Brazilian Journal of Medical and Biological Research (2006) 39: 305-312
ISSN 0100-879X

Effect of unsaturated fatty acids on myocardial performance, metabolism and morphology

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Research supported by FAPESP (No. 02/02704-0).

Received April 26, 2005
Accepted October 11, 2005

Abstract

Diets rich in saturated fatty acids are one of the most important causes of atherosclerosis in men, and have been replaced with diets rich in unsaturated fatty acids (UFA) for the prevention of this disorder. However, the effect of UFA on myocardial performance, metabolism and morphology has not been completely characterized. The objective of the present investigation was to evaluate the effects of a UFA-rich diet on cardiac muscle function, oxidative stress, and morphology. Sixty-day-old male Wistar rats were fed a control (N = 8) or a UFA-rich diet (N = 8) for 60 days. Myocardial performance was studied in isolated papillary muscle by isometric and isotonic contractions under basal conditions after calcium chloride (5.2 mM) and ß-adrenergic stimulation with 1.0 µM isoproterenol. Fragments of the left ventricle free wall were used to study oxidative stress and were analyzed by light microscopy, and the myocardial ultrastructure was examined in left ventricle papillary muscle. After 60 days the UFA-rich diet did not change myocardial function. However, it caused high lipid hydroperoxide (176 ± 5 *vs* 158 ± 5, $P < 0.0005$) and low catalase (7 ± 1 *vs* 9 ± 1, $P < 0.005$) and superoxide-dismutase (18 ± 2 *vs* 27 ± 5, $P < 0.005$) levels, and discrete morphological changes in UFA-rich diet hearts such as lipid deposits and mitochondrial membrane alterations compared to control rats. These data show that a UFA-rich diet caused myocardial oxidative stress and mild structural alterations, but did not change mechanical function.

Key words
- Fatty acids
- Cardiac function
- Oxidative stress
- Morphology
- Catalase
- Superoxide dismutase

Introduction

The effects of many kinds of diets on health have been studied (1-5). Relationships have been established between different kinds of diets and their influence on cardiovascular events. Nutrition rich in saturated fatty acids is one of the most important reasons for atherosclerosis and the reduction of coronary reserve (3,6-8).

Unsaturated fatty acids (UFA) have been used to replace saturated fatty acids because they have an anti-atherogenic effect (3-5,9-12) and protect the heart against acute myo-

cardial infarction (12,13). However, UFA-rich diets can cause morphological cardiac injuries such as necrosis (14) and abnormal muscular striation (15). Also, it has been shown that UFA-rich diets cause myocardial oxidative stress (13,16).

The literature has not completely defined the relationship between UFA-rich diets and heart function. Some investigators have not detected shortening changes in isolated rat hearts (17) or left ventricle (LV) papillary muscle (1,18), while others have detected depressed mechanical function in isolated rabbit (19) and rat (20) LV papillary muscles.

The objective of the present study was to obtain information on the influence of a UFA-rich diet on myocardial performance in isolated cardiac muscle in different inotropic states. We also characterized myocardial oxidative stress and morphology by light and electron microscopy.

Material and Methods

Animal model and experimental protocol

Sixty-day-old male Wistar rats were fed a commercial rat chow (C, N = 8) or a UFA-rich diet (F, N = 8) and water *ad libitum*. The UFA-enriched diet contained 19.39% fat (73.57% UFA and 26.43% saturated fatty acids) and the control diet had 2.30% of fat (58.52% UFA and 41.38% saturated fatty acids). The UFA diet contained a higher proportion of linoleic acid (C: 3.20% and F: 45.04%) and a lower amount of carbohydrate (C: 60.80% and F: 44.96%), protein (C: 21.88% and F: 16.40%) and vitamins and minerals (C: 5.64% and F: 4.70%). The preparation of the UFA-rich diet is described below. Rats were maintained on this dietary regimen for 60 days and then sacrificed. The animals were housed in individual cages in a room with a controlled temperature of 23ºC on a 12-h light:dark cycle. Rats were weighed once a week. Initial body weight, final body weight (FW), LV weight, right ventricle (RV) weight, and the LV/FW and RV/FW ratios were measured in the rats used in the functional study. Animal food consumption was measured once a week.

All experiments and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health and were approved by the Ethics Committee of the Botucatu School of Medicine, UNESP, Botucatu, SP, Brazil.

Diet preparation

The UFA-rich diet was prepared with commercial rat chow. Thus, all diets provided sufficient vitamins, minerals, essential amino acids, and lipids. Corn oil was the UFA source (21,22). We used a mixture of 220 mL corn oil and 30 mL coconut oil per 1000 g of rat chow (23). The mixture was then passed through a pelleting machine. The pellets were dried in a circulating air oven for 24 h at 70ºC. After drying, the UFA-rich diet was stored at 6ºC for use during the experiment (24).

Both diets were analyzed in the Laboratório de Tecnologia dos Produtos Agropecuários da Faculdade de Ciências Agronômicas (UNESP). The fat was extracted with petroleum ether in a Soxhlet extractor (25). The lipids were extracted by the method of Folch et al. (26). The fatty acid composition was determined after esterification with methanol sulfuric acid and the fatty acid methyl esters (26) were analyzed with a CG 17 A chromatograph (Shimadzu Corporation Analytical Instruments Division, Kyoto, Kansai, Japan) using a 35-m DB-Wax column (J & W Scientific, Folsom, CA, USA) and a flame ionization detector.

Functional study

Intrinsic cardiac contractile performance was evaluated by studying isolated LV papillary muscle (27). Briefly, rats were anes-

thetized intraperitoneally with 50 mg/kg pentobarbital sodium and sacrificed; their hearts were quickly removed and placed in oxygenated Krebs-Henseleit solution at 28ºC. The LV anterior or posterior papillary muscles were dissected free, mounted between 2 spring clips, and placed vertically in a chamber containing Krebs-Henseleit solution at 28ºC and oxygenated with a mixture of 95% O_2/5% CO_2, pH 7.38. The muscles were stimulated at 12 contractions/min with a voltage 10% above threshold. The spring clip on the upper end of the muscle was attached to a low inertia DC pen motor (model G100-PD; General Scanning, Watertown, MA, USA) and the lower clip to a load cell (model DSC-3; Kistler-Morse, Redmond, WA, USA). A digital computer with an analog-to-digital interface was used to control the tension or length of the preparation. Tension and length data were sampled at 1 kHz and stored on disk for later analysis.

After mounting, the muscle was allowed to equilibrate for 30 min and then gradually stretched. Tension values were plotted against length to peak active tension (AT) and L_{max} was defined as muscle length at peak AT. The preparation was allowed to stabilize for an additional 15 min while physiologically sequenced contractions were performed. Isometric contraction parameters were determined, including AT, g/mm^2, defined as peak isometric tension minus resting tension, resting tension (RT, g/mm^2), time to peak tension (TPT, ms), peak isometric tension development rate (+dT/dt, g (mm^2)$^{-1}$ s^{-1}), maximum tension decline rate (-dT/dt, g (mm^2)$^{-1}$ s^{-1}), and time from peak tension to 50% relaxation (RT_{50}, ms). The stiffness constant (k_m) was calculated from $\log(S_m) = B + k_m E_m$, where S_m is the stiffness calculated from resting tension in different muscle lengths normalized to cross-sectional area (CSA), E_m is the natural muscle strain ($E_m = 1n(L_m/L_{0.1})$), where L_m is the instant muscle length and $L_{0.1}$ is the length with stress of 0.1 g/mm^2. After isometric contraction, the muscles were analyzed in isotonic contraction against a weight that was able to keep the resting muscle at L_{max}. The isotonic parameters were maximum shortening velocity (-dL/dT, ML/s) and maximum relaxation velocity (+dL/dT, ML/s). Analyses were performed under basal conditions (1.25 mM Ca^{2+}), with 5.2 mM Ca^{2+}, and with 1.0 µM isoproterenol.

The parameters used to characterize papillary muscle were length (mm), weight (mg) and CSA (mm^2). L_{max} *in vitro* was measured with a Gartner cathetometer (Chicago, IL, USA). The muscle portion between the two spring clips was cut and weighed after drying on filter paper. CSA was calculated as the relation between weight and L_{max}, considering that papillary muscle is cylindrical and its specific weight is constant.

To compare the mechanical function between different muscle lengths, isometric and isotonic parameters were normalized to CSA and L_{max}.

Biochemical study

Myocardial oxidative stress. Five animals from each group were used for biochemical study. The heart was removed and cardiac adipose tissue was discarded. LV samples of 200 mg were weighed and homogenized in 5 mL 0.1 M cold sodium phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA). Tissue homogenates were prepared with a motor-driven Teflon glass Potter Elvehjem (Piracicaba, SP, Brazil) tissue homogenizer (for 1 min, at 100 rpm) immersed in ice water. The homogenate was centrifuged at 10,000 rpm for 15 min and the supernatant was used to determine total protein (28), lipid hydroperoxide, total antioxidant status (29), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT).

Lipid hydroperoxide was measured by hydroperoxide-mediated Fe^{2+} oxidation under acid conditions (30). Samples were added

to reaction mixtures containing 100 µM xylenol orange, 250 µM $FeSO_4$, 25 mM H_2SO_4, and 4 mM butylated hydroxytoluene in 90% (v/v) methanol. The mixtures were incubated for 30 min at room temperature prior to measurement at 560 nm.

GSH-Px was assayed using 0.15 M sodium phosphate buffer, pH 7.0, containing 5 mM EDTA, 0.1 mL 0.0084 M NADPH, 0.005 mL GSSG-reductase (Sigma, St. Louis, MO, USA), 0.01 mL 1.125 M NaN_3 (sodium azide), and 0.1 mL 0.15 M GSH (31). SOD activity was determined by the ability of the enzyme to inhibit reduction of nitro blue tetrazolium (Sigma). Nitro blue tetrazolium reduction rate in the absence of tissue was used as a reference. One unit of SOD was defined as the amount of protein needed to decrease the reference rate to 50%. All data are reported as SOD units per mg protein (32). CAT activity was determined at 240 nm with sodium phosphate buffer, pH 7.0 (33).

Enzyme activities were determined using a microplate reader (Bio-Tech Instruments Inc., Winooski, VT, USA). Spectrophotometric determinations were performed with a Pharmacia Biotech spectrophotometer (974213, Cambridge, England). All reagents were purchased from Sigma. The extinction coefficients were 6.22 mM/cm for NADH at 340 nm and 13.6 mM/cm for DTNB at 412 nm.

Serum lipid determination

After decapitation, total blood was placed in a centrifuge tube and allowed to clot to obtain serum. Serum total lipids, triacylglycerol, cholesterol, and HDL-cholesterol were determined with enzyme kits (CELM, Modern Laboratory Equipment Company, São Paulo, SP, Brazil). LDL-cholesterol was calculated using the Friedewald formula (34).

Morphological study

Five animals from each group were used for the histological study. After anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneally), hearts were excised and ventricles were separated. The LV free wall was cut into fragments and chilled in n-hexane at -70ºC in liquid nitrogen. Serial transverse 8-µm sections were cut with a cryostat at -20ºC and stained with hematoxylin and eosin. For ultrastructural study, small pieces of the LV papillary muscle were fixed in Karnowsky's fixative (0.12 M phosphate, pH 7.2) for 1-2 h and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h. After dehydration in a graded ethanol series, samples were embedded in epoxy resin. Ultrathin sections were cut from selected areas with a diamond knife, double-stained with uranyl acetate and lead citrate, and examined with a Philips EM 301 electron microscope. LV myocyte cross-sectional area was measured using a compound microscope attached to a computerized imaging analysis system (Image-Pro Plus 3.0, Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Data are reported as means ± SD or median ± semi-range for non-normal distribution. Data regarding morphological, biochemical and basal mechanical parameters were assessed by the Student *t*-test for independent samples. Data reporting the intensity of the response to inotropic maneuvers are presented as relative change from the baseline. The intensity of the response to calcium elevation and isoproterenol addition was compared between groups using the Mann-Whiney test. The level of significance was set at $P < 0.05$ in all analyses.

Results

The UFA diet did not change the general characteristics of the animals, such as FW (C: 434 ± 32 and F: 429 ± 39; $P > 0.05$), LV/

FW (C: 1.96 ± 0.08 and F: 2.00 ± 0.25; P > 0.05) and RV/FW (C: 0.57 ± 0.05 and F: 0.59 ± 0.05; P > 0.05). CSA (C: 1.06 ± 0.31 and F: 1.06 ± 0.19; P > 0.05) and L_{max} (C: 6.85 ± 0.87 and F: 6.72 ± 1.09; P > 0.05) were similar in both groups, but the rats treated with a UFA-rich diet showed a lower mean food consumption (C: 162 ± 12 and F: 130 ± 13; P < 0.01).

There were no significant differences in mechanical data (AT, RT, TPT, +dT/dt, -dT/dt, RT_{50}, *k*m, -dL/dT, and +dL/dT) obtained under basal conditions and with inotropic stimulation with calcium change from 1.25 to 5.2 mM and 1.0 µM isoproterenol between groups.

Lipid hydroperoxide concentration in cardiac tissue was higher in group F. Total antioxidant status, SOD, and CAT activities were lower in group F (Table 1). Higher lipid hydroperoxide and decreased antioxidant defenses indicated that there was myocardial oxidative stress in group F rats. Table 2 shows that triacylglycerol was lower and LDL-cholesterol was higher in the F group.

The histopathological study of group F LV myocardium was normal under light microscopy. Ultrastructural analysis revealed only mild lipid accumulation among myocytes and mild mitochondrial membrane injuries in group F (Figure 1).

Discussion

The aim of the present investigation was to evaluate the effects of a UFA-enriched diet on myocardial function, oxidative stress, and morphology. Papillary muscle preparations permit the measurement of cardiac muscular ability to develop force and to shorten, independent of changes in cardiac load and heart rate that might modify mechanical performance of the myocardium *in vivo*. Inotropic stimulation allows the identification of alterations in contraction and relaxation phases that cannot be observed under basal conditions and helps in the understanding of the mechanisms involved in myocardial function alterations.

The present results showed that rats fed a diet rich in UFA had the same body weight as controls. This occurred because the rats on the hypercaloric diet ate less than the animals on normal chow. These data are in agreement with previous reports that used the UFA-rich diet in different experiments (1,15,18,20).

Table 1. Effect of diet on the biochemical characteristics of the myocardium.

	Groups	
	C	F
TP (g%)	21 ± 3	24 ± 1
LH (nmol/g tissue)	158 ± 5	176 ± 5*
TAS (g%)	35 ± 6	15 ± 2*
SOD (U/mg protein)	27 ± 5	18 ± 2*
GSH-Px (U/mg tissue)	22 ± 2	22 ± 1
CAT (kat.f)	9 ± 1	7 ± 1*

Data are reported as means ± SD for 5 animals in each group. C = control group; F = group treated with a diet rich in unsaturated fatty acids; TP = total protein; LH = lipid hydroperoxide; TAS = total antioxidant status; SOD = superoxide-dismutase; GSH-Px = glutathione peroxidase; CAT = catalase.
*P < 0.05 compared to control group (Student *t*-test).

Table 2. Effect of diet on serum lipids.

	Groups	
	C	F
TL (mg/dL)	351 ± 70	329 ± 45
TG (mg/dL)	125 ± 21	83 ± 23*
TC (mg/dL)	62 ± 8	64 ± 9
HDL (mg/dL)	27 ± 7	25 ± 7
LDL (mg/dL)	11 ± 11	19 ± 10*

Data are reported as means ± SD for 8 animals in each group. C = control group; F = group treated with a diet rich in unsaturated fatty acids; TL = total lipids; TG = triacylglycerol; TC = total cholesterol; HDL = HDL-cholesterol; LDL = LDL-cholesterol.
*P < 0.05 compared to control group (Student *t*-test).

Triacylglycerol decreased and LDL-cholesterol increased in the animals submitted to a UFA-rich diet. The reduced carbohydrates in the UFA diet could explain the decreased serum triacylglycerol levels in animals fed this diet (3). However, increased LDL-cholesterol was observed because the UFA-enriched diet contained more fat than the control diet, a result also obtained in a study using the same diet (24).

In the present investigation, the modest morphological changes of the myocardium, such as lipid deposit and mitochondrial membrane injuries, were only observed under electron microscopy while no alterations were seen under the light microscope. This contrasts with previous studies that observed necrosis (14) and abnormal myocardial striation (15) using different kinds of UFA-rich diet. According to Lamers et al. (14), the mechanism by which an imbalance in dietary fatty acids produces myocardial lesions is completely unknown. However, others have suggested that these cardiac morphological injuries can be caused by oxidative stress induced by a diet rich in UFA (24,35,36). In the present study, we observed myocardial oxidative stress and light morphological injuries induced by the UFA-enriched chow. Increased dietary levels of UFA can cause these lipids to be incorporated into membrane phospholipids, leading to oxidative stress (24,35,36).

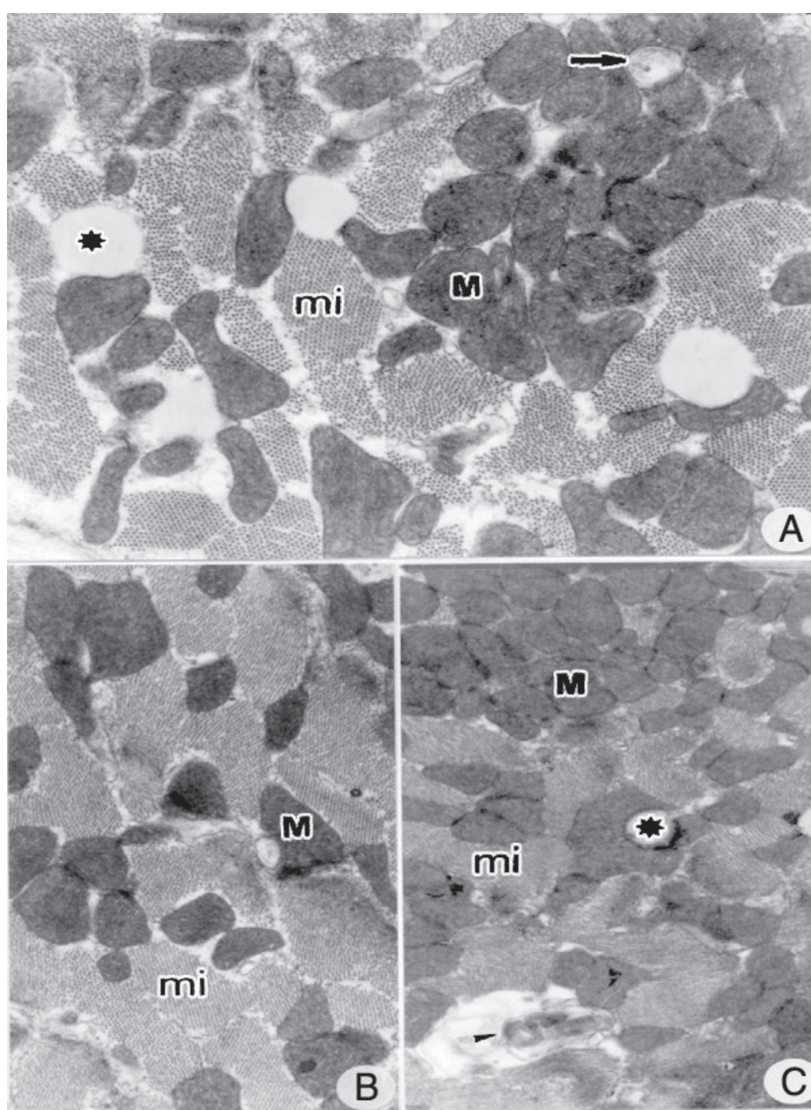

Figure 1. Cardiac striated muscle from the group receiving a UFA-rich diet. *A*, *B* and *C* = Myofibrils (mi). Normal mitochondria (M). Mitochondria with loss of cristae in *A* (arrow). Lipid vesicles in *A* and *C* (*). Myeloid bodies in *C* (arrowhead). A: 4600X; B: 3400X; C: 2650X.

Although we observed oxidative stress and other recent investigations have established that free radicals may be important contributors to cardiac dysfunction and myocardial damage (13,37-39), in the present study myocardial mechanical function was not altered by the diet. Kako and Vasdev (1) also observed that a high UFA diet did not change the mechanical function of isolated papillary muscle. Under basal conditions, De Wildt and Speijers (17) demonstrated that a UFA-rich diet did not influence the contractility of isolated rat hearts. On the other hand, the same investigators noted a loss of myocardial function under inotropic stimulation with isoproterenol.

Charnock et al. (18) showed that rats on a UFA-rich diet had the same mechanical function as control rats. When the papillary muscles were under higher calcium concentrations, the response was better in rats receiving the UFA-rich diet, as shown by increased developed tension. Peterson et al. (19) reported that a high UFA diet can cause an important loss of contractility, with longer

times to develop tension and lower velocities during the contraction phase. ChemLa et al. (20) observed that UFA-rich diets modify the relaxation properties of the rat myocardium and believed this was due to a defect in the handling of calcium, but there are no data confirming this hypothesis.

The present study shows that, although a diet rich in UFA caused mild myocardial ultrastructural alterations and oxidative stress, it did not alter intrinsic cardiac muscle performance.

Acknowledgments

We thank J.C. Georgette and M. Bruno for expert technical assistance, and Colin E. Knaggs (Faculdade de Medicina de Botucatu) for revising the English text.

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