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Myocardial dysfunction induced by food restriction is related to morphological damage in normotensive middle-aged rats

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Summary

Previous works from our laboratory have revealed that food restriction (FR) promotes discrete myocardial dysfunction in young rats. We examined the effects of FR on cardiac function, *in vivo* and *in vitro*, and ultrastructural changes in the heart of middle-aged rats. Twelve-month-old Wistar-Kyoto rats were fed a control (C) or restricted diet (daily intake reduced to 50% of the control group) for 90 days. Cardiac performance was studied by echocardiogram and in isolated left ventricular (LV) papillary muscle by isometric contraction in basal condition, after calcium chloride (5.2 mM) and beta-adrenergic stimulation with isoproterenol (10^{-6} M). FR did not change left ventricular function, but increased time to peak tension, and decreased maximum rate of papillary muscle tension development. Inotropic maneuvers promoted similar effects in both groups. Ultrastructural alterations were seen in most FR rat muscle fibers and included, absence and/or disorganization of myofilaments and Z line, hyper-contracted myofibrils, polymorphic and swollen mitochondria with disorganized cristae, and a great quantity of collagen fibrils. In conclusion, cardiac muscle sensitivity to isoproterenol and elevation of extracellular calcium concentration is preserved in middle-aged FR rats. The intrinsic muscle performance depression might be related to morphological damage.

Introduction

Food restriction (FR) induces beneficial health effects, such as increased longevity [1], carcinogenesis inhibition [2], reduced acute toxicity of drugs [3], and an improved ability to deal with a wide range of toxic processes from endogenous, physical, biological, and chemical agents [4]. Recent research in our laboratory has shown that FR promotes left ventricular dysfunction [5] and increases contraction and relaxation times in isolated papillary muscle of young normotensive and hypertensive rats [6–9]. The mechanisms behind the changes in myocardial function with FR are still unknown. Several factors may contribute to cardiac dysfunction; they include alteration in excitation–contraction coupling, intrinsic changes in contractile proteins, impaired autonomic modulation during stress, insufficient energy supply, extracellular matrix changes, and myocyte dropout [10]. In our laboratory, we have observed striking myocardial ultrastructural alterations [9] and calcium handling and

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beta-adrenergic system changes [8] in young Wistar-Kyoto rats submitted to 50% FR.

Based on our results in young rats, this study investigated the effects of chronic FR on cardiac and myocardial functions in middle age rats, evaluated *in vivo* by echocardiogram and *in vitro* by left ventricular papillary muscle preparation. We also studied myocardial sensitivity to inotropic stimulation and myocardial ultrastructure. Our data show that muscle sensitivity to isoproterenol and elevation of extracellular calcium concentration is preserved in FR rats and suggests that intrinsic muscle performance depression might be related to morphological damage.

Materials and methods

Animal model and experimental protocol

Male Wistar-Kyoto rats (WKY), 12 months of age were divided in two groups: control or a FR diet. The control group (C, $n=11$) was fed Purina rat chow (3.76% fat, 20.96% protein, 52.28% carbohydrate, 9.60% fiber, and 13.40% humidity) and water *ad libitum*. The food restricted group (FR, $n=8$) received 50% of amount of food consumed by the control group. Food consumption for the control group was measured daily and used to calculate food amount for the FR group. Rats were maintained on this dietary regimen for 90 days. Food restricted and control rats were weighed once a week. In this study the term FR is used synonymously with chronic semistarvation, on the basis of the fact that calories are severely restricted and that the animals are deprived of food for almost 24 h between feedings. Although the FR group received 50% the amount of food consumed by control rats, the Purina rat chow ingested (http://www.agribrands.com.br) by the FR group contained sufficient amounts of proteins, vitamins, and minerals for rat maintenance according to Nutrient Requirements of Laboratory Animals [11]. All rats were housed in individual cages in an environmentally controlled room with a temperature of 23 ± 3 °C and 12 h light:dark cycle. Initial and final body weight (IBW and FBW, respectively), left ventricular weight (LVW), right ventricular weight (RVW), left ventricular weight-to-final body weight ratio (LVW/FBW), and right ventricular weight-to-final body weight ratio (RVW/FBW) were measured.

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

Cardiac functional evaluation

Echocardiographic study

Echocardiogram was performed in all animals at the end of the experimental period to evaluate heart structure and function, by using a commercially available echocardiographic machine Sonos 2000 (Hewlett-Packard Medical Systems, Andover, MA, USA) equipped with a 7.5 MHz phased array transducer. Imaging was performed with a 60° sector angle and 3 cm imaging depth. Rats were anesthetized by intramuscular injection with a mixture of ketamine (50 mg/kg) and xylazine (1 mg/kg). Two-dimensionally targeted M-mode echocardiograms were obtained from short-axis views of the left ventricle (LV) at or just below the tip or the mitral-valve leaflets, and were recorded on black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 100 mm/s. All LV tracings were manually measured with a caliper by the same observer, and according to the leading-edge method of the American Society of Echocardiography. Measurements are the mean of at least five consecutive cardiac cycles. LV end-diastolic dimension (LVDD) and posterior wall thickness (LVWT) were measured at maximum diastolic dimension, and the end-systolic dimension (LVSD) was taken at maximum anterior motion of posterior wall. Left atrial dimension (LAD), aortic dimension (AOD), early peak transmitral flow velocity to late peak transmitral flow velocity ratio (Mitral E/A), and heart rate (HR) were also measured. Relative wall thickness (RWT) was determined by LVWT/LVDD. Left ventricular systolic function was assessed by calculating the fractional shortening index $FS=[(LVDD-LVSD)/LVDD \times 100]$.

Isolated muscle performance

Myocardial performance was evaluated by studying isolated papillary muscle from the LV as

described in detail previously [6, 7, 9]. Briefly, at the time of study, rats were killed and their hearts were quickly removed and placed in oxygenated Krebs–Henseleit solution at 28 °C. Trabecular carneae or papillary muscle was dissected free, mounted between two spring clips, and placed vertically in a chamber containing Krebs–Henseleit solution at 28 °C and oxygenated with a mixture of 95% O_2 and 5% CO_2 (pH 7.38–7.42). The spring clips were attached to a Kyowa model 120T-20B force transducer and to a lever system, which allowed adjusting muscle length. Preparations were stimulated 12 times/min at a voltage 10% above threshold.

After a 60-min period during which the preparations were permitted to shorten while carrying light loads, muscles were loaded to contract isometrically and stretched to the apices of their length–tension curves. After a 5-min period, during which preparations performed isotonic contractions, muscles were again placed under isometric conditions, and the apex of the length–tension curve (L_{max}) was determined. A 15-min period of stable isometric contraction was imposed prior to the experimental period. One isometric contraction was then recorded for later analysis. The mechanical behavior of papillary muscle was evaluated in basal condition and after inotropic maneuvers: increase in extracellular Ca^{2+} concentration from 1.25 to 5.2 mM and during beta-adrenergic stimulation with 1 μM isoproterenol.

The following parameters were measured from isometric contraction: peak developed tension (DT, g/mm^2), resting tension (RT, g/mm^2), time to peak tension (TPT, ms), maximum rate of tension development (+dT/dt, g/mm^2/s), maximum rate of tension decline (−dT/dt, g/mm^2/s), and time from peak tension to 50% relaxation (RT_{50}, ms). At the end of experiment, the muscle length at L_{max} was measured and the muscle between the two clips was blotted dry and weighed. Cross-sectional area (CSA) was calculated from the muscle weight and length by assuming cylindrical uniformity and a specific gravity of 1.0. All force data were normalized for the muscle cross-sectional area.

Morphological study

Three animals from each group were used for ultrastructural analysis. Small fragments of the LV papillary muscle were fixed in Karnovsky's fixative (0.12 M phosphate, pH 7.2) for 1–2 h followed by post fixation in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h. After dehydration in a graded ethanol series, the fragments were embedded in epoxy resin. Ultra-thin sections were double-stained with uranyl acetate and lead citrate and examined in an electron microscopy (Phillips EM 301).

Statistical analysis

Values are shown as means ± SD for normal distribution or median ± semi-range for non-normal distribution. Data from morphological and basal mechanical parameters were compared by Student's *t*-test. The intensity of response (Δ) to inotropic maneuvers was presented as relative change (%) from baseline data and was calculated as follows: $\Delta = (M2-M1)/M1 \times 100$, where M1 was the value in basal condition and M2 was the value after calcium or isoproterenol addition. The Δ values were compared by Mann–Whitney test. Significance was considered at $p < 0.05$.

Results

General characteristics of rats

Table 1 summarizes the influence of FR on food consumption, body weight, and heart weight. FR decreased 43% of FBW, and 30% of LVW and RVW. For this reason, LVW/FBW and RVW/FBW ratios were significant increased by FR.

Functional study

Tables 2–5 show data from cardiac function obtained by echocardiographic study and isolated papillary muscle preparation. In echocardiographic study, LVDD/FBW, LVSD/FBW, LAD/FBW, and AOD/FBW were significantly increased in FR than C rats. LVWT, RWT, FS, Mitral E/A, and HR did not change with food restriction (Table 2). Mechanical function of papillary muscle in basal condition showed significant reduction in +dT/dt, and significant increase of TPT in FR rats than control animals (Table 3). Extracellular calcium elevation promoted same intensity of response in DT, RT, +dT/dt, TPT,

Table 1. Food consumption and general characteristics of rats.

	Control ($n=11$)	FR ($n=8$)
Food consumption (g/day)	23.88 ± 2.77	11.92 ± 1.40***
Food consumption (kcal/day)	65.67 ± 7.62	32.78 ± 3.85***
IBW (g)	568 ± 46	538 ± 53
FBW (g)	577 ± 36	332 ± 34***
LVW (g)	0.91 ± 0.05	0.64 ± 0.05***
RVW (g)	0.27 ± 0.03	0.19 ± 0.02**
LVW/FBW (mg/g)	1.57 ± 0.05	1.91 ± 0.15***
RVW/FBW (mg/g)	0.48 ± 0.06	0.58 ± 0.08**

Values are means ± SD; n, number of animals; FR, food restriction; IBW, initial body weight; FBW, final body weight; LVW, left ventricle weight; RVW, right ventricle weight.
**$p < 0.01$; ***$p < 0.001$ versus control; (Student's t-test).

Table 2. Echocardiographic data.

	Control ($n=11$)	FR ($n=8$)
LVDD/FBW (mm/kg)	15.30 ± 1.12	21.94 ± 3.10***
LVSD/FBW (mm/kg)	8.34 ± 0.99	12.86 ± 2.51***
LVWT (mm)	1.51 ± 0.22	1.37 ± 0.18
RWT	0.35 ± 0.07	0.36 ± 0.05
LAD/FBW (mm/kg)	8.05 ± 1.15	13.01 ± 1.21***
AOD/FBW (mm/kg)	6.98 ± 0.85	10.75 ± 1.16***
FS (%)	45.38 ± 5.92	41.68 ± 4.06
Mitral E/A	1.65 ± 0.27	1.68 ± 0.18
HR (bpm)	231 ± 24	214 ± 18

Values are means ± SD; n, number of animals; FR, food restriction; FBW, final body weight; LVDD, left ventricular diastolic dimension; LVSD, left ventricular end-systolic dimension; LVWT, left ventricular posterior wall thickness; RWT, relative wall thickness; LAD, left atrial dimension; AOD, aortic dimension; FS, left ventricular fractional shortening; Mitral E/A, early peak transmitral flow velocity to late peak transmitral flow velocity; HR, heart rate; bpm, beats per minute.
***$p < 0.001$ versus control (Student's t-test).

−dT/dt, and RT_{50} in both groups (Table 4). After isoproterenol addition, only RT response was significantly decreased in FR. Other parameters did not change with isoproterenol in both groups (Table 5).

Morphological study

The papillary muscle of control animals showed normal morphological aspects that included fibers presenting sarcoplasm filled with myofibrils, well-defined sarcomeres, mitochondrias presenting numerous lamellar cristae, plasma membrane with regular aspect, and central nucleus with loose chromatin. Sarcoplasmic reticulum vesicles were observed between the myofibrils. Muscle fibers were surround by capillaries, interstitial cells, and collagen fibrils in connective tissue.

Food restriction caused alterations in papillary muscle fiber ultrastructure. These alterations were present in most muscle fibers and included deep infolding of the plasma membrane, reduction of sarcoplasmic content due to the loss and/or disorganization of myofilaments and Z line,

Table 3. Data from isolated muscle preparation.

	Control ($n=11$)	FR ($n=8$)
DT (g/mm^2)	7.15 ± 1.44	6.44 ± 1.00
RT (g/mm^2)	1.27 ± 0.29	1.16 ± 0.24
+dT/dt (g/mm^2/s)	62 ± 8	52 ± 8**
TPT (ms)	194 ± 14	212 ± 15**
−dT/dt (g/mm^2/s)	21 ± 4	19 ± 3
RT_{50} (ms)	225 ± 22	249 ± 37
CSA (mm^2)	1.20 ± 0.23	1.16 ± 0.26

Values are means ± SD; n, number of animals; FR, food restriction; DT, peak developed tension; RT, resting tension; +dT/dt, maximum rate of tension development; TPT, time to peak tension; −dT/dt, maximum rate of tension decline; RT_{50}, time from peak tension to 50% relaxation; CSA, muscle cross-sectional area.
**$p < 0.01$ versus control (Student's t-test).

Table 4. Extracellular calcium change effect in isolated muscle preparation.

	Control ($n=11$)	FR ($n=8$)
DT	0.0 ± 9.0	1.0 ± 7.5
RT	2.0 ± 9.0	0.0 ± 13.5
+dT/dt	15.0 ± 16.0	12.0 ± 25.0
TPT	−8.0 ± 20.0	−7.0 ± 11.0
−dT/dt	20.0 ± 27.54	18.5 ± 19.5
RT_{50}	−21.0 ± 47.0	−21.0 ± 7.0

Values (median ± semi range) are expressed as relative change (%) from baseline data (extracellular calcium changed from 1.25 to 5.2 mM); FR, food restriction; n, number of animals; DT, peak developed tension; RT, resting tension; +dT/dt, maximum rate of tension development; TPT, time to peak tension; −dT/dt, maximum rate of tension decline; RT_{50}, time from peak tension to 50% relaxation (Mann–Whitney test).

Table 5. Isoproterenol stimulation in isolated muscle preparation.

	Control ($n=11$)	FR ($n=8$)
DT	0.0 ± 32.0	14.5 ± 48.5
RT	−6.0 ± 27.0	−9.0 ± 10.5*
+dT/dt	17.0 ± 17.0	13.0 ± 22.0
TPT	−12.0 ± 2.0	−12.5 ± 11.0
−dT/dt	35.0 ± 37.0	42.0 ± 30.0
RT_{50}	−23.0 ± 6.5	−29.0 ± 18.5

Values (median ± semi range) are expressed as relative change (%) from baseline data; FR, food restriction; *n*, number of animals; DT, peak developed tension; RT, resting tension; +dT/dt, maximum rate of tension development; TPT, time to peak tension; −dT/dt, maximum rate of tension decline; RT_{50}, time from peak tension to 50% relaxation.
*$p < 0.05$ versus control (Mann–Whitney test).

hyper-contracted myofibrils, presence of polymorphic and swollen mitochondria with disorganized cristae. The sarcoplasmic reticulum had normal aspect. The space between muscle fibers was increased and filled by a large quantity of collagen fibrils.

Discussion

The aim of this study was to investigate the effect of food restriction on cardiac and myocardial function in middle-aged rats, and analyze the myocardial sensitivity to inotropic stimulation and morphological pattern of the myocardium. Papillary muscle preparations allow measurement of the cardiac muscular ability to develop force and shorten, independent of changes in cardiac load and heart rate that might modify mechanical performance of the myocardium *in vivo*. The intensity response to inotropic stimulation allows detect alterations in contraction and relaxation phases that cannot be observed under basal condition and helps in the understanding of mechanisms involved in myocardial functional alterations.

The food restriction decreased final body weight more intensively than cardiac weight. Therefore, LVW/FBW and RVW/FBW ratios were increased in FR rats. This shows a sparing of left and right ventricles relative to body weight with FR, and is commonly seen in small animals, such as the rat [5, 8, 9, 12, 13].

The echocardiographic study did not shown left ventricular function alterations in middle-aged FR rats. Other authors have evaluated the relationship between FR and cardiac function in young and middle-aged rats. Chang et al. [14] observed that FR for alternative days over 6 months prevented or delayed myocardial contractility impairment analyzed by hemodynamic measurements in middle-aged and senescent rats. Hilderman et al. [15] showed depression of heart function in adult rats receiving 6 g or 12 g of food during 14 or 28 days. Haddad et al. [16] observed a lower functional state of left ventricular pressure and maximal rate of pressure rise in young female rats submitted to 50% or 75% FR. Okoshi et al. [5] found ventricular dysfunction in 5-month-old rats submitted to 50% FR for 90 days. McKnight et al. [17] observed depressed left ventricular systolic pressure in adult rats after 14 days of 75% FR.

The impairment of isolated papillary muscle preparation (+dT/dt and TPT) in basal condition observed in this experiment, suggests that FR promoted myocardial systolic dysfunction. Previous studies on isolated heart or papillary muscle in young rats submitted to FR showed increases in contraction and relaxation times [7–9, 18, 19].

As previously mentioned, several factors could contribute to myocardial dysfunction included calcium handling and/or beta-adrenergic pathway changes [10]. As both control and food restricted groups had similar intensity response to high dose calcium and isoproterenol, we can speculate that calcium handling and beta-adrenergic system pathways were preserved in FR rats. Klebanov et al. [18] verified an enhanced sensitivity to low perfusate calcium (0.85, 1.0, 1.5, and 3.0 mM) and isoproterenol (1, 3, and 10 nM) in isolated heart of rats submitted to long-term 40% FR. As Klevanov et al. [18] used low calcium and isoproterenol doses, in contrast to our protocol (5.2 mM calcium and 1 μM isoproterenol), the discrepant results could be due to the different drug concentrations.

The important morphological changes observed in our work might lead myocardial dysfunction in food restricted rats (Figure 2). FR caused severe morphological damage in most papillary muscle fibers, involving sarcolemma, myofilaments, sarcoplasm, mitochondria, and the extracellular matrix. These morphological damages were more intense and diffuse that was observed in our previous work in FR young

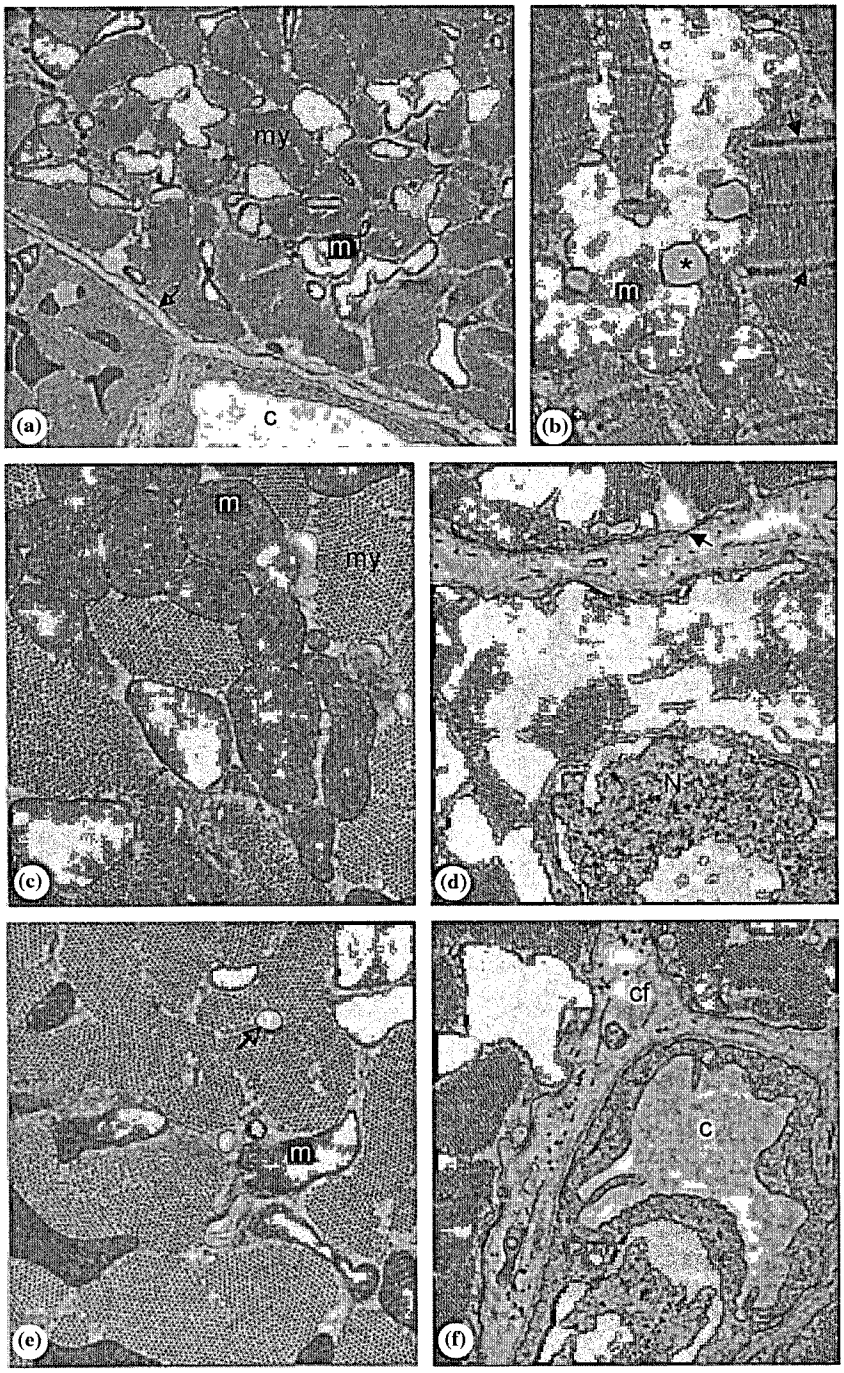
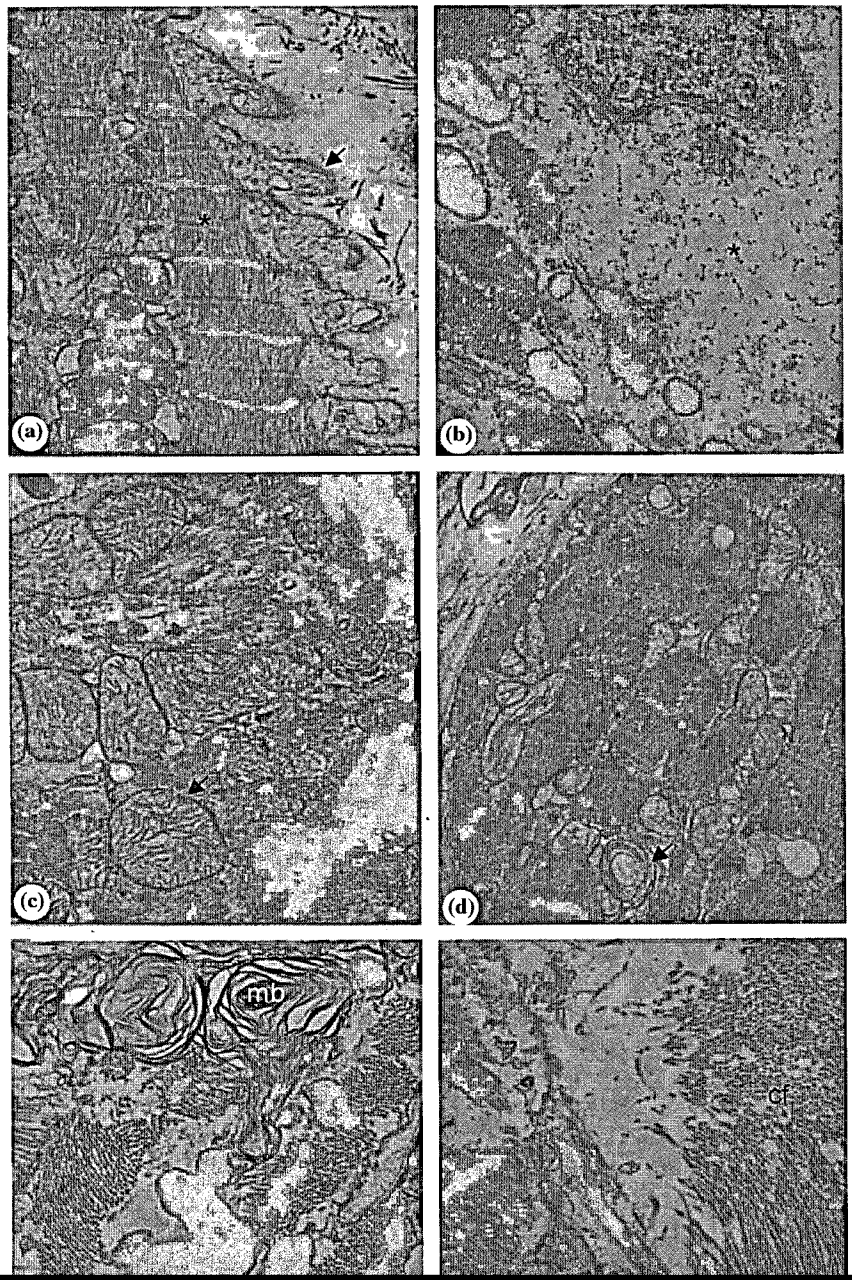


Figure 1. Electron micrographs of the myocardial cells in the control rats. (a) sarcoplasm filled with myofibrils (my) and mitochondria (m). Plasm membrane (arrow) and capillary (c) (9750×). (b) Mitochondria (m) and well-defined sarcomeres (between arrows). Lipid droplets (*) (17,000×). (c) Mitochondria presenting numerous lamellar cristae (m) and myofibrils (my) (23,000×). (d) Plasma membrane with regular aspect (arrow) and central nucleus with loose chromatin (N) (17,000×). (e) Mitochondria presenting numerous lamellar cristae (m) and sarcoplasmic reticulum vesicles (arrow) between the myofibrils (23,000×). (f) Muscle fibers surrounded by capillaries (c) and collagen fibrils of the connective tissue (cf) (17,000×).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WKY rats [9]. Disorganization and lack of myofibrils, myofilaments, and Z disc, and disconnection between myocytes can hamper the coordinated transmission of muscular contraction and reduce myocardial performance [20]. Moreover, the large increase in myocardial collagen fibrils between muscle fibers seen in this study may be involved in the development of myocardial dysfunction in FR rats, since fibrosis can impair cardiac performance [21].

Several authors have shown that chronic FR induces a significant reduction in cardiac isomyosin V1 expression in rats, which is offset by an increase in the two lower ATPase (V_2 and V_3) isoforms [16, 22]. The myosin isoform V_3 was demonstrated to cause duration of displacement events and force transients longer than V_1 isoform [23]. The shift in the myocardial isoenzyme towards the slow V_3 isoform may in part explain the increased of time to peak tension in FR rats [22].

In this study, FR in middle-aged rats promoted myocardial dysfunction without changing ventricular function. These different results may be due that muscle performance changes can be detected earlier than ventricular function alterations. Another explanation is that, heart function was evaluated only by fractional shortening, and it was not determined time to peak shortening and shortening velocity.

Although this work demonstrates that severe FR deteriorates myocardial function, other studies shows that less severe forms of FR (40%) improves ischemic tolerance and preserves the preconditioning response in middle-aged and senescent rats [24–26].

In summary, this investigation shows that a 90-days period of food restriction promotes morphological damage and myocardial systolic dysfunction in middle-aged rats. Our data show that muscle sensitivity to isoproterenol and elevation on extracellular calcium concentration is preserved in FR rats and suggests that intrinsic muscle performance depression might be related to the morphological damage.

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