Combined effects of age and diet-induced obesity on biochemical parameters and cardiac energy metabolism in rats

Luiz Gustavo de Almeida Chuffa\textsuperscript{1} and Fábio Rodrigues Ferreira Seiva\textsuperscript{2}

\textsuperscript{1}Department of Anatomy, \textsuperscript{2}Department of Chemistry and Biochemistry, Bioscience Institute, Univ. Estadual Paulista, UNESP, Botucatu, Zip Code: 510; P.O Box: 18618-970, São Paulo, Brazil

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Obesity is often associated with decreased fat oxidation and aging is a well-recognized risk factor for cardiovascular disease. This study investigated calorimetric and morphometric parameters, as well as the glucose levels, lipid profile and cardiac energy metabolism in young and old, controls and obese rats. The animals were divided into four groups: Group I (GI): young rats fed normal diet for 75 days; Group II (GII): young rats fed hypercaloric diet (HD) for 75 days; Group III (GIII): old rats fed normal diet for 510 days; and Group IV (G IV): old rats fed HD for 510 days. The following analyses were performed: calorimetric, glucose and lipid concentrations, atherogenic index (AI), total antioxidant substances (TAS), fat depots, cardiac lipid hydroperoxide (LH) and cardiac lactate dehydrogenase (LDH), citrate synthase (CS), phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH) activities. Older animals were heavier than young and the hypercaloric animals were heavier than controls. Animals from GIV had lower fat oxidation than GIII, which in turn, had higher fat oxidation than GI. Total cholesterol, LDL-C and all fat depots were higher in the GII, as compared to GI. The GIV rats had higher VLDL, retroperitoneal fat, serum lipids and cardiac glycogen levels than GII. Furthermore, GIV rats had higher fat depots, triacylglycerol, total cholesterol and VLDL than GIII. Animals from GII and -IV showed higher LH and AI than age-matched controls. Older hypercaloric rats also had higher TAS than older control rats, which also had lower LH and TAS than younger control rats. Aged animals had increased CS and LDH and decreased PFK and PDH activities. Additionally, GIV rats exhibited an increase in PDH activity, compared to GIII. We conclude that the consumption of HD coupled with aging leads to impaired basal and cardiac metabolism.

Keywords: Obesity, ageing, Dyslipidemia, Cardiac metabolism, Oxidative stress.

Human population is experiencing a marked increase of its elderly segment around the world. It is estimated that the persons aged 65 yrs and older will account to 71 million over the next 20 yrs in North America\textsuperscript{1}. There are several studies focusing early onset of aging which provides novel information associated with metabolic status including exacerbated production of reactive oxygen species (ROS)\textsuperscript{2}.

ROS are physiologically produced during normal cell metabolism and can react with biomolecules such as DNA, lipid and proteins, thereby altering their structure and function. The aging process also brings many changes in body composition, such as augment in fat deposits and alterations of lipid profile that may lead to obesity development. These changes are likely due to the alterations in energy balance, with a positive and negative energy balance, leading to weight gain and weight loss, respectively\textsuperscript{3}. In addition, growing evidences suggest that aging is directly related with impaired glucose metabolism, such as absence of meal-induced insulin sensitization\textsuperscript{4}. Thus, nutritional programs, such as dietary restriction are strongly recommended, in order to extend maximum life-span\textsuperscript{5}.

Among health problems affecting elderly people, cardiovascular diseases are at the top of the list, including the final stage of heart failure\textsuperscript{6}. Also, there is a well-established relationship between obesity and higher risks for developing cardiovascular disease. In aged rodents, a negative correlation between cardiac performance and aging is severely aggravated after 52 weeks-old and hyperinsulinemia, hyperlipidemia and adiposity index are enhanced by sucrose supplement.

*Corresponding author:
Phone: +55 (14) 3811-6040; Fax: +55 (14) 3811-6361. E-mail: guchuffa@yahoo.com.br

Abbreviations: AI, atherogenic index; CS, citrate synthase; HD, hypercaloric diet; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; LDH, lactate dehydrogenase; LH, lipid hydroperoxide; OGTT, oral glucose tolerance test; PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; RMR, resting metabolic rate; RQ, respiratory quotient; TAS, total antioxidant substances; TC, total cholesterol; TG, triacylglycerol; VLDL, very low-density lipoprotein; VCO2, average carbon dioxide production; VO2, average oxygen consumption.
along with aging. Notably, when food intake exceeds energy expenditure, the retained energy is stored as fat depots. Conversely, when young rat calorie intake is more than necessary to maintain vital functions, there is an increase in the metabolic rate and release of surplus energy in the heat form, but in older rodents or even in humans these mechanisms can fail, so that rodents and human would develop a spontaneous obesity as they grow older. This metabolic disorder becomes more evident after observing that aged rats even receiving diets containing low fat acids tend to acquire obesity. Taken together, few studies have reported the combined effect of aging and hypercaloric diet (HD) on the regulation of body weight, lipidaemia, glucose and cardiac metabolism.

The western diet, which is a palatable carbohydrate- and fat-enriched diet, is one of the main contributors for the higher incidence and prevalence of overweight, obesity and related dysfunctions, such as type 2 diabetes, hypertension and heart failure. In this regard, it is clear that aging and obesity share some features often overlapping each other. In order to identify possible consequences of aging and obesity on the risk factors for cardiovascular disease, this study has been aimed to determine calorimetric and morphometric parameters, as well as the glucose levels, lipid profile and cardiac energy metabolism in young and old, controls and obese rats.

**Materials and Methods**

**Experimental design and dietary contents**

Thirty-two male Wistar rats were individually housed in polypropylene cages in an environmentally-controlled clean air room with temperature of 22 ± 3°C, 12-h light-dark cycle and relative humidity of 60 ± 5%. The animals were randomly divided into four groups (n = 8/group): Group I (GI): 75 days of age, fed standard chow and water; Group II (GII): 75 days of age, fed hypercaloric diet (HD) plus 30% sucrose in its drinking water; Group III (GIII): 510 days of age, fed standard chow and water; and Group III (GIV): 510 days of age, fed HD plus 30% sucrose in its drinking water. During 75 days of experiment, all animals received water ad libitum and the controls groups’ standard rodent chow (3074 SIF, Purina Ltda., Campinas, SP, Brazil) containing (by weight) 19.90% protein, 30.03% carbohydrate, 3.80% fat, 13.36% fiber, and 3.00 kcal/g of metabolizable energy.

The hypercaloric group was fed hypercaloric chow obtained after mixing 203.7 g sucrose and 88.26 g of soybean oil to 1000 g of a previously triturated standard chow. The dietary ingredients were homogenized in 60°C warm distilled water and the homogenate was used to prepare the pellets. The proportions of dietary protein, carbohydrate, fat and fiber were determined according as described previously. The hypercaloric chow contained (by weight) 18.00% protein, 47.59% carbohydrate (20.37% sucrose), 11.41% fat, 5.43% fiber, and 3.64 kcal/g of metabolizable energy.

Food intake and drinking consumption were evaluated daily at the same time (9–0 h), as the difference between food and drinking solution given and the leftover. The body weights were determined once a week. Food intake and caloric values of chows and sucrose solution (4 kcal/g), were used to obtain total energy intake (EI, kcal/day). The body mass index (BMI) (g/cm²) = body weight/total length² and Lee index (g/cm) = 3√ body weight/total length were also calculated. All experiments and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health and was approved by the Ethics Committee of the Botucatu School of Medicine, UNESP, Botucatu, SP, Brazil.

**Indirect calorimetry**

After 70 days of beginning the experiment, rats were placed into metabolic chambers (airflow = 1.0 l/min) in a computer-controlled indirect calorimeter (CWE, Inc, St. Paul, USA) to determine the fed calorimetric parameters. For assessment of fasting calorimetric parameters, rats were fasted overnight (12–14 h) and placed into metabolic chambers. Respiratory quotient (RQ) and energy expenditure, namely resting metabolic rate (RMR) were measured using a respiratory-based software program (software MMX, CWE, Inc., USA). Average oxygen consumption (VO2) and carbon dioxide production (VCO2) were integrated over periods of 15 min. Carbohydrate and fat oxidation were calculated from the non-protein oxygen consumption and the amount of oxygen consumed per g of oxidized substrate.

**Glycemic profile and oral glucose tolerance test (OGTT)**

After 60 days of experiment, the animals in fasted state (12–14 h) had the blood taken from a tail vein and glucose concentrations were determined by an
automatic glucose analyzer (Boehringer Mannheim, Eli Lilly Ltda, São Paulo, Brazil). On the last day of treatment (75 days), rats were fasted overnight (12–14 h) and submitted to the oral glucose tolerance test (OGTT). Glucose was given (3 g/kg body weight) by gavage as a 20% aqueous solution. Blood glucose levels were measured before glucose administration (basal level) and at 30, 60 and 120 min after glucose ingestion\textsuperscript{20}. After the OGTT, all animals were anesthetized with 0.1 ml i.p. of 1% sodium pentobarbital for measurement of the abdominal circumference and body length, following to euthanasia by decapitation\textsuperscript{12}.

**Fat depots and lipid profile**

Adipose tissue was isolated and weighed from the epididymal, visceral and retroperitoneal fat pads, resulting in isolated and total fat depots. Total blood was collected using a funnel into a centrifuge tube and allowed to clot to obtain the serum. Serum samples were used for triacylglycerol, total cholesterol and its fraction determinations (Test kit CELM, Modern Laboratory Equipment Company, Sao Paulo, Brazil). The atherogenic index (AI) was determined as total cholesterol/HDL ratio\textsuperscript{9}.

**Myocardial analysis**

The heart was rapidly removed and weighed. The left ventricle was isolated and stored at -86°C for further analysis. For total protein and enzymes determinations\textsuperscript{13}, samples of 200 mg left ventricle were homogenized in 5 ml of a cold 0.1 M phosphate buffer, pH 7.4. Tissue homogenates were prepared in a motor driven Teflon glass Potter Elvehjem, tissue homogenizer (1 min, 100 × g). The homogenate was centrifuged at 10,000 × g for 15 min and the supernatant was used for determination of several cardiac key metabolic enzymes.

Cardiac lipid hydroperoxide (LH) was determined with 100 µl of sample and 900 µl of a reaction mixture containing 0.25 mM FeSO\textsubscript{4}, 25 mM H\textsubscript{2}SO\textsubscript{4}, 0.1 mM xylene orange, and 4 mM butylated hydroxytoluene in 90% (v/v) methanol. Total antioxidant substances (TAS) were also quantified in cardiac tissue through a test kit (Randox Laboratories Ltd., Crumlin, Co., Antrim, UK). The mixtures were incubated for 30 min at room temperature before reading at 560 nm\textsuperscript{14}.

Cardiac energy metabolism was assessed by lactate dehydrogenase (LDH; E.C.1.1.1.127), citrate synthase (CS; E.C.4.1.3.7), phosphofructokinase (PFK; E.C.2.7.1.11) and pyruvate dehydrogenase (PDH; E.C. 1.2.1.51) activities\textsuperscript{15}. The assay medium for LDH contained 50 mM Tris-HCl buffer, pH 7.5, 0.15 mM NADH and 1 mM pyruvate. For CS activity, the assay medium consisted of 50 mM Tris-HCl, pH 8.1, 0.3 mM acetyl-CoA, 0.1 mM DTNB (5,5′-dithiobis-2-nitrobenzoic acid) and 0.5 mM oxaloacetate. PFK was assayed using 50 mM Tris-HCl buffer, pH 8.0, 10 mM MgCl\textsubscript{2}, 1 U glycerol-3-phosphate dehydrogenase, 1 U aldolase, 2.5 U triosephosphate isomerase, 0.12 mM NADH, 0.75 mM ATP and 6 mM fructose-6-phosphate. The PDH activity was determined using 2.5 mM NAD, 0.2 mM thiamin pyrophosphate, 0.1 mM coenzyme-A, 0.3 mM dithiothreitol, 5 mM pyruvate, 1 mM MgCl\textsubscript{2} and 1 mg/ml of BSA in 0.05 M potassium phosphate buffer, pH 7.8\textsuperscript{15}.

The enzyme activities were performed at 25°C using a microplate reader (µ Quant-MQX 200 with Kcjunior software coupled to computer system control, Bio-Tec Instruments, Winooski, Vermont, USA) and then normalized to total protein. The spectrophotometric determinations were performed in a Pharmacia Biotech spectrophotometer with temperature controlled cuvette chamber (UV/visible Ultrospec 5000 with Swift II Applications software to computer system control, 974213, Cambridge, England, UK).

**Statistical analysis**

Analysis of variance for two factors (Two-way ANOVA) was used to examine the effects of diet and age. All comparisons were performed by Tukey test and the results expressed as the Mean ± SD. Statistical significance was set at \(P<0.05\). SigmaStat software for windows (Jandel Corporation, San Rafael, CA, USA) and SigmaPlot version 11.0 were used to access statistics and graphic design.

**Results**

**Nutritional and morphometrical parameters**

Figure 1 represents the body weight gain during the experimental period. Older animals had higher initial body weight than young animals and GI, -II and -IV showed higher body weight at the end of experiment. Animals fed HD had lower food consumption and higher BMI and Lee index, compared to their controls (Table 1). Glucose levels were augmented only in GIV group compared with GII and GIII groups.

**Calorimetric parameters**

Group II showed lower RMR/body weight, VO2/body surface and carbohydrate oxidation than GI.
Group III had higher VO2/body weight, VO2/body surface and fat oxidation, compared with GI. Fat oxidation was only statistically different between G IV and III, which was lower in the former (Table 2).

Glucose tolerance test, lipid profile and fat depots

All animals responded to glucose overload augmenting the glucose levels, but after 120 min, only GI had glucose returned to basal levels. Both GIII and -IV showed higher glucose levels than their controls after 30 and 60 min (Fig. 2). Group II had elevation in total cholesterol, LDL-C and higher fat depots (isolated and total content), compared with GI, and similar results were found after comparing GIV with group GIII, besides the GIV had also higher TG and VLDL concentrations. Group IV showed higher TG, HDL-cholesterol and VLDL-C and lower LDL-C than GII (Table 3).

Cardiac oxidative stress and energetic metabolism

Animals from GII presented higher LH levels and AI compared with GI and GIII showed reduced LH and TAS levels, when compared with GI. The aged group fed hypercaloric diet had higher LH, AI and TAS, compared to GIII (Fig. 3A, B and C). Group II exhibited a reduction of stored cardiac glycogen,
while GIV had elevated glycogen in comparison to their controls (Fig. 3D). Group II exhibited the PFK and PDH activities, respectively reduced and increased, compared with GI. Groups III and -IV showed higher CS and LDH activities and lower PFK and PDH activities than GI and -II, respectively. Obese and aged animals displayed higher PDH activity compared to only aged animals (Table 4).

Table 2—Calorimetric data

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
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<tr>
<td>RQ</td>
<td>0.88±0.22</td>
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<tr>
<td>RMR/body weight (kcal/h.kg)</td>
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<tr>
<td>VO2/body weight (ml/h/g)</td>
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<td>VCO2/body weight (ml/h/g)</td>
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<td>VO2/body surface (ml/h/g0.75)</td>
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<tr>
<td>Carbohydrate oxidation (mg/min)</td>
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</tr>
<tr>
<td>Fat oxidation (mg/min)</td>
<td>4.47±2.55</td>
</tr>
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*p<0.05 vs GI; b p<0.05 vs GII; c p<0.05 vs GIII.

Group I: young rats receiving standard chow + water; Group II: young rats receiving hypercaloric diet and 30% sucrose in its drinking water; Group III: old rats receiving standard chow + water; Group IV: old rats receiving hypercaloric diet and 30% sucrose in its drinking water.

RQ: respiratory quotient; RMR: resting metabolic rate; VO2, average oxygen consumption; VCO2, average carbon dioxide production.

Fig. 3—(A): Cardiac lipid hydroperoxide (LH) levels; (B): Atherogenic index (AI); (C): Total antioxidant substances (TAS); and (D): Cardiac glycogen levels [Data expressed as the mean ± SD. Two-way ANOVA with Tukey’s post-hoc test. GI (Group I): rats with 75 days of age, receiving standard chow + water; GII (Group II): rats with 75 days of age, receiving HD and 30% sucrose in its drinking water; GIII (Group III): rats with 510 days of age, receiving standard chow + water; (Group IV) GIV: rats with 510 days of age, receiving HD and 30% sucrose in its drinking water. *p<0.05 vs. GI group; †p<0.05 vs. GII group; ‡p<0.05 vs. GIII group].
Discussion

Several abnormalities that come along with aging have been extensively studied in the recent years. Among these alterations, obesity development and cardiovascular diseases have drawn much attention. The modern way of life that includes carbohydrates and fats-rich diets, is one of the main causes for the growing number of people with overweight around the world. Obesity is considered to be a major cause of increased cardiovascular mortality and an independent risk factor for the development of heart failure. In animal models, obesity is associated with functional and structural alterations into the heart, such as left ventricle hypertrophy, cardiac fibrosis, myocardial lipid accumulation (i.e., lipotoxicity) and other metabolic changes which may lead to cardiomyopathy.

Animals from GIII exhibited higher body weight at the end of the experiment differently from GI (Fig. 1), indicating that in young rats, the metabolic parameters, such as the RMR might have contributed to the control of body weight gain. Some studies have reported metabolic alterations during aging process, but without mentioning body weight gain. In this study, older animals had higher initial body weight than those younger ones. This finding could be associated with the lower RMR in animals aged 510 days (Fig. 2). RMR translates the amount of necessary energy for the maintenance of basal metabolism and reduction of RMR indicated that less energy was being used, thereby providing more energy to be stored. Corroborating this finding, there are some studies reporting the reduction of RMR during aging. Body weight gain may be understood not only as the consequence of a positive energy balance, but also as the mechanism by which energy is eventually stored. In fact, conversion of carbohydrates into lipids is an anabolic process.
that only occurs after glycogen stores has been replenished. Therefore, some dietetic components, such as carbohydrate and lipids play important roles on body weight control and obesity development, since they utilize different pathways to provide energy generation\(^9\),\(^{21}\).

Animals fed HD, independently of age, had lower food consumption than controls, which was associated with the energy amount of HD (average of 3.64 kcal/g HD vs 3.0 kcal/g control diet). Also in these animals, there was a compensatory effect of additional calories from the sucrose solution. When energy ingestion exceeds energy expenditure, the surplus energy can be stored as fat depots, characterizing obesity. Animals receiving HD showed higher abdominal circumference, BMI and Lee index, when compared to control of the same age. These parameters were accompanied by higher total body fat and specific fat depots found in hypercaloric groups. Notably, elevation in fat deposits, mainly visceral fat, increases the risk for cardiovascular diseases\(^{22}\).

In this study, significant differences related to fat accumulation and aging were not observed and also, it might suggest that HD consumption had anticipated age-related effects on body fat gain\(^14\). Solomon et al.\(^{15}\) reported a reduction of lipid oxidation in obese humans and likewise, this effect was observed in GIV, indicating that lower lipid oxidation in addition to lower RMR might have contributed to the increase in fat depots, consequently enhancing the risk for cardiovascular disease. Surprisingly, animals with advanced age and receiving control diet showed changes in fat oxidation mimicking that of HD consumption.

We also Evaluated fasting glycemia and the curve response after a glucose overload test (OGTT). After 60-day experiment, fasting glucose was not altered in GI, -II and -III, however, as expected, the GIV showed elevated values which could be attributed to both carbohydrate-rich diet and older age-related glucose metabolism impairment. Groups II, -III and -IV had higher circulating glucose after 30 min of glucose overload. At the end of 120 min, glucose levels returned to the basal level only in the GI. These findings suggested that both young animals fed HD and control aged animals developed a hyperglycemic state, associated with insulin resistance and/or glucose intolerance\(^{23,24}\). Although fasting glucose levels were not altered, the OGTT simulates a post-absorptive state, in which the production and release of insulin and its responsiveness become necessary\(^21\). Moreover, our data indicated that both aging and HD consumption might interfere with the mechanisms related to glucose uptake.

Cardiac oxidative stress and energetic metabolism was also investigated to clarify the effects of aging, as well as the HD intake. Regardless of age, our data demonstrated that ingestion of cholesterol and sucrose-rich diet was linked to oxidative process caused by ROS generation. One possible explanation was that cholesterol and sucrose circulating levels would be uptaken by cardiac muscle, thus increasing the oxidative damage, resulting in higher LH levels. Earlier study\(^{14}\) has shown that fatty acid-rich diet disrupts the mitochondrial respiratory capacity in liver and skeletal muscle; we also found the similar response in cardiac muscle. Additionally, group III showed lower LH levels and TAS (which both enzymatic and non-enzymatic antioxidants compounds), corroborating previous data that showed a decrease in antioxidant defenses during aging\(^{25}\).

TG concentration as well as total cholesterol and its fractions were not altered by age. Interestingly, advanced age is related to a lipoprotein profile that is typical of low risk for developing atherosclerotic disease, such as increased HDL-C, HDL\(_2\)-C and HDL-C/cholesterol ratio\(^{22}\). It is well-known that several factors may influence the lipid metabolism, such as physical activity, diet, body composition, environmental factors and medication, but in this study, the factor “diet” was determinant for dyslipidemia. Animals from GII had elevation in total cholesterol, LDL-C levels and atherogenic index, indicating young animals consuming HD having an increased risk of developing cardiovascular disease.

When young and old animals fed HD were compared, reduced LDL-C and increased HDL-C levels were observed in GIV, indicating aged animals having protective mechanisms that minimized the damage arising from lipids metabolism. Although cells have natural mediators against harmful effects of diets components, e.g., up-regulation of hepatic P450-enzymes that reduce cholesterol toxicity and activation of hepatic LXR\(_\alpha\), which can protect against atherosclerotic process\(^{22,26}\), the “diet” factor is strongly associated with high risk of cardiovascular disease. Taken together, RMR reduction associated with glycemic disturbances and increased body fat depots could be used as indicative of obesity during aging process and consequently to prevent the onset of cardiovascular disease.
Cardiac energy metabolism was studied through activities of key enzymes, such as CS (the first enzyme of Krebs cycle that imports acetyl-CoA), LDH and PFK1 (both biomarkers of glycolytic pathway) and PDH which converts pyruvate into acetyl-CoA. The CS and LDH activities were enhanced only by age and became even more evident after combination with HD. Animals from group II showed increased activity of PDH, probably due to higher amounts of sucrose in diet, further elevating the production of acetyl-CoA to be oxidized in the Krebs cycle. In addition, PFK1 activity was reduced in GII animals due to increased citrate, a potent allosteric inhibitor of PFK. The higher concentration of cardiac glycogen found in GIII and -IV confirmed this finding, and also, it could represent an adaptive response to the higher levels of circulating lipids\(^7\). Elevation in energy amounts or insulin levels probably resulted in glycogen synthesis and storage by cardiac tissue.

The cardiac metabolism has been shown to promote lower glucose oxidation in aged rats, which was associated with reduced PFK and PDH activities\(^{21}\). Notably, the increased CS activity in aged animals might be due to the influx of acetyl-CoA that comes from fatty acid metabolism. High citrate levels might alter the equilibrium towards pyruvate formation and energy generation for myocardial contractile function.

Elevation in lactate levels that occurs during aging might alter the equilibrium towards pyruvate formation through an anaplerotic reaction and pyruvate finally replaces oxaloacetate by promoting high CS activity\(^{28}\). A low rate of glucose catabolism explained the stored cardiac glycogen found in GIII, since the non-oxidized glucose can be stored as glycogen. These findings demonstrated that cardiac muscle of young animals, in response to HD intake was able to make metabolic adjustments, which were not observed in aged animals.

In conclusion, the study demonstrated that HD consumption and aging process impaired, non-synergistically, basal and cardiac metabolism. Combination of HD with age negatively modulated the glycemic profile and cardiac energy production. Interestingly, aging contributed to correct adverse effects of hypercaloric diet, mainly those related to lipids metabolism.

Acknowledgments

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