UNIVERSIDADE FEDERAL DO MARANHÃO – UFMA

CENTRO DE CIÊNCIA BIOLÓGICAS E DA SAÚDE – CCBS

CURSO DE MEDICINA

"POST-WEANING EXPOSURE TO HIGH-SUCROSE DIET LEADS TO EARLY CARDIOMETABOLIC SYNDROME AND DIABETIC CARDIOMYOPATHY ONSET"

Gilberto de Holanda Lopes Filho

SÃO LUÍS – MA

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Trabalho de conclusão de curso apresentado à Faculdade de Medicina – UFMA, como requisito básico para obtenção de grau médico.

Orientador: Prof. Dr. Antonio Marcus de Andrade Paes. Departamento de Ciências Fisiológicas – DFC – UFMA.

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“A maioria das gaivotas não se preocupa em aprender mais do que o mínimo, no que se refere a voar – como ir da praia até a comida e voltar. Porém, Fernão Capelo Gaivota não era uma ave igual às outras [...]. Para esta gaivota, o que importava não era a comida, mas o voo.

Podemos nos erguer da ignorância, [...] podemos aprender a voar! [...] o mundo é o desconhecido.”

- Richard Bach, Fernão Capelo Gaivota.

.....

“A mente que se abre a uma nova ideia jamais retorna ao seu tamanho original.”

- Albert Einstein

RESUMO

Nas últimas décadas, o aumento na prevalência de doenças metabólicas correlacionou-se com o aumento do consumo de açúcar refinado, principalmente entre os jovens. Assim, as doenças cardiovasculares, principal causa de morbimortalidade nessa população, pode ocorrer de forma precoce e levar a desfechos cardiovasculares negativos mais prematuros. Recentemente, a cardiomiopatia diabética (DCM) surgiu como uma nova entidade clínica diretamente relacionada ao diabetes (DM), com subsequente progressão para insuficiência cardíaca, na ausência de fatores de risco cardiovascular clássicos. Neste contexto, o objetivo deste estudo foi investigar os efeitos da exposição a uma dieta rica em sacarose (HSD) enriquecida em 25% sobre parâmetros cardiometabólicos e histologia do tecido cardíaco desde os 21 (pós-desmame) até 120 (jovens adultos) dias de vida em ratos *wistar* machos.

Nossos resultados apontaram que, a partir dos 60 dias de vida, os animais HSD apresentaram aumento da adiposidade visceral (~ 25% aumento no tecido adiposo peri-epididimal) na ausência de sobrepeso e desenvolveram os 3 principais marcadores do DM: hiperglicemia, resistência insulínica e hipertrigliceridemia. Ao fim da intervenção dietética, os animais HSD também desenvolveram hipertensão sistólica e diastólica e marcadores histológicos de remodelamento patológica nas amostras de ventrículo esquerdo, como fibrose perivascular e hipertrofia dos cardiomiócitos.

Em conclusão, observamos que o consumo de sacarose durante os primeiros dias de vida pode levar ao início precoce da síndrome cardiometabólica e também induzir anormalidades miocárdicas compatíveis com uma fase de transição entre mecanismos pró-adaptativos e remodelamento patológica, que é característico do estágio pré-clínico da DCM. Além disso, nosso estudo sugere que o prolongamento do período de acompanhamento ou o aumento da concentração de sacarose na dieta pode levar a um fenótipo completo de DCM neste modelo animal.

Palavras-chave: Dieta Rica em Açúcar; Sacarose; Diabetes; Cardiomiopatia Diabética; Síndrome Cardiometabólica.

ABSTRACT

In the last decades, the increase in prevalence of metabolic disease correlated with increase in refined sugar intake, mainly among youth. This way, cardiovascular disease, main cause of morbimortality in this population, may have early onset and lead to premature negative cardiovascular outcomes. More recently, diabetic cardiomyopathy (DCM) emerged as a new clinical entity directly caused by diabetes (DM), with subsequent progression to heart failure in absence of classic risk factors. In this context the aim of this study was to investigate the effects of exposure to a 25% enriched high-sucrose diet (HSD) over cardiometabolic parameters and cardiac tissue histology since 21 (post-weaning) to 120 (young adults) days of life in male wistar rats.

Our results pointed that since 60 days of life, HSD animals presented increased visceral adiposity (~25% increased peri-epidydimal adipose tissue), without increased body weight and developed the main 3 markers of DM hyperglycemia, insulin resistance, hypertriglyceridemia. In the end of dietary intervention, HSD animals also developed systolic and diastolic hypertension and histological markers of pathological remodeling in ventricle samples, such as perivascular fibrosis and cardiomyocyte hypertrophy.

In conclusion, we observed that sucrose intake during early life nutrition may lead to premature onset of cardiometabolic syndrome and also induce myocardial abnormalities compatible with a transition from pro-adaptative mechanisms to pathological remodeling process, characteristic of a pre-clinical stage of DCM. Also, our study suggests that prolongation of follow up period or increasing sucrose concentration in diet may lead to a full DCM phenotype in this animal model.

Key-words: High-sugar Diet; Sucrose; Diabetes; Diabetic Cardiomyopathy; Cardiometabolic Syndrome.

SUMÁRIO

"POST-WEANING EXPOSURE TO HIGH-SUCROSE DIET LEADS TO EARLY CARDIOMETABOLIC SYNDROME AND DIABETIC CARDIOMYOPATHY ONSET"

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INTRODUCTION

Over the past few decades, the unhealthy dietary pattern and the sedentary lifestyle have contributed to the increased prevalence of type 2 diabetes (DM2). According to International Diabetes Federation (2017), the problem has reached pandemic proportions, with around 425 million of adults with DM2 worldwide (1). Along with this scenario, it has been evidenced a global excessive consumption of added-sugar foods, especially among the younger population, which are more susceptible to consume palatable aliments.

accordingly, they presents the higher rates of dietary sugar intake – beyond the World Health Organization recommendation of no more than 10% of the daily caloric intake (ideally less than 5%) (2,3).

Consequently, the exposure to high dietary sugar during early life nutrition may induce a deleterious metabolic programming pattern, thus, leading to enhanced risk of developing metabolic disorders as DM2 and adverse cardiovascular outcomes during youth life and adulthood (4,5). Indeed, several studies have already demonstrated the causal relationship between consumption of foods with added fructose-containing sugars (mainly presented as sucrose and high fructose corn syrup - HFCS), and DM2 in animal and human models, with high or isocaloric sugar enriched diets (6–10).

While glucose is the main sugar that circulates physiologically in the blood, about 90% of the fructose component is absorbed in the liver and plays a central role in the pathophysiology of DM2 related to the excessive consumption of sugar-rich diets (11,12). Glucose and fructose are metabolized by different enzymes in the liver, where, in comparison to the glycolytic pathway, the fructolytic pathway has more intense activity, being, in contrast, insensitive to the cellular energy status and, in addition, neither allosterically regulated nor feedback inhibited (12–14). Therefore, the liver generates a large amount of substrates for the intermediary metabolism of carbohydrates and lipids, interfering with the regulatory signaling pathways and also leading to the activation of the transcription factors involved in lipogenic and gluconeogenic pathways, ultimately resulting in the three main components of DM2: hyperglycemia, insulin resistance and hypertriglyceridemia (14–16).

On the other hand, is widely known that DM2 is an independent cardiovascular risk factor. Actually, diabetic individuals are 2 to 3 times more prone to have cardiovascular disease (CVD), which, in turn, is the main cause of death in this population, thus becoming an important public health problem with high morbimortality rates and significant impact on countries economic production and health care costs (1).

In regard to the direct heart damage in diabetic patients, it has been recently recognized an individual clinical entity known as “Diabetic Cardiomyopathy” (DCM), which is characterized by abnormalities in myocardial structure and function, expressed as early subclinical impairment in diastolic relaxion. In later stages, occur subsequent development of systolic dysfunction and progression to symptomatic heart failure (HF) in the absence of coronary artery disease, hypertension, severe valvar disease or other classic cardiovascular risk factors such as age, obesity, smoking and dyslipidemia (17,18).

Actually, diastolic dysfunction is present in about 25-60% (18) of DM2 patients, among which, the prevalence of HF ranges around 19-36% (19) with a described HF incidence of 39% (19). In fact, each rise of 1% in hemoglobin A_{1c} is independently associated with an 8% increased HF risk, suggesting that glycemic control disruption plays a central role in the onset and pathophysiology of DCM (19,20). In addition to hyperglycemia, systemic insulin resistance, increased fatty acids production and impaired myocardial insulin signaling are the major metabolic abnormalities in DM2 involved in the pathogenesis of DCM and are all positively correlated with the excessive dietary intake of refined carbohydrate, specially fructose-containing sugars (19,21).

Taking this in account, the studies have demonstrated that the rise in DM2 prevalence occurred in parallel with the enhanced dietary sugar intake and support the arguments that hepatic fructose metabolism is a central link between DM2 and DMC, independently of body mass index (BMI), obesity and caloric intake (21).

Thus, the aim of this study was to investigate and characterize the role of an early exposure to high-sucrose diet in inducing metabolic abnormalities compatible with a DM2 status and the development of histological markers of pathological cardiac remodeling associated with the onset of DCM in male rats.

METHODS

Animals, experimental groups and study design

For this study, Wistar-Hannover rats were provided by Federal University of Maranhão animal facility house and all the experimental protocols were approved by the Committee for Ethics and Welfare on Animal Use – CEUA, under register number 23115.007690/2016-10.

After weaning, (postnatal day 21), the animals were randomized in two groups: control group (CTR, *n*=8), fed with standard chow (Nuvital®, Nuvilab, Brazil) and the high-sucrose diet group (HSD, *n*=8), fed with a 25% enriched sucrose chow manufactured as previously described (22). The animals were maintained under environmental controlled conditions (temperature: 22 ± 3 °C; humidity: 60 % and 12h light/dark cycle) with airflow and free access to water and chow for 14 weeks, until the postnatal day 120 (young adults).

During the dietary intervention period, body weight and food intake were measured twice a week. Additionally, blood samples were collected after 8h fasting period for biochemical measurements (glycemia, triglyceridemia and serum total cholesterol levels) at 30, 60, 90 and 120 postnatal days.

At the end of the follow-up (postnatal day 120), after 8 hours fasting, the animals were anesthetized (40:10 mg/kg ketamine:xylazine solution) and euthanized by decapitation. By laparotomic surgical approach were obtained organs and tissue samples for weighing, morphometric and histological analysis (to know: peri epidydimal, retroperitoneal and mesenteric fat tissues, central lobe of the liver, soleus and gastrocnemius skeletal muscles and heart) as well as blood samples for biochemical assessment.

Diets composition

The CTR chow, according to manufacturer, is composed by: 55.4% total carbohydrate (10% sucrose), 21% protein, 5.2% total lipids, totaling 3.52 kcal/g. In the other hand, the HSD is composed by: 65% total carbohydrate (25% sucrose),12.3% protein, 4.3% total lipids, totaling 3.48 kcal/g (22). Thus, although the HSD presents higher carbohydrates proportion, both chows have very similar caloric composition, allowing a focused evaluation of effects macronutrients *per si* in animal metabolism, without interference of a primary dietary energy surplus.

Assessment of glucose-insulin axis function

In order to assess glucose-insulin axis, at 30, 60, 90 days of life and in the last week of follow up, were performed intraperitoneal glucose tolerance test (ipGTT) and intraperitoneal insulin tolerance test (ipITT). For ipGTT, animals undergone an 8h fasting period prior to administration of glucose at dose of 2 g/kg. For glycemia measurement with a glucometer (Accucheck Active®, Roche

Diagnostic, Germany), tail vein blood drops were collected immediately before (time 0) and 15, 30, 60 and 120 min after glucose load as previously described by our research group (9,10). For the ipITT, animals were fed and received 1 UI/kg insulin (Humulin 70/30®, Lilly, USA) and, then, blood samples were collected at time 0, 5, 15, 18 and 20 min for glycemic measurements (23). The insulin resistance was evaluated with the Fasting triglycerides-glucose (TyG) index calculation [ln (fasting glucose (mg/dL) × fasting triglyceride (mg/ dL)) / 2], recently suggested to be a marker of hepatic insulin resistance (9,10) and as a potentially useful tool for predicting T2DM development in clinical practice (24).

Cardiovascular characterization

The Cardiovascular data were obtained by Panlab® non-invasive blood pressure measurement device, model LE 5002, used to measure systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR). For this purpose, the animals were immobilized with a containment device after they have passed through a 3-5 minutes period in a chamber heated to 40ºC so that dilation of the blood vessels of the tail occurs. A reliable value corresponds to the average of 3 consecutive measurements.

Histology Samples Preparation

For this purpose, cross-sectional cut of the ventricle was made at the midpoint between the cardiac apex and the atrioventricular groove, at the level of papillary muscle. The samples were fixed in 4% paraformaldehyde for 24 hours, after which, underwent dehydration process and were embedded in paraffin blocks. Then, slices of 5µm thickness were obtained from the embedded samples. For visualization of the muscular components (in red) and of the fibrotic

tissue (in blue), it was used Masson's Trichrome staining (MTS) technique, as described elsewhere (25,26).

Diabetic Cardiomyopathy Histological Parameters

To investigate and characterize the pathological cardiac remodeling features associated with DCM, the slides of ventricle samples were photographed using an optical microscope coupled to a Nikon Eclipse Ti-U image acquisition system. The images obtained were then analyzed using the Fiji-ImageJ v1.52 software with semi-automated methodology standardized by our research team for determination of the following parameters: total myocardial area, left ventricular free wall thickness, perivascular fibrosis, interstitial fibrosis, and cellular hypertrophy.

Initially the images were processed to standardize and equilibrate levels of brightness and contrast. In order to assess the rates of interstitial and perivascular fibrosis, the Trainable Weka Segmentation plug-in, free provided by the Fiji-ImageJ software developer, was used to create an algorithm-based color classifier for automatic color segmentation in MTS samples (red MTS for muscle cardiomyocyte fibers = set as red color; blue MTS for fibrous tissue = set as green color; and the white image background = set as purple color). The correspondence of staining colors and the color segments generated by the algorithm can be very precisely adjusted manually, until a reliable result is achieved. After this step, as a result, the Trainable Weka Segmentation plug-in generated a "color classifier" file that can be loaded and applied on all the sample images automatically. In the sequence, the new produced image files are processed by a "color deconvolution" tool, responsible for the separation of each

color component in new image files: fibrous tissue (green), cardiomyocyte muscle fibers (red) and background (purple).

We standardized our methodological approach combining the most described protocols in literature regarding automated and semiautomated histologic analysis of interstitial and perivascular fibrosis (25,27–32), whose efficacy has been already shown to be reliable and in accordance to a pathologist characterization (25,26). For a detailed comprehension of the image processing and software analysis view FIG.1. The percentage of perivascular fibrosis was calculated as the ratio between vessel area (μm^2) and perivascular fibrosis area (μm^2). And the percentage of interstitial fibrosis was obtained as the ratio between the area of interstitial fibrosis (μm^2) and the cardiomyocytes total area (μm^2). For evaluation of muscle hypertrophy, 20x magnification images of cardiomyocytes were obtained on topography of the papillary muscle and the mean cross-sectional area (μm2) of 100 cardiomyocytes per sample was calculated (33,34).

Statistical Analysis

Statistical analysis was performed using Graph-Pad Prism 7.0 software (GraphPad Software Inc., USA) and data was expressed as mean ± SEM and compared by means of unpaired t test (one-tailed) for a significant difference of 5% ($p<0.05$).

RESULTS

Early exposure to high-sucrose diet leads to metabolic abnormalities compatible with a type 2 diabetes phenotype, without the occurrence of weight gain

At the end of dietary intervention, both animal groups presented similar body mass (FIG.2 – A) and food intake (FIG.2 – B), however, the

morphometric analysis of the collected tissue samples evidenced that HDS animals had ~25% increased peri-epididymal adipose tissue(HSD= 1,75 ± 0,06 *vs*. CTR= 1,40 ± 0,08; p= 0,0045), while their muscular mass (by means of soleus muscle) was ~9,5% decreased (CTR= 0,042 ± 0,001 *vs.* HSD= 0,038 ± 0,0006; p= 0,0405) compared to CTR group (TAB.1), indicating a body mass redistribution, characterized by increased adiposity and a lower muscle mass. This find is in agreement with the absence of weight difference between HSD and CTR groups, once they had a similar food intake. Also, there was no difference in relative mass of heart or liver, suggesting that the metabolic alterations were not intense or prolonged enough to promote structural alterations at tissue levels in these organs.

In addition, post-weaning exposure to HSD was able to induce metabolic disruption by impairing glucose and lipids homeostasis, with serum biochemical profile assessment showing the occurrence of hyperglycemia (HSD= 92,63 ± 1,832 *vs.* CTR= 85,25 ± 1,048; p= 0,0018) (FIG.3 – A,B,C) and hypertriglyceridemia (HSD= 131,4 ± 24,75 *vs.* CTR= 75,46 ± 15,48; p= 0,0380) (FIG.3 – D,E,F) in the experimental HSD group, without differences in total cholesterol levels (FIG.3 – G,H,I).

In regard to glucose-insulin axis function evaluation CTR and HSD animals were subjected to ipGTT, ipITT and TyG index calculation. In the ipGTT, after administration of glucose bolus, both groups reached glucose peak levels within 15 min and returned to baseline in 2 hours. The area under curve calculation (ipGTT AUC) indicated that the HSD animals had higher glucose intolerance when compared to CTR group (HSD= 168,65 ± 17,30 *vs.* CTR= 100,27 ± 14,91; $p<0,05$) (FIG.4 – A).

Accordingly, during the ipITT, the HSD animals showed an impaired response to insulin administration, indicating that these animals had increased systemic insulin resistance compared to CTR, as demonstrated by the ipTT AUC calculation (HSD= 131,4 ± 24,75 *vs.* CTR= 75,46 ± 15,48; p= 0,0380) (FIG.4 – B). Once there is a significant association between high sugar intake and increased hepatic triglycerides production with insulin resistance (13,24), the TyG index was calculated and showed that HSD animals had hepatic insulin resistance compared to CTR (HSD= 8,57 ± 0,21 *vs*. CTR= 7,92 ± 0,20; $p < 0,05$) (FIG.4 – C,D,E).

In summary, our results evidenced that the post-weaning exposure to HSD was able to promote increased visceral adiposity and loss of lean mass without changes in food intake or body weight and also promote metabolic disruption, with impaired glucose and lipids homeostasis resulting in hypertriglyceridemia, hyperglycemia and hepatic and systemic insulin resistance, the main markers of DM2 onset.

High-sucrose diet induces alterations in cardiovascular physiology leading to increased arterial pressure values

In the last week of follow up, the mean of heart rate (HR), mean arterial pressure (MAP), systolic (SBP) and diastolic (DSP) blood pressure were obtained to evaluate the effects of the experimental high-sucrose diet on the cardiovascular parameters (FIG.5). As result, the HSD animals exhibited higher levels of systolic (HSD= 157,9 ± 3,812 *vs.* CTR= 130,3 ± 7,612; p= 0,0158) (FIG.5 – B) and diastolic (HSD= 128,9 ± 5,547 *vs.* CTR= 107,8 ± 3,323; p= 0,0155) (FIG.5 – D) blood pressures compared to control, while no differences were observed in HR or MAP.

This way, post-weaning intake of the high-sucrose diet can induce early onset of cardiovascular disturbs, resulting in increased systolic and diastolic blood pressure, consequently promoting a higher risk of cardiovascular fatal and non-fatal events in younger ages.

High-sucrose-induced DM2 is associated with histological alterations compatible with pre-clinical stage of DCM

In order to investigate the occurrence of tissue level concentric hypertrophy characteristic of later stages of DCM (19), we evaluated, using the histological slides images, the total myocardial area (FIG.6) and also the thickness of left ventricle free wall (FIG.7) and found that, nevertheless, there were no significant differences between the experimental groups. So, we proceeded to investigate earlier pathologic structural and cellular abnormalities in the ventricle samples.

In this regard, our histological analysis showed that, although there was no evidence of interstitial fibrosis (FIG.8) compatible with significant diastolic relaxation impairment, there was higher ratios of perivascular fibrosis (HSD= 3,174 ± 0,4327 *vs.* CTR= 1,442 ± 0,2473; p= 0,0042) (FIG.9) and cardiomyocyte cellular hypertrophy (HSD= 767987 ± 49121 *vs.* CTR= 570501 ± 29097; p= 0,0043) (FIG.10), which, in turn, are findings related to initial stages of DCM, during transition from pro-adaptative to maladaptive alterations.

Thus, the early exposition to our experimental high-sucrose diet was capable to induce pre-clinical DCM in young adult (120 days of life) male rats, showing that DM2 target-organs lesions had early onset and can, consequently, evolve to clinical heart failure in lower ages than expected.

DISCUSSION

The focus of this study was to evaluate the effects of an early exposition to a 25% sucrose enriched diet in the cardiometabolic status and over the cardiac ventricular tissue histological parameters in order to find out if this dietary intervention would anticipate the onset of DM2 and its associated target-organs injuries, such as DCM.

In this context, we observed that, by the end of 120 days of life, both animal groups presented similar body weight and food intake. In fact, literature data indicate that the effects of sucrose-enriched diets on body weight mainly depends on the caloric composition (isocaloric or hypercaloric diets), sucrose concentration, the type of sucrose intake (added to chow or to drinking water) and period of exposition (35–37). As a result of the diversity in methodological approaches, there are divergent metabolic phenotype characterization, but, nevertheless, the evidences indicate that high fructose-containing sugar diet is reliable method to early induce a lean DM2 phenotype (35) with progressive development of obesity, hypertension, dyslipidemia and hyperuricemia, thus, further configuring a complete metabolic syndrome phenotype in both animals and human studies (12,36,38).

In agreement with this, in our study, tissue and organ morphometric analysis showed that, despite the absence of difference in weight, HSD animals presented a redistribution of body composition, characterized by increased visceral adiposity and reduced lean mass, suggesting that a longer period of dietary intervention would, as well, result in higher body weight compared to CTR.

In regard to food intake, we found no differences between experimental groups and the literature evidences are also controversial. Even though the high palatability of added sugars foods can activate the neural reward systems and

lead to hyperphagia (39), some studies have reported different results in regard to food intake (10,40,41). Actually, there are several controversies in respect to fructose and/or glucose actions over the hypothalamic regulation of appetite and satiety and its modulation by diet composition, single effects of glucose and fructose on insulin and anorexigenic and orexigenic hormone responses, period of dietary exposition and metabolic profile (42–44).

It is though that the initial stimulus to enhanced added-sugar food intake, after a period of time, can undergo a neuroadaptative process mediated by the organism response to increased sugar intake and its metabolic products, thus, leading to abnormalities in hypothalamic appetite regulation and suppression of reward system as a protective mechanism against the metabolic stress state (42,45–47).

More recently, it has been suggested the important role of the carbohydrate-induced expression of Fibroblast Growth Factor (FGF)-21 as a modulator of sugar intake and sweet taste preference by inducing satiety signals to suppress sugar intake and also stimulating lipids beta-oxidation, emerging, this way, as a potential protective mechanism against excessive sugar intake and metabolism disruption associated to fructose component of sucrose (16,48)

In the metabolic context, fructose hepatic metabolism is thought to be the main factor associated to DM2 development. In contrast to glucose, that is widely distributed through circulation and absorbed by insulin sensitive tissues such adipocytes and muscles, around 90% of ingested fructose is absorbed by the liver, where it is rapidly metabolized into fructose-1-phosphate by fructokinase. While the glycolytic pathway is strictly regulated by hormones, cell energy status and subjected to several rate-limiting steps, the fructolytic pathway has more

intense metabolic activity (fructokinase is around 10 times more active than glucokinase), is independent of hormonal, insensitive to cell ATP/ADP ratios, it is not feedback inhibited and converges with end step of glycolysis pathway in trio-phosphates production bypassing all the regulatory and limiting steps of glucose degradation.

As consequence, fructolysis generates large amount of substrates that can be used to ATP production or, given the excess trio-phosphates availability, in the intermediate metabolism to produce carbohydrates and lipids via gluconeogenesis and DNL pathways, respectively. In addition to that, fructose is reported to be a potent inducer of lipogenesis and gluconeogenesis programming in the liver, via activation of transcription factors SREBP-1c (Sterol Regulatory Element-Binding Protein – 1c) and ChREBP (Carbohydrate-Responsive Element-Binding Protein), further contributing for enhanced hepatic glucose production and triacylglycerol exportation as VLDL molecules, thus resulting in hyperglycemia and hypertriglyceridemia, typical features of DM2.

In agreement, our study evidenced that since 60 days of life the animals exposed to HSD presented both hyperglycemia and hypertriglyceridemia when compared to CTR group. In recent study of our group, Flister et. al. (49), using the same diets composition, evaluated metabolic parameters and markers of hepatic DNL and stress-sensitive pathways at 30, 60 and 90 days of exposition and found that at 30 days of exposition, HSD animals presented fasting hyperglycemia, hyperinsulinemia, increased visceral adiposity without weight gain, impaired glucose tolerance and increased levels of ChREBP as well as markers of ER stress and pro-adaptative UPR activation pattern. Once several steps of DNL occur in ER membrane and excessive FA production also leads to

misfolded protein accumulation and activates UPR, whose all three arms are involved in lipogenic regulation. In this same work (49), at 60 days of dietary intervention, animals developed full metabolic syndrome phenotype, with increased TAG, levels been associated with hepatic and systemics insulin resistance onset and transition to a pro apoptotic UPR programing activation, resulting in hepatic oxidative damage by 90 days of exposure, showing that this HSD-fed rat is an reliable animal model of DM2 and metabolic syndrome in a time-dependent way.

Interestingly, glucose induced pancreatic insulin release also play role as lipogenic stimuli in liver, thus increasing DNL (14,16). Under upregulated lipogenic states, accumulation of DNL byproducts, such as ceramides and DAG, in hepatocytes have been reported to be central players in development of hepatic insulin resistance via activation of atypical forms of PKC that impairs IRS-PIK3-Akt signaling pathway (50–52). In accordance, when evaluating glucose-insulin axis, we found, as expected, that, increased visceral adiposity and hypertriglyceridemia, indicators of increased sucrose-driven lipogenesis, were accompanied by hepatic and systemic insulin resistance development, evidenced, respectively, by TyG calculation, increased insulin insensitivity in ipITT and increased glucose intolerance.

In comparative study regarding fructose *vs*. glucose hepatic metabolism, Geidl-Flueck and Gerber (14) reunited evidence indicating that fructose may increase hepatic lipogenic programing also in an insulin-independent way, given that LIRKO (Liver Insulin Receptor Knockout) animals, presented high levels of ChREBP and SREBP-1c, as well as DNL enzymes after fructose feeding, even in isocaloric dietary interventions. In addition, Softic et al. (16) demonstrated that

liver fructokinase (a.k.a. ketohexokinase) knockdown was able to improve hepatic lipid intermediates accumulation, liver steatosis and insulin signaling, thus evidencing that fructose is the main pathological component of sucrose contributing to DM2 development.

Another particular feature of fructose hepatic metabolism is the generation of UA, driven by the purine pathway, in response to ADP rise as result of intense consumption of ATP by fructokinase (13,14), consequently leading to serum hyperuricemia, which is considered an additional component of metabolic syndrome as well as a key risk factor for cardiovascular and renal diseases (53,54).

Considering all this, HSD-fed rat figures as a reliable physiological model to induce DM2 metabolic phenotype (hyperglycemia, hypertriglyceridemia and insulin resistance), regardless lack of initial weight gain, with time-dependent progression to a full metabolic syndrome phenotype, as previously demonstrated by our work group (10,40,49) and other experimental studies of animal models of insulin resistance (35) and metabolic syndrome (36). In sequence, to evaluate if this model is also able to induce DM2-related cardiovascular complications, we evaluated cardiovascular parameters and histological markers of cardiac microvascular dysfunction and tissue markers of DCM onset.

Indeed, when investigating the deleterious effects of fructose intake over whole body and local tissues and organs functions, Zhang, Jiao & Kong (55) reunited experimental evidence showed that, in cardiovascular system, high levels of UR and FFA was associated with increased ROS production, increased FA uptake, enhanced AGEs production, higher vascular tonus, local inflammatory

activation and hypertension, as well as lower insulin sensitivity, less glucose usage and impaired endothelial-dependent vasorelaxation.

In accordance, in our study, by the end of follow up period, the HSD animals exhibited systolic and diastolic hypertension, in comparison to CTR group, at 120 days of life, thus proving that exposition to fructose-containing sugars in early life nutrition lead to premature onset of cardiovascular disease by the age of young adults. Despite divergent results concerning fructose-induced hypertension, comparative studies (36,56,57) have already reinforced this animal model as a reliable promoter of hypertension. Contrary findings been associated to insufficient sucrose/fructose dietary concentrations and/or to insufficient exposure time(36,57).

Corroborating our findings, Villegas-Romero et al. (58) studied the effects of post-weaning exposure to 30% fructose-enriched drinking water in both short term (critical post-weaning window: from 12 to 28 days of life) and long term (until 7 months age) and found that both groups developed, in early adulthood, arterial hypertension in similar levels and that it was associated with decreased endothelial production of NO by eNOS, increased ROS production, decrease of antioxidant systems and impaired endothelial-dependent vasorelaxation in a context of insulin resistance and hyperinsulinemia, whereas long term exposure was also related to increased TG and vasoconstrictor molecules production (ET-1). In addition, other experimental studies have also demonstrated the important roles of oxidative stress and inflammation (59), increased RAAS (Renin-Angiotensin-Aldosterone System) (60,61), hyperuricemia (59,62) and also impaired baroreflex sensitivity and sympatho-vagal imbalance (63,64) in the physiopathology of fructose induced Hypertension.

Although the mechanisms of fructose-induced hypertension have not been completely clarified, literature points that the unique ability of fructolytic pathway to increase uric acid production figures as a central factor (65,66) in inducing endothelial dysfunction, vascular oxidative stress, inflammation and vessel smooth muscle cells proliferation, thus leading to increased vascular tonus, impaired endothelium-dependent vasorelaxation, arterial stiffness and resulting in hypertension and increased atherogenic potential (53,56,67). Moreover, hyperglycemia, hyperinsulinemia, oxidative stress and consequent increase in sympathetic nervous system activity, renin-angiotensin system activation and increased production of vasoconstrictors are also implicated in hypertension development after fructose-containing sugars intake (56,67,68).

In fact, fructose-induced excessive UA production is a potent intracellular mediator of oxidative stress, once it can activate NADPH oxidase, thus, increasing ROS production and also reducing, in circulation and target-organs, NO bioavailability via endothelial nitric oxide synthase (eNOS) uncoupling, leading to generation of peroxynitrite ($ONOO^-$), and setting a vicious cycle of oxidative stress that impairs even more endothelial function (53,69,70). In addition, redox-sensitive activation of NF-kB in endothelial cells results in a pro-inflammatory status that favors monocytes infiltration, activate growth factors and induce VSMC proliferation, pro-inflammatory cytokines release and arterial stiffness, thus, increasing vascular tonus and accelerating atherogenesis (59,71).

Other features of sucrose/fructose-induced DM2, such as insulin resistance, hyperinsulinemia, hyperglycemia and increased visceral adiposity are also implied in hypertension through several mechanisms (56,68), such AGE formation (72), increased sympathetic nervous system activity (63,73), RAAS

system (60,74), and increased production of other vasoconstrictors like endothelin-1 (58,75).

In regard to cardiac tissue involvement, although long-term hypertension, by itself, is associated with cardiac alterations and development of concentric hypertrophy, DM2-associated abnormalities can directly lead to pathological cardiac remodeling, further resulting in DCM phenotype.

In this sense, Kamide et al (61) demonstrated that, in fructose-fed rats that presented hypertension, increased sympathetic activity and left ventricle hypertrophy, the isolated pharmacological control of blood pressure was not able to improve ventricular alterations while, Ang II AT1 receptor blockage was not only able to reduce arterial pressure levels but also improved heart hypertrophy, suggesting that, cardiac alterations, actually, were less dependent of systemic hypertension and more linked to metabolic disruption context induced by fructose intake, in which RAAS systemic and local activation has been described.

Given the high energy demand and intense metabolic activity of cardiac muscle, any disturbance in metabolic status and substrates availability or usage may directly affect myocardial structure and function, leading to DCM onset (76–78). In physiological conditions, the main energy source in the heart is β-oxidation of fatty acids (accounting around 60-70% of energy production), followed by glycolytic metabolism, that responds for 30-40% of ATP synthesis (77,79). However, in diabetic subjects, the rise in circulation FA availability and uptake by the heart and the impaired utilization of glucose as an energetic substrate as consequence of DM2-induced insulin resistance are pointed, in fact, as the main initiating factors associated with DCM development (80,81).

Under these conditions, the cardiac metabolism becomes more limited to use FA as substrate and mostly relies in β-oxidation to ATP production, reflecting, this way, loss of "metabolic flexibility" – the cardiomyocytes capacity to use different molecules as substrates, including fatty acids, glucose, lactate, ketone bodies and amino acids – to equilibrate energy demand and production, in order to guarantee contractile efficiency and adequate cardiac output to systemic circulation (76,79,82).

While this shift to FA utilization it initially takes place as an adaptative mechanism, given the fact that β-oxidation produces larger amounts of ATP than glycolytic pathway, it occurs under costs of higher oxygen consumption and further results in uncoupling of energy production and metabolic demand, progressively resulting in cardiac contractile dysfunction. Actually, high circulation levels of FA are found to upregulate membrane expression of FA transport proteins in DM2, such FABP and CD36, leading to excessive FA uptake by cardiomyocytes.

The increase in intracellular fatty acids, in addition, leads to activation of nuclear transcription factor PPARα (peroxisome proliferator-activated receptor α), a key regulator of enzymes involved in mitochondrial β-oxidation, that promotes FA utilization and, thus, make temporarily possible to maintain energy production during the adaptative phase of DCM (78,83,84). Moreover, PPARα mediate PDK4 (Pyruvate Dehydrogenase Kinase 4) activation, that suppress PDH (Pyruvate Dehydrogenase), a key enzyme in glycolytic pathway, consequently contributing to greater impairment in cardiomyocyte glucose utilization (84–86).

However, FA uptake markedly overloads mitochondrial oxidative capacity, thus, leading to mitochondrial structural and functional alterations, resulting in electron transport chain inefficiency (20,87). In this scenario, dysfunctional mitochondrial is associated with increased ROS production, oxidative stress, impaired Ca^{++} handling, myocardial lipids accumulation and lipotoxicity, then, been pointed as the major contributor to contractile dysfunction in DCM (19,88–90).

Following that, FA accumulate in cytosol, where they are directed to lipogenic pathways and promote myocardial lipids accumulation as TAG molecules (cardiac steatosis), as well as accumulation of lipotoxic byproducts, such as ceramides and diacylglycerols, that are associated with development of cardiac insulin resistance, via impairment of insulin receptor activity (83,87,91).

In parallel, increased myocardial lipids synthesis raises cytoplasmatic levels of malonyl-CoA, that decrease FA transport through mitochondrial membrane via inhibition of carnitine acyl transferase I transporter (CAT I), thus, contributing to energy depletion, decreased cardiac efficiency and further progression to systolic dysfunction (91–93). Also, accumulation of ceramides and DAG have been associated with cardiomyocytes apoptosis, myocardial fibrosis, hypertrophy, impaired diastolic filling and contractile dysfunction (87,91).

Taken together, oxidative stress, lipotoxicity, mitochondrial dysfunction, hyperuricemia and NK-kB mediated pro-inflammatory programming activation impairs ER function and lead to accumulation of misfolded proteins, thus triggering the UPR. Under persistent metabolic cellular stress, UPR shifts to pro-apoptotic response, leading to cardiomyocytes death (81,94).

As well, intracellular calcium homeostasis is key factor underlying cardiac excitation-contraction coupling, and reuptake of cytosolic Ca^{++} to its main storage site, the ER, is fundamental for cardiomyocyte diastolic relaxation (20,95). In DCM rodent models, it has been described impaired activity of SERCA2a (ATP-dependent sarcoplasmic reticulum Ca^{++}-ATPase 2a), sarcolemma Na^{+}/Ca^{++} exchanger and also decreased mitochondrial Ca^{++} uptake, resulting in increased cytosolic Ca^{++} concentrations, slower Ca^{++} transients, prolongation of intracellular Ca^{++} decay and impaired Ca^{++} reuptake by sarcoplasmic reticulum (20,83,95), consequently promoting prolonged action potential duration and diastolic relaxation time and, by this means, contributing to impaired ventricular relaxation seen in early stages of DCM(19).

In our study, after the dietary intervention, histological analysis showed that HSD animals presented increased perivascular fibrosis and cardiomyocytes hypertrophy, although they did not develop interstitial fibrosis or hypertrophy at tissue level, thus, characterizing a pre-clinical stage of early onset DCM. In agreement with this, the literature points that cellular and structural alterations, that further results in diastolic dysfunction, only occurs after the transition from a pro-adaptative to a maladaptive cardiac response in consequence of intense and/or prolonged metabolic stress in the diabetic heart.

Similarly, in early stage DM2 rats fed with 10% fructose in drinking water for 6 weeks, Lou et al. (93), showed that tissue cardiac structural changes are preceded by fatty acid metabolism alterations, related to increased myocardial oxidative stress, inflammation, metabolic inflexibility, mitochondrial overload. Moreover, Nunes et al (96), Zhang et al. (97) and Xie (98) demonstrated, in

different experimental studies, that, increasing fructose concentration and time of exposure is able to induce cardiomyocyte hypertrophy and cardiac fibrosis.

In this context, among the resulting cardiac structural abnormalities, perivascular fibrosis and hypertrophy figure among the earliest findings. Mechanistically, cardiomyocytes hypertrophy occurs as an adaptative response, but, in the presence of prolonged mechanical or physiological stress, it may undergo a maladaptive phenotype, leading to deterioration of cardiac function (77,99,100).

Hypertrophy and perivascular fibrosis usually present together in consequence of inflammation, increased activity of metalloproteinases, stimulation of pro-fibrotic mediators and growth factors (100–102). More recently, DM2 driven activation of systemic and tissue renin-angiotensin-aldosterone system have also been demonstrated to activate signaling pathways involved in cardiomyocytes hypertrophy as well as perivascular and interstitial fibrosis (19,101,103). By its turn, perivascular fibrosis represents an important cardiac microvascular complication of DM2 and may lead, along with endothelial dysfunction, to a decrease in oxygen and glucose delivery in coronary circulation, thus progressively impairing cardiac efficiency (101,104). In fact, cardiomyocyte hypertrophy and fibrosis have been associated with diastolic dysfunction in animal models of DCM, including fructose intake dietary models (83,97,105,106).

CONCLUSIONS

Taken together, literature evidence and the findings pointed by our study show that post-weaning exposition to high-sucrose diet represents a reliable physiological model of DM2 onset with early development of DCM in its initial pre-clinical phase. In this context, fructose hepatic metabolism, due its lack

of inhibitory regulation, higher activity and insensitivity to cellular energy status, play a key role in the development of DM2-driven metabolic abnormalities. Fructose component of sucrose not only provide higher amounts of substrates, but also acts as a potent inductor of hepatic DNL and gluconeogenesis, resulting in hyperglycemia, hypertriglyceridemia, insulin resistance and, in addition, to hyperuricemia even in the absence of weight gain.

Moreover, DM2 metabolic alterations promotes vascular oxidative stress, eNOS uncoupling, pro-inflammatory programming activation, endothelial dysfunction, VSMCs proliferation, higher production of vasoconstrictor molecules such as Ang II and ET-1 and increased sympathetic activity. As consequence, increased vascular tonus, impaired endothelium-dependent relaxation and arterial stiffness, thus, result in arterial hypertension. In addition, the increased FA supply and impaired usage of glucose by cardiac metabolism leads to loss of cardiac metabolic flexibility, restrings ATP production to FA β-oxidation, activate PPARα transcription factors, impairing even more glucose metabolic pathway and progressively overloading mitochondrial metabolic capacity, resulting in mitochondrial dysfunction, that are thought to be the central initial factors in DCM physiopathology.

Considering this, dietary interventions to reduce sucrose/fructose containing diets consumption and a bigger comprehension of hepatic and cardiac metabolic pathways cross-talk emerge as potential therapeutic targets to decrease DM2 associated cardiovascular diseases, DCM development and to avoid progression to symptomatic heart failure, thus reducing mortality rates in this population. In the same way, it is essential to increase investigation of initial

alterations, establish consensual diagnostic criteria and focused therapeutic approach during the initial pre-clinical phases of DCM.

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CONFLICT OF INTERESTS

Authors declare the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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FIGURES, TABLES AND SUBTITLES

FIG.1 – Histopathological approach for software-based processing and semi-automated analysis of left ventricle tissue samples.

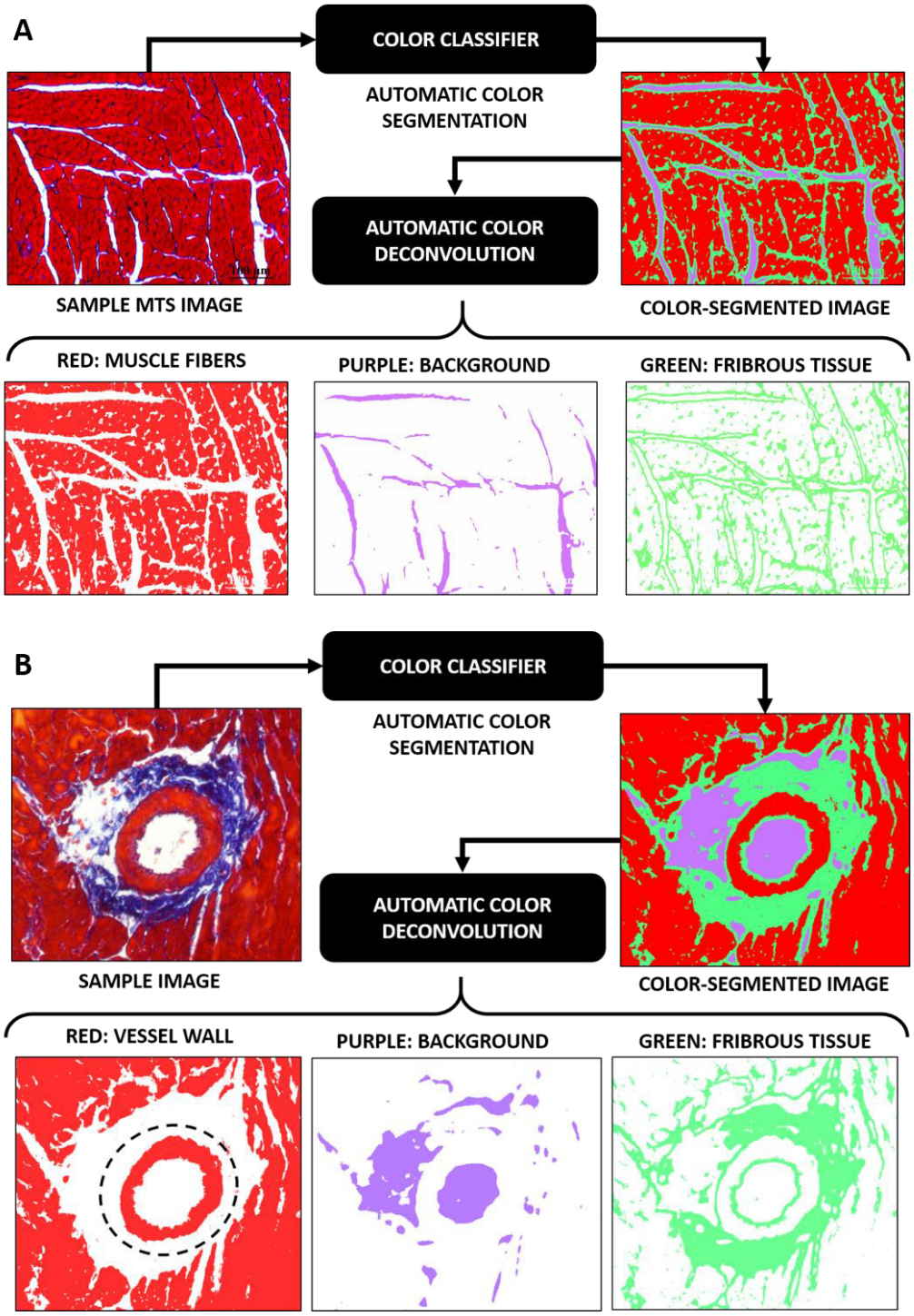

FIG.1 – Histopathological approach for software-based processing and semi-automated analysis of left ventricle tissue samples. The "Color Classifier" file generated with "Trainable Weka Segmentation" plug-in was applied on the histological samples images to identify and classify the correspondent pre-set components of Masson's Trichrome Staining (MTS). In the sequence, the "Color Deconvolution" plug-in was used to separate the components of interest: muscle fibers/vessel wall and fibrous tissue in different images. **A.** Ventricle cross-sectional samples for calculation of interstitial fibrosis ration in a 10x augment with 250µm scale bar. **B.** Ventricle microvasculature vessel sample for calculation of perivascular fibrosis ratio in 10x augment with 250µm scale bar.

FIG.2 – Body Weight and Food Intake

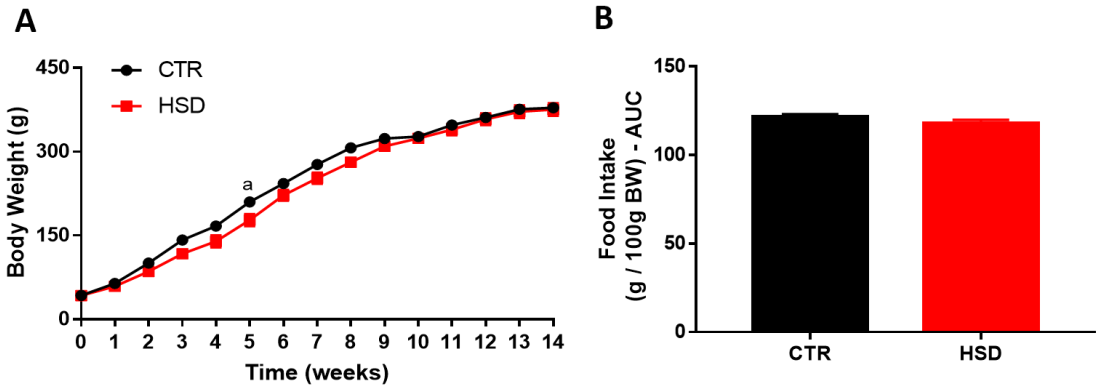

FIG.2 – Body Weight and Food Intake. A. Weekly evaluation of body weight (g). **B.** Area under curve of food intake follow up. The bars represent mean ± SEM. Statistical analysis was performed with (n=8/group) one tailed unpaired t test. The letters indicate statistically significant differences ($p<0,05$) vs. [a]CTR.

FIG.3 – Biochemical Characterization

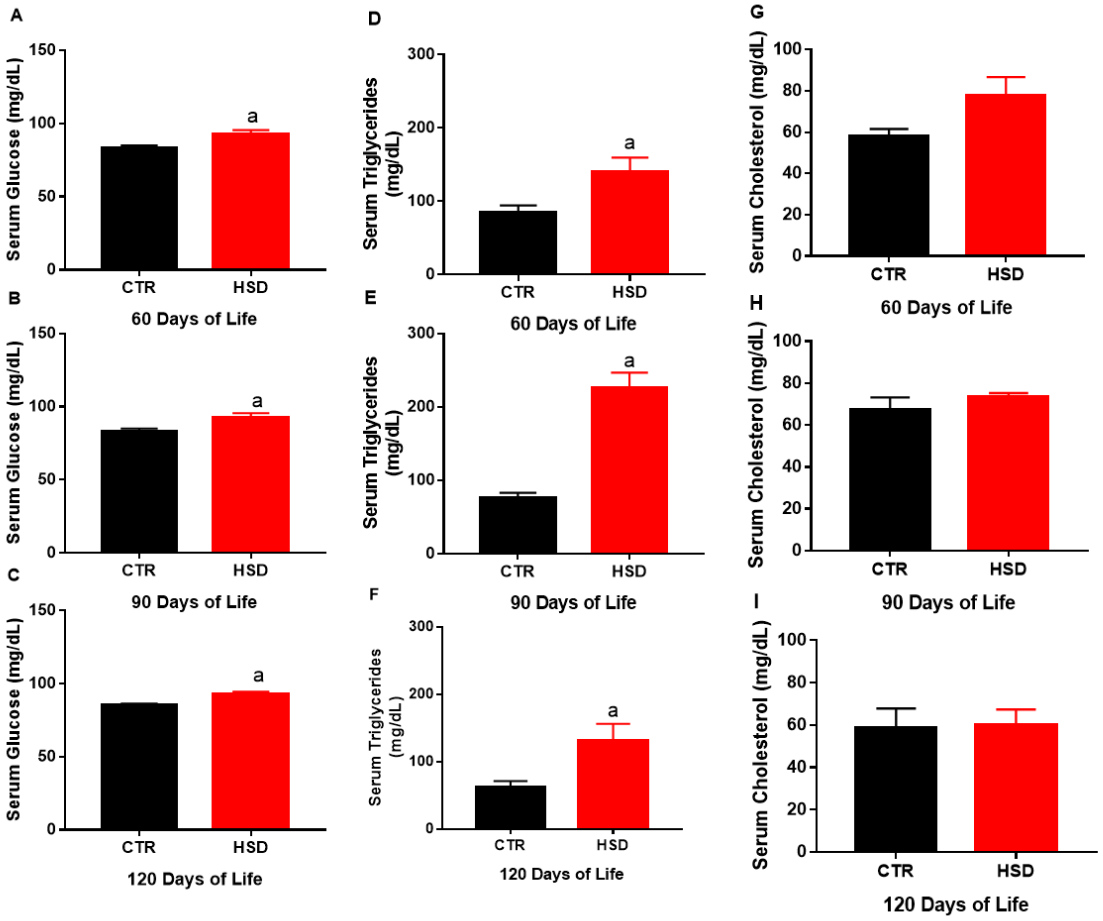

FIG.3 – Biochemical Characterization. A,B,C. Fasting serum glucose levels (mg/dL). **D,E,F**. Fasting triglycerides levels (mg/dL). **G,H,I**. Fasting cholesterol levels (mg/dL). Biochemical parameters were evaluated at 60, 90 and 120 days of life. Bars represent mean ± SEM. Statistical analysis (n=8/group) was performed with one tailed unpaired t test. The letters indicate statistically significant differences ($p<0,05$) vs. ^{a}CTR.

FIG.4 – Evaluation of Insulin-Glucose Axis

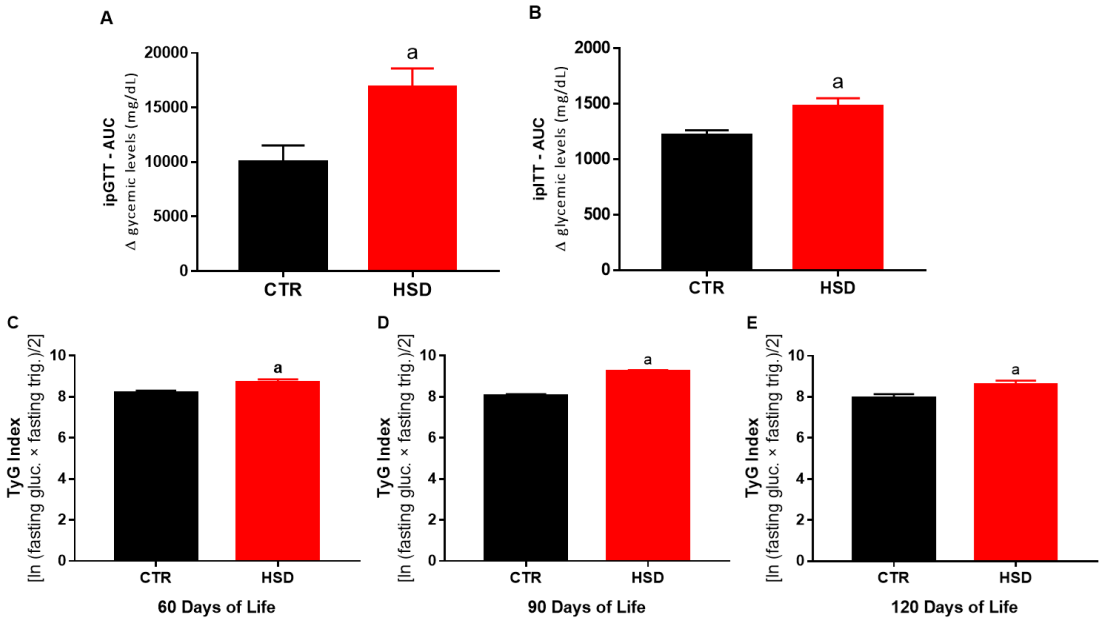

FIG.4 – Evaluation of Insulin-Glucose Axis. A. Area under curve of serum glucose levels variation during intraperitoneal glucose tolerance test (ipGTT) at the end of follow up period. **B**. Area under curve of variation of serum glucose levels during intraperitoneal insulin tolerance test (ipITT) at the ende of follow up period. **C,D,E.** TyG index [ln (fastingglucose fasting triglyceride)/2] calculated at 60, 90 and 120 days of life. Bars represent mean ± SEM. Statistical analysis (n=8/group) was performed with one tailed unpaired t test. The letters indicate statistically significant differences ($p<0,05$) vs. [a]CTR.

FIG.5 – Cardiovascular Parameters Characterization

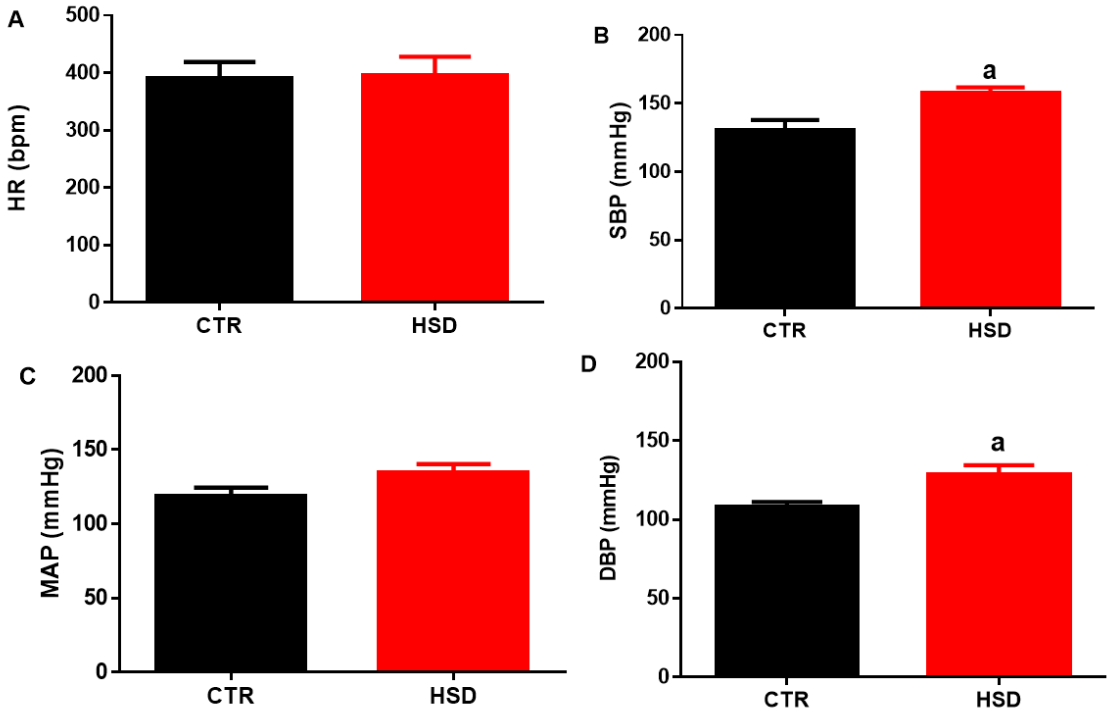

FIG.5 – Cardiovascular Parameters Characterization. A. Heart rate (HR) in bpm. **B**. systolic Blood Pressure (SBP). **C**. Mean arterial Pressure (MAP). **D**. Diastolic Blood Pressure (DBP). All the cardiovascular measures were performed in the last week of dietary intervention. Bars represent mean ± SEM. Statistical analysis (n=8/group) was performed with one tailed unpaired t test. The letters indicate statistically significant differences ($p<0,05$) vs. ^{a}CTR

FIG.6 – Total Myocardial Area

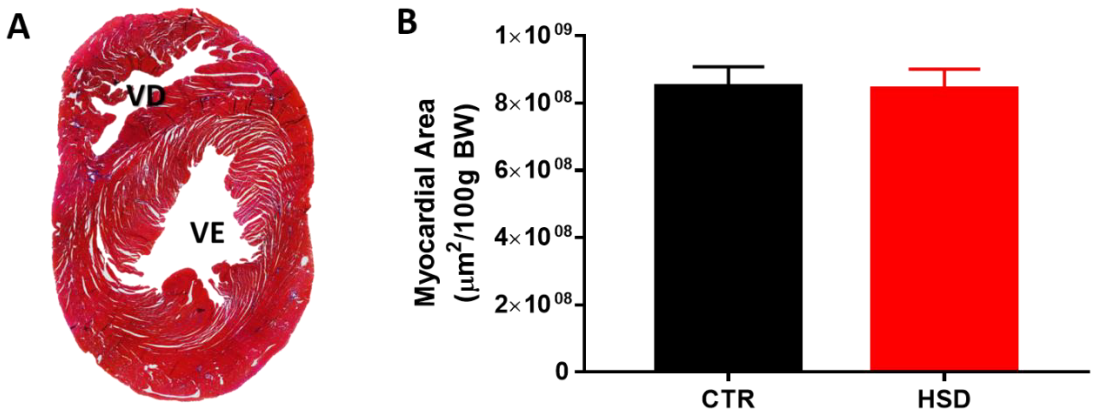

FIG.6 – Total Myocardial Area. A. Image of ventricle cross sectional area in 2x augment (250μm scale). **B.** Bars represent mean ± SEM of total myocardial area of the ventricle cross sections (n=5/group). Statistical analysis was performed with one tailed unpaired t test. The letters indicate statistically significant differences (p<0,05) vs. [a]CTR.

FIG.7 – Left Ventricle's Free Wall Thickness

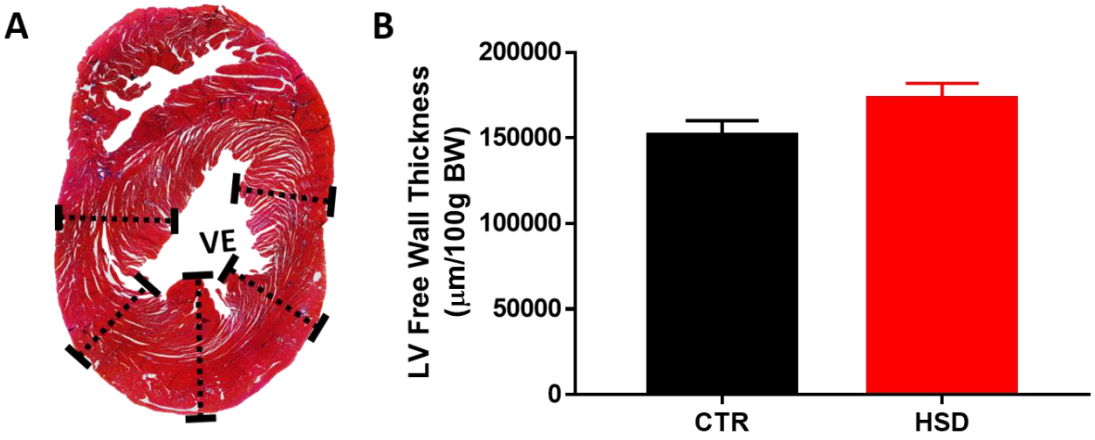

FIG.7 – Left Ventricle's Free Wall Thickness. A. Image of ventricle cross sectional area in 2x augment (250µm scale), indicating the points of measurement for obtention of mean thickness of left ventricle free wall. **B.** Bars represent mean ± SEM of left ventricle's free wall thickness (n=5/group). Statistical analysis was performed with one tailed unpaired t test. The letters indicate statistically significant differences (p<0,05) vs. [a]CTR.

FIG.8 – Myocardial Interstitial Fibrosis Ratio

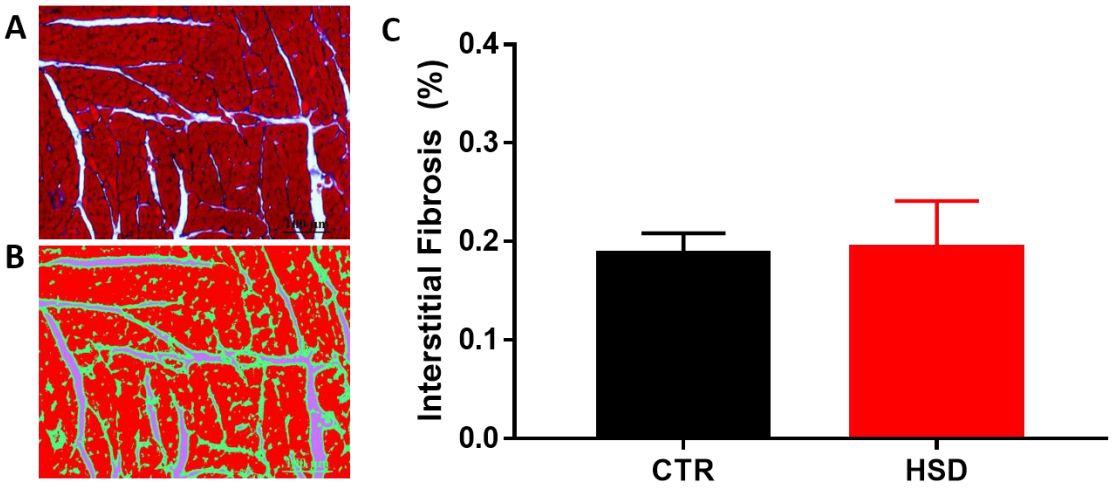

FIG.8 – Myocardial Interstitial Fibrosis Ratio. A. Image of Masson's Trichrome Staining (MTS) ventricle sample cross section at level of papillary muscle in 10x augment (250µm scale). **B.** Image of ventricle sample cross section at level of papillary muscle generated with automated color segmentation plug in. **C.** Bars represent mean ± SEM interstitial fibrosis ratio (n=5/group). Statistical analysis was performed with one tailed unpaired t test. The letters indicate statistically significant differences (p<0,05) vs. [a]CTR.

FIG.9 – Myocardial Perivascular Fibrosis Ratio

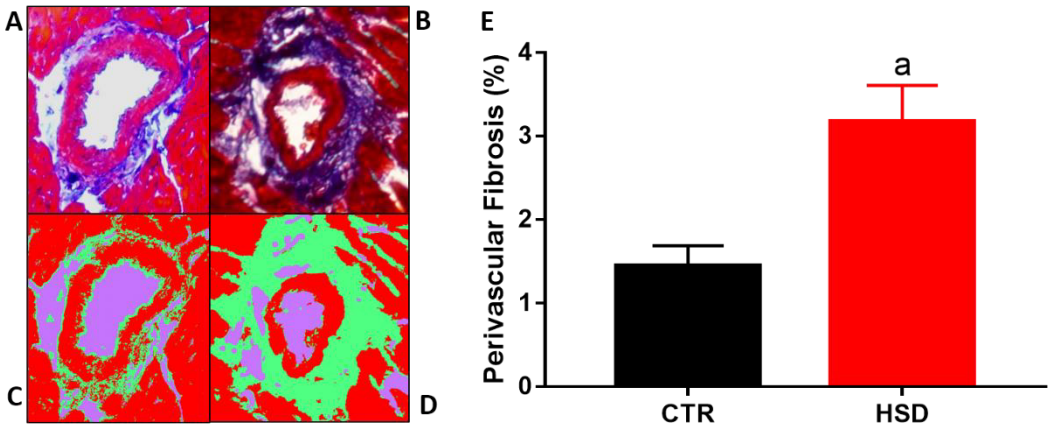

FIG.9 – Myocardial Perivascular Fibrosis Ratio. A, B. Images of vessel and adjacent fibrous tissue of, respectively, CTR and HSD Masson's Trichrome Staining (MTS) samples in 10x augment (250µm scale). **C**, **D.** Images obtained with automated color segmentation plug in of CTR and HSD, respectively. **C.** Bars represent mean ± SEM perivascular fibrosis ratio (n=5/group). Statistical analysis was performed with one tailed unpaired t test. The letters indicate statistically significant differences (p<0,05) vs. [a]CTR.

FIG.10 – Evaluation of Cardiomyocyte Hypertrophy

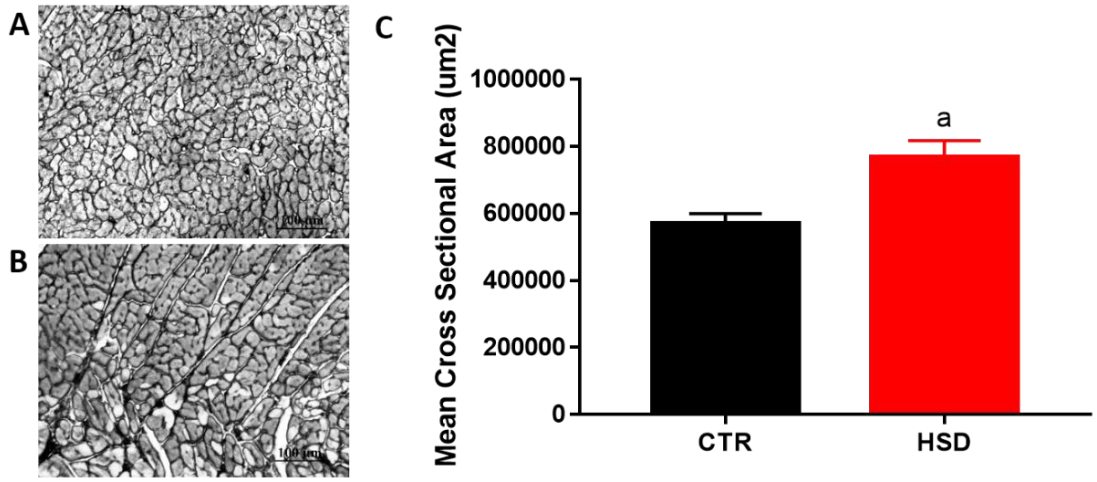

FIG.10 – Evaluation of Cardiomyocyte Hypertrophy. A, B. Original images of left ventricle cross section samples at level of papillary muscle, processed to better evidence cardiomyocytes limits. **C.** Bars represent mean ± SEM of measurements of 100 cardiomyocyte area per sample in μm^2 (n=5/group). Statistical analysis was performed with one tailed unpaired t test. The letters indicate statistically significant differences ($p<0,05$) vs. [a]CTR.

TAB.1 – MORPHOMETRIC ANALYSIS OF COLLECTED TISSUES

Tissues weight (g/100g BW)	**CTR**	**HSD**
Periepididymal Fat	1,40 ± 0,08	**1,75 ± 0,06[a]**
Retroperitoneal Fat	1,32 ± 0,11	1,57 ± 0,08
Mesenteric Fat	1,03 ± 0,06	1,29 ± 0,11
Heart	0,33 ± 0,01	0,34 ± 0,01
Liver	2,92 ± 0,06	3,13 ± 0,09
Soleus Muscle	0,042 ± 0,001	**0,038 ± 0,0006[a]**
Gastrocnemius Muscle	0,59 ± 0,0306	0,61 ± 0,01

TAB.1 – MORPHOMETRIC ANALYSIS OF COLLECTED TISSUES. The numbers represent mean ± SEM of relative tissue weight normalized to 100g of body weight. Statistical analysis was performed with one tailed unpaired t test and the letters indicate statistically significant differences ($p<0,05$) vs. [a]CTR.

ANEXOS

1. REGRAS DA REVISTA PARA ELABORAÇÃO DO MANUSCRITO:

"JOURNAL OF DEVELOPMENTALORIGINS OF HEALTH AND DISEASE"

You can find the Journal of Developmental Origins of Health and Disease instructions for contributors here:

(http://assets.cambridge.org/DOH/DOH_ifc.pdf) (111.761 KB)

INSTRUCTIONS TO AUTHORS

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Journal of Developmental Origins of Health and Disease (J DOHaD) is the official scientific journal of the International Society for Developmental Origins of Health and Disease (DOHaD).

JDOHaD publishes leading research in the field of Developmental Origins of Health and Disease (DOHaD). The Journal focuses on the environment during early pre-natal and post-natal animal and human development, interactions between environmental and genetic factors, including environmental toxicants, and their influence on health and disease risk throughout the lifespan. JDOHaD publishes work on developmental programming, fetal and neonatal biology and physiology, early life nutrition, especially during the first 1,000 days of life, human ecology and evolution and Gene-Environment Interactions. JDOHaD also accepts manuscripts that address the social determinants or education of health and disease risk as they relate to the early life period, as well as the economic and health care costs of a poor start to life. Accordingly, JDOHaD is multi-disciplinary, with contributions from basic scientists working in the fields of physiology, biochemistry and nutrition, endocrinology and metabolism, developmental biology, molecular biology/ epigenetics, human biology/ anthropology, and evolutionary developmental biology. Moreover clinicians, nutritionists, epidemiologists, social scientists, economists, public health specialists and policy makers are very welcome to submit manuscripts.

The journal includes original research articles, short communications and reviews, and has regular themed issues, with guest editors; it is also a platform for conference/workshop reports, and for opinion, comment and interaction.

Categories of papers

Original research articles - This category is intended for full-scale basic, clinical or epidemiological studies including large controlled trials. Articles may contain

up to 5,000 words (not including references, figures and tables) and should include an abstract of up to 250 words and 3-5 key words. (Exceptions to the length limitation will be considered for unusually large or complex studies.)

Brief reports - This category is for smaller, self-contained laboratory or clinical studies or analyses. Papers in this category may contain up to 2,500 words (not including references, figures and tables) and should include a maximum of 25 references, up to 2 illustrations (figures or tables), an abstract of up to 150 words and 3-5 key words.

Rapid communications - This category is for 'fast-breaking' new work, which is of great potential interest and can be succinctly presented.

Authors who wish to submit a rapid communication must first send an abstract to the Editor-in-Chief, Michael Ross (DOHaDeditor@cambridge.org) for approval of submission in this category. Submissions that do not have prior approval will be reviewed on the regular track.

Papers in this category may contain up to 2,500 words (not including references, figures and tables) and should include a maximum of 25 references, up to 2 illustrations (figures or tables), an abstract of up to 150 words and 3-5 key words. Rapid communications will be reviewed and published on a "fast track" basis.

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All individuals included as authors of papers must have contributed substantially to the scientific process leading up to the writing of the paper. Such contribution includes the conception and design of the project, the performance of experiments and the analysis and interpretation of data. In addition, the author should have made a substantial contribution to drafting or critical revision of the manuscript for important intellectual content.

We are aware that authors sometimes receive assistance from technical writers, language editors and/or writing agencies in preparing manuscripts for publication. Such assistance must be noted in the cover letter and in the Acknowledgements section along with a declaration that the author(s) are entirely responsible for

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Failure to acknowledge assistance from technical writers, language editors and/or writing agencies in preparing manuscripts for publication in the Acknowledgements section may lead to disqualification of the paper.

Under no circumstances will DOHaD accept submissions by writing or editorial agencies on behalf of authors and there will be no correspondence with writing or editorial agencies regarding submitted or revised manuscripts.

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The Editor-in-Chief will acknowledge receipt of the manuscript, provide it with a manuscript reference number and assign it to an Associate Editor and to reviewers. The reference number of the manuscript should be quoted in all correspondence with the / DOHaD Office and Publisher.

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The following instructions must be followed carefully:

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Word Perfect or other word-processor files or Macintosh-based files are not acceptable.

* Tables should be placed at the end of the document and not within the text.

* Do not use "enter" in order to start a new page. "Hard" page or section breaks must be used.

* A cover letter should be attached as a separate file. In the cover letter, the category under which the manuscript is submitted should be indicated and the corresponding author identified, including phone number, fax number and electronic mail address. The cover letter must include a statement regarding authorship (see Authorship section above).

* File-names should indicate the name of the first author of the paper or an abbreviated version thereof and the content of the file (text, tables, figures).

* Printed copies of the manuscript, tables and figures are not required and should not be sent.

Please note that correspondence regarding submitted and revised manuscripts will be with the Corresponding Author only.

Revised\Final Submission

* The uploaded manuscript must be in the form of a Word for Windows file with figures (prepared as instructed below) in separate files. Word Perfect or other word- processor files or Macintosh-based files are not acceptable.

* Do not use "enter" in order to start a new page. "Hard" page or section breaks must be used.

Figures should be prepared using appropriate formats and saved as TIFF or JPEG files. It is essential that JPEGs are greater than 320dpi. PowerPoint files or figures "pasted" into Word files are not acceptable for revised or final submissions.

File names should indicate the manuscript number assigned by the journal and the content of the file (text, figures).

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Manuscripts submitted to / DOHaD including those for supplements will be reviewed by at least two external reviewers and evaluated by an Associate Editor. Authors are requested to suggest up to 4 reviewers who are especially qualified to referee the work and would not have a conflict of interest. Please provide the names, email addresses, fax numbers and mailing addresses of the suggested reviewers. If authors prefer that a particular reviewer do not evaluate the paper, they may indicate this request with appropriate justification, which will be treated confidentially. Suggestions and requests regarding reviewers will be considered by the Editor without obligation to accept them.

Authors should note that manuscripts may be returned after initial review by the Editors if the paper is deemed unlikely to be reviewed favorably. This rapid rejection process enables the author to submit promptly to publication elsewhere.

Every effort will be made to provide the author with a review within six weeks of receipted of the manuscript. If the Editor requests that revisions be made to a manuscript before publication, a maximum of 3 months shall be allowed for preparation of the revision, except in unusual circumstances.

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The manuscript should be typed double-spaced throughout on 'Letter' or A4 paper. Pages should be numbered sequentially beginning with the Title Page. Margins should not be less than 2.5cm on all sides and the font should be clearly legible and uniform throughout.

A ***Short Title*** of up to forty-five characters should be provided on the title page and should be repeated at the top right of every following page. The names of the authors (e.g. Smith et al. or Smith and Jones) should be given at the top left of every page besides the title page.

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