failure with impaired mitochondrial respiration *J Biol Chem* 2006 ;281:33789–01. [PubMed: 16959785]
25. Kaufman BA, Kolesar JE, Perlman PS, Butow RA. A function for the mitochondrial chaperonin Hsp60 in the structure and transmission of mitochondrial DNA nucleoids in *Saccharomyces cerevisiae*. *J Cell Biol* 2003;163:457–61. [PubMed: 14597775]
26. Raucci A, Cugusi S, Antonelli A, Barabino SM, Monti L, Bierhaus A, et al. . A soluble form of the receptor for advanced glycation endproducts (RAGE) is produced by proteolytic cleavage of the membrane-bound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10). *FASEB J* 2008 ;22:3716–27. [PubMed: 18603587]
27. Chuah YK, Basir R, Talib H, Tie TH, Nordin N. Receptor for advanced glycation 449 end products and its involvement in inflammatory diseases. *Int J Inflam* 2013 ; ;2013:403460
28. Higuchi MD, Benvenuti LA, Martins Reis M, Metzger M. Pathophysiology of the heart in Chagas' disease: current status and new developments. *Cardiovasc Res* 2003 ;60:96–07. [PubMed: 14522411]
29. Aon MA, Bhatt N, Cortassa SC. Mitochondrial and cellular mechanisms for managing lipid excess. *Front Physiol* 2014; 5:282 [PubMed: 25132820]
30. Green A, Rumberger JM, Stuart CA, Ruhoff MS. Stimulation of lipolysis by tumor necrosis factor- α in 3T3-L1 adipocytes is glucose dependent. *Diabetes* 2004 ;53:74–81. [PubMed: 14693700]
31. Fisher-Wellman KH, Ryan TE, Smith CD, Gilliam LA, Lin CT, Reese LR, et al. A direct comparison of metabolic responses to high-fat diet in C57BL/6J and C57BL/6NJ mice. *Diabetes* 2016 ;65 :3249–61. [PubMed: 27495226]

Figure 1. Diet regulates the morphology of the heart during early (A, 100DPI) and late (B, 150DPI) stages of chronic infection (n=8).

A. MRI analysis both at diastole and systole condition showed significantly decrease in the left ventricle internal diameter (LVID) in the infected HFD fed mice compared with infected RD fed mice at early chronic stage of infection d100pi. The infected HFD mice showed no significant difference to uninfected RD mice (LVID systole). MRI analysis both at diastole and systole condition showed significantly decrease in the right ventricle internal diameter (RVID) in the infected RD fed mice compared with uninfected RD fed mice at d100pi. However, the infected HFD mice showed no significant difference to uninfected RD mice with RVID measurements at d100pi.

B. MRI analysis both at diastole and systole condition showed a significant decrease in the left ventricle internal diameter (LVID) and wall thickening of the ventricles in the infected HFD mice compared to infected RD mice at late stages of chronic infection (d150pi). More importantly, the infected HFD mice showed significantly increased RVID both at diastole and systole condition compared to all the other experimental groups at d150pi. The error bars represent standard error of the mean. * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ compared to uninfected RD mice. # $p < 0.05$, ## $p < 0.01$ or ### $p < 0.001$ compared to infected RD mice.

Figure 2. Histology of the myocardium of mice during early (A) and late (B) stages of chronic infection (n = 8, minimum five images/section were analyzed).

(A) H&E staining showed significantly increased inflammatory infiltrates (arrow) in the hearts of infected mice relative to uninfected mice fed on either RD or HFD and in uninfected HFD mice hearts compared to the hearts of uninfected RD fed mice at d120pi.

(B) H&E staining showed significantly more damage (inflammation and hypertrophy) in the hearts of infected HFD fed mice compared to the infected RD fed mice in RV at d160pi. The hearts of infected HFD mice showed increased levels of lipid droplets and capillary size in LV compared to the infected RD mice (Additional images are presented in supplemental Fig. 2). (arrow – inflammatory infiltrates, arrow head –hypertrophy and bar –100µm).

Figure 3. Histological grading of the H&E sections.

Histological grading of cardiac pathology was carried out in the experimental groups and classified in terms of degree of inflammation (inflammatory infiltrates), hypertrophy, presence of lipid droplets/granules and increase of capillary size. Each class was graded on a six point scale ranging from 0 to 5+ as discussed in the Methods section, and presented as a bar graph. The error bars represent standard error of the mean. * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ compared to uninfected RD 495 mice. # $p < 0.05$, ## $p < 0.01$ or ### $p < 0.001$ compared to infected RD mice.

A **B**

Figure 4. HFD reduces cardiac ApoB levels during chronic infection.

(A) IHC analysis using anti-ApoB staining demonstrated a significant decrease in the levels of ApoB both in the RV and LV of infected HFD fed mice compared with uninfected (RD and HFD) mice during early chronic infection (d120pi) (n = 8).

(B) Cardiac ApoB levels significantly decreased in both the RV and LV of infected HFD mice compared to the infected RD mice at later stages of chronic infection (d160pi) as indicated by IHC analysis (n=8). These images were quantitated and presented as bar graphs in Supplemental Figure 3.

Figure 5. Immunoblot analysis of the hearts demonstrated that HFD enhances disruption of cardiac lipid metabolism during chronic Chagas disease.

(A) Protein levels of Abca1, CETP and PPARα were altered in the hearts during chronic *T. cruzi* infection. The levels of these proteins significantly decreased in infected HFD mice compared to infected RD mice during the later stages of chronic infection (d160pi) (n=8).

(a) Fold changes in the protein levels of Abca1, CETP and PPARα were normalized to GDI expression and represented as the bar graph.

(B) The cardiac levels of lecithin-cholesterol acyltransferase (LCAT) and the key proteins involved in fatty acid metabolism such as fatty acid synthase (FAS), acyl-coA synthase (ACSL1), and ATP citrate lyase were significantly decreased in the infected HFD fed mice compared to infected RD fed mice during late chronic stage (d160pi) (n=8).

(b) Fold changes in the protein levels of LCAT, FAS, ACSL1, and ATP citrate lyase were normalized to GDI expression and represented as the bar graph.

The error bars represent standard error of the mean. * p < 0.05, ** p < 0.01 or *** p < 0.001 compared to uninfected RD mice. # p < 0.05, ## p < 0.01 or ### p < 0.001 compared to infected RD mice.

Figure. 6: Immunoblot analysis of the hearts demonstrated that HFD affects the mitochondrial respiratory response and cardiac inflammation during chronic Chagas disease.

(A) Protein levels of SDHA1 and HSP 60 were altered in the hearts during chronic *T. cruzi* infection. The levels of SDHA1 proteins significantly decreased in infected HFD mice compared to infected RD mice during the later stages of chronic infection (d160pi) (n=8).

(a) Fold changes in the protein levels of SDHA1 and HSP60 were normalized to GDI expression and represented as the bar graph.

(B) Protein levels of RAGE (52kD) and cleaved RAGE (48 and 25kD) were altered in the hearts during chronic *T. cruzi* infection. The levels of cleaved RAGE (25kD) were significantly increased in the infected HFD mice compared to infected RD mice at early chronic stage (d120pi), but were then significantly reduced during the later stages of chronic infection (d160pi) (n=8).

(b) Fold changes in the protein levels of RAGE (52kD) and cleaved RAGE (48 and 25kD) were normalized to GDI expression and represented as the bar graph.

(C) Protein levels of TNF α and IFN γ were altered in the hearts during chronic *T. cruzi* infection. The levels of TNF α and IFN γ proteins significantly decreased in the infected HFD mice compared to infected RD mice during the later stages of chronic infection (d160pi) (n=8).

(c) Fold changes in the protein levels of TNF α and IFN γ were normalized to GDI expression and represented as the bar graph.

The error bars represent standard error of the mean. * p < 0.05, ** p < 0.01 or *** p < 0.001 compared to uninfected RD mice. # p < 0.05, ## p < 0.01 or ### p < 0.001 compared to infected RD mice.