

**HISTOCHEMICAL RESPONSES OF RATS EXERCISED IN TWO WEEKLY FREQUENCIES AND INGESTING STANDARD OR HYPERCALORIC DIET**

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**Abstract.** This study investigated if overfed rats present morphological and histochemical muscle adaptation similar to normally fed, both submitted to two different weekly frequencies of training. Thirty male Wistar rats were fed either with standard chow (SCØ) or with hypercaloric diet (HCØ). They were subdivided into six subgroups: sedentary (SCØ and HCØ), trained twice/week (SC2 and HC2) and trained five times/week (SC5 and HC5). The trained groups swam 60 min/day, during 10 weeks. Twenty four hours after the last training, samples of Gastrocnemius were excised and stained with HE, NADH-TR and m-ATPase, and the capillary density was calculated. Total heart mass (HM) and the mass of atrium (AM), left (LV) and right (RV) ventricles were excised and weighted. The comparisons were made by ANOVA and by Covariance analysis, adjusting the variables by body weight. The results showed that the HCØ achieved higher BM, however, absolute HM did not differ post training. Irrespective of the diet, rats that were trained twice a week presented significantly greater increase in the AM. In general, the SC5 and HC5 groups showed higher HM, LV, RV, proportion of oxidative fibres and capillary density, compared to the sedentary and twice week trained groups. A higher proportion of injuries (splitting) was noted in the HC2 and HC5 compared to SC2 and SC5. These results indicate that the frequency of training influenced the skeletal and heart adaptation and larger changes were observed in the 5x/week group, which ingested the standard diet. The 5x/week training groups also presented large amount of muscle fibres damage.

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## Introduction

In the past decades overweight and obesity has become the most prevalent nutritional disorder around the world, and a growing body of knowledge from clinical, epidemiological and experimental research, has revealed the consequences of the excess of body fat on health [18].

Some studies suggest that obese subjects present lower fat oxidation [13,37]. This has been also observed in obese-prone normal-weight subjects submitted to a fat-rich diet [1]. Although the reasons for this process are still unclear [28] some evidence supports the hypothesis that higher proportion of Glycolytic muscle fiber [14] is a key factor in the aetiology of obesity [37].

Regular physical activity (PA) promotes metabolic adaptations in skeletal muscle, which includes better contraction ability and modifications in the fibre type [5,8,24], vascular remodeling with the formation of new capillaries [16] and higher activity of oxidative enzymes [28]. All these modifications result in both an improvement of the functional capacity [10] and a higher fat oxidation rate, which occur similarly in exercised obese and normal weight subjects [29].

The low functional capacity demonstrated by sedentary normal-weight subjects, is also observed, to a greater extent, in those subjects who present an excessive body fat [30]. The adaptive structural and functional changes in the ventriculoarterial tree, exert an impact in the O<sub>2</sub> consumption and, consequently, in the performance of any physical activity [23].

Even though the exercise is of fundamental importance to the cellular adaptation in the control of fat metabolism and in the cardiovascular function, in normal weight and overweight subjects, it is still unclear, if these adaptations occur in the same way when weekly training of varying frequencies is applied.

In a recent review of the literature no studies were found that focused on the cellular and cardiac adaptations of subjects, ingesting normal and hypercaloric diets, being submitted to different week frequency of training.

The purpose of this study was to investigate if overfed rats present morphological and histochemical muscle adaptation similar to normally fed rats. the investigation was also interested in the interaction of overnutrition with exercise. In this study the groups were submitted groups of rats on either hypercaloric or normal diets to different weekly frequencies of endurance training.



## Materials and Methods

The ethical committee of Universidade do Oeste Paulista approved all procedures of this research.

Thirty male Wistar rats (*Rattus norvegicus*), aged 90 days, from Central Biotério of Sao Paulo State University (UNESP), Campus of Botucatu, were used in this study. The animals were maintained in the Biotério of UNESP, Campus of Presidente Prudente, place where the experiment was made, housed five per cage in a temperature-controlled room (24° C) and a 12-h light/dark cycle.

The mice were fed with one of two diets as follows: One group (n=15) received standard chow pelleted (NUVILAB) produced by Nuvital Nutrients Ltda. (Colombo, PR, Brazil), with 22% of protein (4.07kcal/g). The other group (n=15), received a hypercaloric diet, formulated by Nutrition and Metabolism Laboratory of Federal University of Sao Carlos (DEFMH/CCBS/UFSCAR), and composed by: 40% of standard chow NUVILAB, and the other 60% included peanuts, milk chocolate, and sweet biscuit. This hypercaloric diet resulted in 20% protein, 32% fat, 48% carbohydrate, and 4% fibre (5.60 kcal/g). The composition of this diet has been described previously [4]. Food and water were available ad libitum.

The rats were assigned in six sub-groups (5 animals each) and as the proposal of the work was to evaluate the adaptations in adult rats, before the beginning of the experimental period, the animals remained sedentary, until their weight reached at least 350 g. This procedure was adopted so that the animals could adapt to the hypercaloric diet and that the natural weight gain expected as a result of the growth, did not have any influence in the result of the study.

After the period of the food adaptation the groups were distributed as follows:

Two control sub-groups remained sedentary, one ingested standard chow pellet (SCØ) and the other ingested the hypercaloric diet (HCØ) throughout the experimental period;

Two sub-groups were exercised two times per week (2x/week) on consecutive days, one ingested a standard chow pellet (SC2) and the other ingested a hypercaloric diet (HC2);

Two groups were exercised five times per week (5x/week) on consecutive days, one ingested a standard chow pellet (SC5) and the other ingested a hypercaloric diet (HC5).

The training consisted of continuous swimming for 60 min/day, on consecutive days, during 10 weeks. This exercise was performed in tanks of water with the capacity for 10 animals, measuring 40x60x80cm. The water temperature was maintained between 32°C and 36°C and was changed daily.



Before initiating the experimental phase all the animals of the trained groups had been submitted to a period of 5 days of adaptation to the water as follows: in the first day the rats had remained 20 min with 15 cm of water in the tank, in second day 30 min with 20 cm of water, in third day 40 min with 30 cm, in the fourth day 50 min with 40 cm, and in fifth day 60 min with 50 cm.

The surgical procedure began twenty-four hours after the last training, with intraperitoneal anaesthesia of sodium pentobarbital (20 mg/kg weight). Samples from consistent midmuscle region of Gastrocnemius muscles (Gast) were excised. After that, all animals were killed with a deep intraperitoneal injection of excessive dose of sodium pentobarbital (60 mg/kg) and the heart was resected and weighted.

The deep cross section (~5mm) of the lateral Gast was resected, rapidly frozen in isopentane-cooled liquid nitrogen and stored at -70 °C pending histochemical assay.

The total heart mass (HM), and the weight of its fractions: atrium mass (AM), left (LV) and right (RV) ventricles were resected carefully and weighted immediately after surgical procedure. The ratio of left ventricular weight/total heart mass (LV/HM) was calculated.

Serial transverse sections of 10 µm-thickness vertical slices of Gast for analyzing fibers and capillaries were cutted in a cryostat (Reichert-Jung Cryocut 1800; Leica) at -20°C.

Some sections were stained with haematoxylin-eosin (HE) and mounted in Permount SP15-500 (Fisher Chemical) histological media. Fibre diameters and the muscle morphometry were measured using a computerized image analysis system LEICA Qwin 2.6 Image Processing and Analysis System (Leica Cambridge, Cambridge, England) coupled to a LEICA DC 300F digital camera. Subsequent sections were reacted for NADH-tetrazolium reductase and myofibrillar ATPase (NADH-TR), after acid (pH 4.2–4.6) and alkaline (pH 9.2–9.4) preincubations [2], for the evaluation of the oxidative and/or glycolytic metabolic ability of muscular staple fibres. This procedure allowed the identification of three different types of staple fibres: Fast-twitch-glycolytic (FG); Fast-twitch-oxidative-glycolytic (FOG) and Slow-twitch-oxidative (SO). This technical procedure was performed as described [9].

The fibre diameter was determined as the average of three measurements made along the length of the fibre (from the calibrated eyepiece) and fibre cross-sectional area was calculated by assuming a circular cross-section [35].

To determine the capillary density, two random histologic samples were stained by the technique of m-ATPase of each animal. The number of blood vessels per unit area was calculated by dividing the total number of blood vessels by the total



area sampled. For each site only visualised whole fibres and the number of capillaries adjacent to them were used. The capillary density was determined by the capillary-to-fiber ratio.

*Statistics:* The results are expressed as means  $\pm$  standard deviation. The comparisons of the absolute data among groups were performed by ANOVA and the adjusted data by body mass by ANCOVA. Both analyses were followed by Least Significant Difference post-hoc test for multiple comparisons. The level of significance was set at  $P < 0.05$ . All analysis was performed using the SPSS software, version 13.0 (SPSS Inc, Chicago, IL).

## Results

**Table 1**

Absolute of weight of rats and heart weight according of different nutritional status and exercise protocols

	SCØ	SC2	SC5	HCØ	HC2	HC5	P value
BM	489.4 $\pm$	518.1 $\pm$	469.6 $\pm$	523.5 $\pm$	538.6 $\pm$	545.1 $\pm$	<0.02
(g)	52.2 <sup>ab</sup>	14.8 <sup>acd</sup>	14.1 <sup>a</sup>	52.4 <sup>acd</sup>	52.0 <sup>bc</sup>	37.3 <sup>cd</sup>	
HM	1.639 $\pm$	1.830 $\pm$	1.749 $\pm$	1.559 $\pm$	1.664 $\pm$	1.744 $\pm$	ns
(g)	0.285	0.426	0.295	0.131	0.184	0.125	
AM	0.309 $\pm$	0.505 $\pm$	0.309 $\pm$	0.396 $\pm$	0.460 $\pm$	0.292 $\pm$	<0.001
(g)	0.070 <sup>a</sup>	0.156 <sup>b</sup>	0.070 <sup>a</sup>	0.090 <sup>ab</sup>	0.052 <sup>b</sup>	0.127 <sup>a</sup>	
LV	0.984 $\pm$	0.989 $\pm$	1.014 $\pm$	0.797 $\pm$	0.870 $\pm$	0.897 $\pm$	ns
(g)	0.081	0.156	0.081	0.339	0.387	0.114	
RV	0.161 $\pm$	0.153 $\pm$	0.188 $\pm$	0.139 $\pm$	0.173 $\pm$	0.259 $\pm$	ns
(g)	0.014	0.050	0.048	0.018	0.046	0.040	
LV/ HM	0.609 $\pm$	0.547 $\pm$	0.589 $\pm$	0.625 $\pm$	0.538 $\pm$	0.614 $\pm$	<0.05
	0.065 <sup>ab</sup>	0.041 <sup>ab</sup>	0.081 <sup>ab</sup>	0.080 <sup>a</sup>	0.024 <sup>b</sup>	0.026 <sup>ab</sup>	

Values are means  $\pm$  standard deviation; SCØ - standard chow fed sedentary group; SC2 - standard chow fed 2x/week trained group; SC5 - standard chow fed 5x/week trained group; HCØ - hypercaloric diet fed sedentary group; HC2 - hypercaloric diet fed 2x/week trained group; HC5 - hypercaloric diet fed 5x/week trained group; BM - body mass; HM - heart mass; AM - atrium mass; LV - left ventricle; RV - right ventricle; LV/HM - left ventricle/heart mass ratio; ns - no statistical differences



At the onset of the study the body weight did not differ significantly between both groups (SCØ415.0±49.8g vs HCØ438.3±41.0g). However, the sedentary groups showed a significant improvement ( $P<0.05$ ) of 17.9% in the standard chow fed group (415.0±49.8g vs 489.4±52.2g), and 19.4% in the hypercaloric fed group (438.3±41.0g vs 523.5±52.4g), in the comparison between the beginning and the end of the experimental period. The BM of all trained groups also presented statistical differences ( $P<0.001$ ) comparing the diet-matched groups at the beginning with the end of the experimental period.

The BM of both trained groups fed with hypercaloric diet showed statistical higher values ( $P<0.02$ ) compared with SC5 and SC, and the HC5 was higher also than SCØ (Table 1).

The possible differences in the heart structure according to food pattern and exercise protocols were investigated by the weight of heart and of its segments.

The absolute values of HM, LV and RV showed no changes with the training in normal fed and hypercaloric fed rats (Table 1). Significant increment in the AM was observed in both 2x/week trained groups, compared with SCØ and with HC5. The LV/HM ratio was significantly lower in the HC2 compared with HCØ.

**Table 2**

Heart weight adjusted by body mass of rats according of different nutritional status and exercise protocols

	SCØ	SC2	SC5	HCØ	HC2	HC5	P value
HM	1.706±	1.823±	1.971±	1.539±	1.604±	1.668±	<0.01
(g)	0.102 <sup>ab</sup>	0.109 <sup>ab</sup>	0.120 <sup>b</sup>	0.098 <sup>a</sup>	0.101 <sup>a</sup>	0.103 <sup>ab</sup>	
AM	0.327±	0.432±	0.374±	0.388±	0.438±	0.264±	<0.005
(g)	0.040 <sup>ab</sup>	0.050 <sup>a</sup>	0.048 <sup>ab</sup>	0.039 <sup>a</sup>	0.040 <sup>a</sup>	0.041 <sup>b</sup>	
LV	1.008±	0.984±	1.078±	0.931±	0.870±	1.029±	<0.01
(g)	0.046 <sup>ab</sup>	0.050 <sup>ab</sup>	0.055 <sup>b</sup>	0.050 <sup>ab</sup>	0.047 <sup>a</sup>	0.060 <sup>b</sup>	
RV	0.171±	0.152±	0.223±	0.136±	0.164±	0.243±	<0.0001
(g)	0.015 <sup>a</sup>	0.016 <sup>a</sup>	0.018 <sup>b</sup>	0.016 <sup>a</sup>	0.015 <sup>a</sup>	0.018 <sup>b</sup>	

Values are means ± standard error; SCØ - standard chow fed sedentary group; SC2 - standard chow fed 2x/week trained group; SC5 - standard chow fed 5x/week trained group; HCØ - hypercaloric diet fed sedentary group; HC2 - hypercaloric diet fed 2x/week trained group; HC5 - hypercaloric diet fed 5x/week trained group; HM - heart mass; AM - atrium mass; LV - left ventricle; RV - right ventricle; LV/BM - left ventricle by body mass

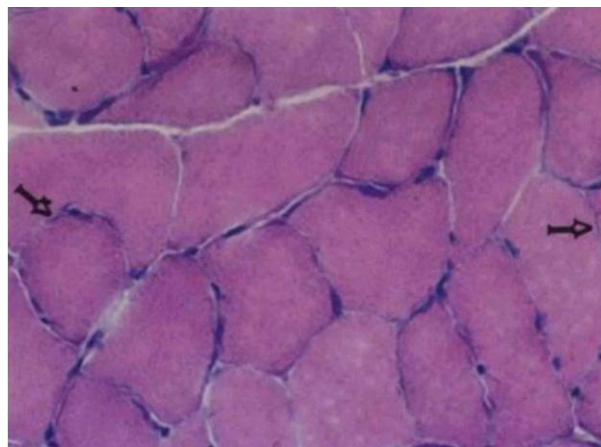
The comparisons of HM, Atrium, LV and DV using ANCOVA adjusting the variables by the weight of the animals at the end experimental period (Table 2), demonstrated that the frequency of training and BM exert influence in the heart adaptation.

The SC5 showed a significant improvement in the HM ( $P < 0.01$ ) in the comparison with HC2 and HC. Both, SC5 and HC5 presented higher LV, compared with HC2 ( $P < 0.005$ ), and higher RV compared with all other groups ( $P < 0.0001$ ). On the other hand, a significant lower value ( $P < 0.005$ ) in the adjusted AM of HC5 compared with SC2, HC2 and HCØ groups was observed. This evidence suggests that, irrespective of the fed or the BM, a higher weekly frequency of exercise promote an improvement in the left and right ventricles and a lower frequency promoted an increase in the AM.

The morphological analysis of the Gast in the sedentary using HE, showed fibres with similar cross-sectional area and the NADH-TR showed predominance of FG fibres.

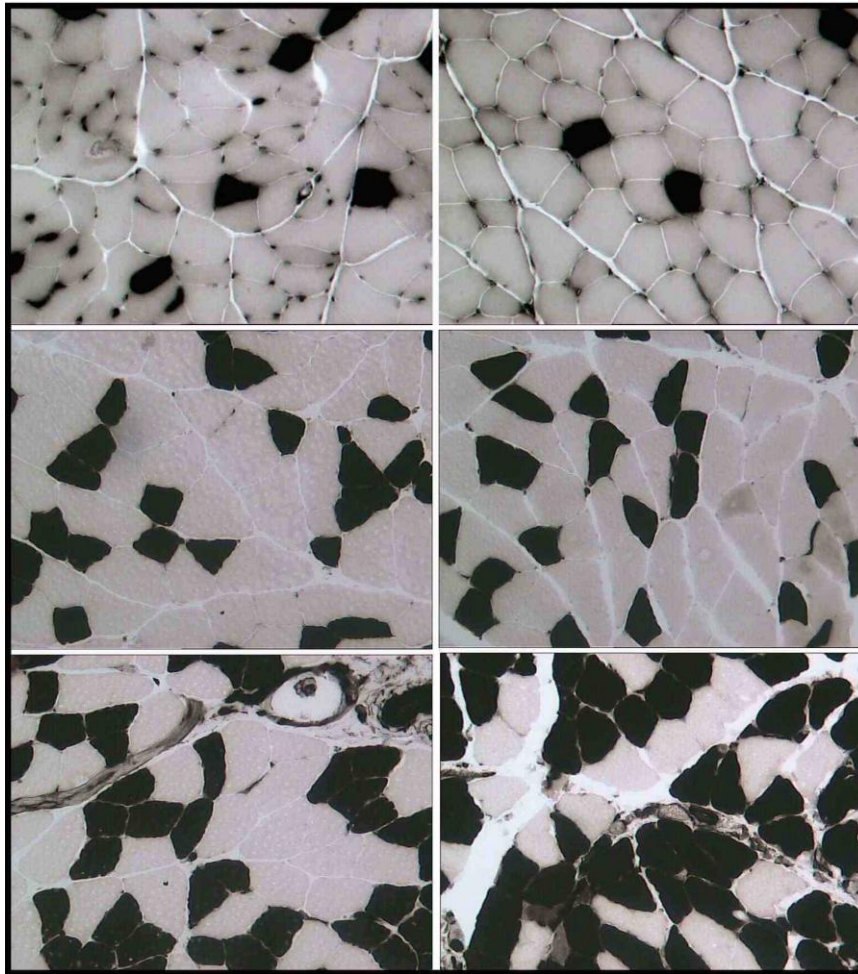
Individual FG and FOG fibres hypertrophy were evident mainly in hypercaloric fed trained rats, and several peripheral nuclei was observed in all trained groups, compared with the sedentary-matched control rats.

The HC2 and HC5 groups presented, parallel with the hypertrophy, a great amount of lateral splitting and denervated fibres, which denote excessive muscle injuries in these groups (Fig. 1).



**Fig. 1**

Cross section of the midmuscle region of Gastrocnemius muscles of 5x/week trained Wistar rats fed with hypercaloric diet (HC5), stained with haematoxylin-eosin (HE). The arrows indicate splitted fibres (original x400)

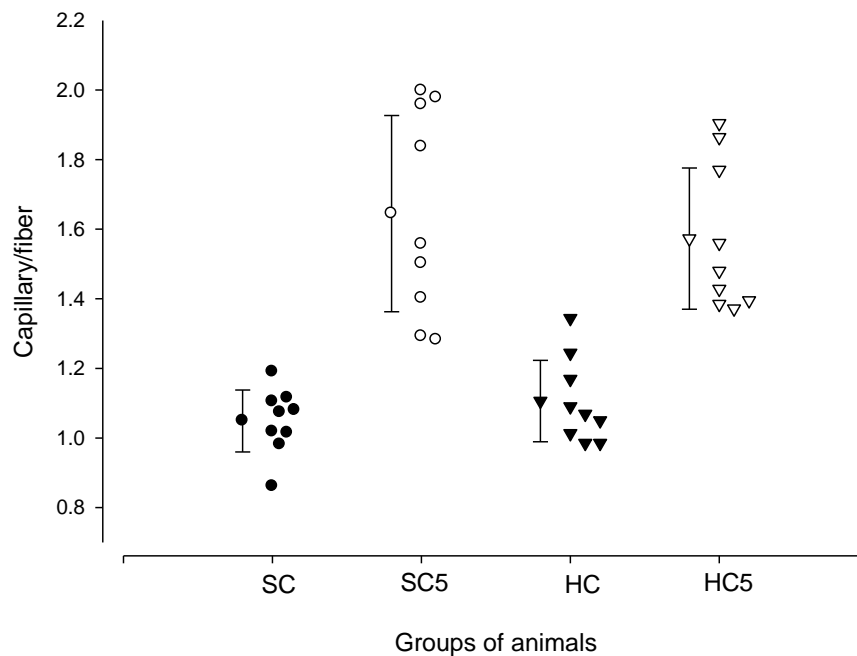
**Fig. 2**

Cross section of the midmuscle region of Gastrocnemius muscles of Wistar rats. Control group fed with standard chow (top left), control group fed with hypercaloric diet (top right), 2x/week trained group fed with standard chow (middle left), 2x/week trained group fed with hypercaloric diet (middle right), 5x/week trained group fed with standard chow (bottom left) and 5x/week trained group fed with hypercaloric diet (bottom right). The type I fibers are darkly stained. ATPase stain preincubated at pH 4.2 was used (original x100)



Fig. 2 shows the changes in the type of the Gast muscles fibres. There was an increase of darkly acid ATPase-stained fibres in trained rats compared to both control groups (SCØ top right and HCØ top left). The groups trained 2x/week (SC2 middle right and HC2 middle left) showed more similar distribution of the fibres. The higher proportion of SO fibres were observed in both 5x/week groups (SC5 bottom right and HC5 bottom left).

As illustrated in Fig. 3, the amount of capillaries around the different fibre types of both 5x/week trained groups showed a significant ( $P < 0.001$ ) improvement (angiogenesis) of ~50% when compared with the matched sedentary control rats. This increase affected SO, FG and FOG fibres and could be observed by the raising of capillary density.



**Fig. 3**

Mean  $\pm$  standard deviation and individual values of capillary density of sedentary (SCØ) and trained 5 times/week (SC5) rats fed with standard chow diet and sedentary (HCØ) and trained 5 times/week (HC5) rats fed with Hypercaloric diet



## Discussion

The important feature of this study is the muscle and heart morphological adaptation as well as the proportion of capillary around the fibers with the prolonged PA in normally fed and overfed rats.

The difference in body mass observed only in the SC5, comparing with the other groups at the end of the experiment, suggest that this change is associated with weekly frequency of training and the kind of nutrition, which probably, promoted a higher energy expenditure and, consequently, lower accumulation of body fat in this group.

Several studies have been showed that changes in the fibre type from Glycolytic to oxidative, can occur with endurance training [6,5,26].

The SC5 and the HC5 presented a rising of SO fibres. These adaptations in the muscle fibres related to exercise frequency are supported by the literature [3,5,6,15,27,33].

The enhancing in the fat oxidation is related with the raising of the oxidative key enzyme activities [38], and the activity of these aerobic enzymes is higher in SO fibres[13].

The small increment in the cross sectional fibre area observed in our trained groups, are compatible with the literature [24,31,34] and it is supposed to be promoted by the higher amount of SO fibres [11] resulting of the rising in the capillary supply [19,36].

Other studies did not show increment in cross sectional area of the fibre with endurance training, but are consistent about the capillary adaptation per unit volume of muscle with exercise. This possible shift of muscle fibre distribution and the raising in the capillary, are supposed to be related with the change in the metabolic supply which are influenced by a better sensibility to insulin [19,36], and a raising in the interleukin-6 activity[22].

Otherwise another important feature found in the literature is that the main histochemical markers to improve the aerobic capacity and fat oxidation are the association of skeletal muscle capillary, muscle fibre distribution, and fibre area and the activity of some key oxidative enzymes[8,9,27,28]. These features confirm our results.

The improvement of capillary density observed in both 5x/week trained groups compared with the sedentary-matched groups, suggest the occurrence of angiogenesis independently of the amount of the ingested calories and the weight of the animals. Similar results were observed in other studies, which indicate that exercise training stimulates angiogenesis[16].



Other studies showed an improvement of capillaries around muscle fibres [7,9,11,16,20] and they speculate that is occur as result of the increasing collateral-dependent blood flow and greater muscle capillarity, which is also associate the improvement in the endurance capacity [17].

The mechanism which regulate the processes to improve the capillary in skeletal muscle are unclear, but data indicate reduced oxygen tension and related metabolic alterations in the skeletal muscle according to different exercise, are the possible stimuli to the increase the endothelial cell-stimulating angiogenic growth factor [7].

Another important feature of this study is that parallels to the histochemical fibre adaptation, was observed lateral split and denervations in the skeletal fibre, mainly in the hypercaloric fed trained groups. These results denote that the overload of this exercise in overweight rats, even without contact with the floor, is intense enough to cause cell injuries.

The 5x/week trained rats with lower weight showed lower muscle injuries (SC5) and those with higher body weight presented higher muscle injuries. These data suggest that the increasing of injuries is associated not only with the frequency of training but also with its intensity, which is higher in the rats with higher body weight. These results had been also achieved in the literature [21], which observed experimentally the occurrence of associated muscular injury with physical activity accompanied of a subsequent cell regeneration [32].

This finding has great implications in relation to initiating exercise programs in sedentary subjects. The functional muscle adaptations for performing prolonged exercise do not occur in conjunction with the commencement of the exercise program, but somewhat delayed [6]. This must be taken into consideration when initiating any exercise program, particularly, in those with an excess of body fat.

The heart adaptation was also object of the present investigation and it was verify that the BM and the frequency of training influence the cardiac adaptation. This adaptation and diastolic function are usually assessed by two or three-dimensional echocardiogram, which do not permit the direct measure of heart structure.

It was decided by the direct weight of heart and of its segments, by the possibility of comparison of the structures according to the exercise frequency and the food pattern. Besides, in the review the literature we have not found studies with this interaction between nutrition and exercise like the present experiment. This measure permitted an analysis of the absolute and relative weight, which allowed a comparison of the heart adaptation in the different experimental conditions proportionally by the BM.



The main adaptation was observed in the heart of both 5x/week trained groups, which showed higher proportional weight of LV and RV compared with HC0 and HC2 and with all other groups, respectively. These results demonstrate that the adaptation occurred in the heart as function of regular exercise, are dependent of the frequency of training and independent of body weight. This in spite of dilatation of LV to be typical in obese person [12].

Results about LV mass and relative wall thickness adaptation in responses from a sub maximal exercise, can be found in the literature [25].

The shift of fibre type, the increase of vascularization and the morphological heart adaptation observed in the five times per week trained rats, also occurred, in a lesser extent, in those which had the same frequency of training but with higher body weight. On the other hand the twice a week exercised groups (both fed with normal and hypercaloric diet) showed neither skeletal muscle nor heart adaptations evidenced by the 5x/week groups.

Finally, this study emphasizes the importance of a better understanding of the mechanisms mediating exercise responsiveness in skeletal muscle, and brings us some evidences which need to be more elucidated about excessive fatigue in those with excess of body weight during exercise, and the adequate intensity and frequency of training for a better cellular adaptation.

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