Effect of swimming associated with diet on the anterior tibial muscle of rats: morphological and hystochemical study

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ABSTRACT

The objective of this study was to investigate the effect of the association of different swimming program frequencies and diets on the characteristics of the anterior tibial muscle of 24 Wistar male rats. These rats were randomly assigned into three groups: untrained (control), trained group (two days/week) and trained group (five days/week). Each group was divided into two groups, which received one of the two normal or high calorie diets. After the training period, muscle samples were collected and frozen at –70°C. Serial cryostat sections (8 μm) were sectioned and submitted to HE stain and to NADH-TR, m-ATPase (pH 4.4) and Sudan Black histochemical methods. The morphology was analyzed and the degree of fiber enlargement (on hypertrophy) evaluated using the lesser fiber diameter method. The data were submitted to analysis of variance (one way ANOVA). Muscle fibers were classified as SO, FOG and FG, presenting a mosaic distribution pattern, which were unchanged in all groups. Muscle fibers revealed a very low hypertrophy in all groups. Initial and final body weight were significantly different in trained groups. In the trained groups, especially in five days/week, muscle fibers revealed higher diameter, splitting and some internal myonucleus. Some atrophic fibers were observed and this observation was suggestive of denervation. The oxidative metabolism was higher in SO and FOG fibers. No significant alterations were observed in muscle contraction ability and the lipids content was intense in SO fibers, moderate in FOG fibers and low in FG fibers. The present study, with this protocol of two days/week and five days/week training caused different types of morphological lesions in fibers. The high calorie diet did not cause statistically significant results in comparison with the normal diet.

INTRODUCTION

In the last years, several researches have emphasized how physical activity has been effective in the treatment and prevention of chronic diseases such as diabetes, cardiovascular diseases and particularly obesity, mainly caused by high caloric diets and sedentary life-style.

In the last decades, a high number of obese individuals has been observed especially in rich countries, such as Canada, New Zealand, United Kingdom and United States. In the Unites States, a variation on the number of obese individuals from 12% to 17.9% in the period from 1991 to 1998 was observed. Data published in 2000 indicated that obese and overweighed adults counted for 54.9% of the American population[1]. Marx[2] estimates that these data doubled in the last 20 years, thus revealing that 30% of the American adults are obese and 35% are overweighed. Children and adolescents are not exempted: 15% are above normal weight.

In Brazil, obesity reaches 41.5% of the population, being strongly associated with dyslipidemia[3].

Considered as multifactor disease since 1985 by the National Institute of Health for increasing the incidence of other chronic-degenerative diseases[3], since 1997, the World Health Organization (WHO) recognized obesity as an universal disease[4].

Obese individuals present higher risk of cardiovascular diseases, hypertension, type 2 diabetes mellitus, dyslipidemia, osteoarthritis, sleep apnea, infertility and some types of neoplasy[1,4-6].

In order to revert this situation, the adoption of methods aimed at the increase of the slim mass with the consequent increase on the metabolic conditioning and the lipids mobilization, especially in fat tissue, liver, heart, skeletal muscles and in the plasma lipoproteins[7], becomes necessary. One of the methods that may lead to this objective is the physical activity[8].

The effect of different training situations – static (vertical staircase) and dynamic (treadmill) – on the long extensor muscles of fingers and soleus muscles in young rats was studied by Melichna et al.[9]. The authors observed that the dynamic training resulted in an elevation on the muscles' oxidative capacity, while in the sedentary group, a preferential increase on the glycolytic feature was seen.

Similar effects were observed by Lopez-Rivero et al.[10] when running exercises with intense training were used in horses.

Fibers modulation was observed by Misumi et al.[11] in horses submitted to swimming exercise associated with running. The results revealed increase of FOG and decrease of FG fibers.

The interaction of hypercaloric diets with physical activity may also contribute for the prevention of dyslipidemia and obesity, as reported by Duarte[12], when submitted rats fed with hypercaloric diet to swimming exercises.

Similar results could be observed by Ross et al.[4], when submitted rats to exercises with or without caloric restriction. Even if significant weight reductions in the group with no caloric restriction was not observed, the authors verified reductions on the abdominal fat tissue, factor associated with cardiovascular diseases and type 2 diabetes mellitus.

In this context, the objective of the present research was to study, in rats fed with normal diet and rats fed with hypercaloric diet, the effect of different swimming training frequencies on the morphology, the hypertrophy degree, the oxidative-glycolytic metabolism, the lipids content and on the slow and fast contractile ability of fibers of the anterior tibial muscle.

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MATERIAL AND METHODS

Twenty-four Wistar male adult rats (*Rattus norvegicus, albinus*) with average body weight of 350 g were used in the present research. The animals were kept in collective cages with four rats in each cage under constant temperature (26°C) and light and dark photoperiods of 12 hours.

Up to the beginning of the experiment, the rats were fed with standard *Purina* ration for rodents. Three weeks before the experiment, 50% of these animals were submitted to cafeteria diet (hypercaloric)[13,14], and the other animals were fed with standard *Purina* ration. Both groups had ration and water *ad libitum*. The research was approved by the Ethics Committee of the Oeste Paulista University (Unoeste), Presidente Prudente, SP.

Rats that received standard diet composed the normal group. The normal and hypercaloric groups were subdivided into untrained (control), trained group (two days/week) and trained group (five days/week) with four rats in each group.

The normal untrained (NU) and hypercaloric (NH) groups, composed of four animals from the normal group and four animals from the hypercaloric group, received ration and water *ad libitum*.

The normal trained and hypercaloric groups were composed of eight animals from the normal group and eight animals from the hypercaloric group, divided into: normal trained two days/week (T2XN), hypercaloric trained two days/week (T2XH), normal trained five days/week (T5XN) and hypercaloric trained five days/week (T5XH).

Half of the animals were fed with standard *Purina* ration containing 3.78 kcal/g. The other half received hypercaloric diet with the following composition: 15 g of standard ration (3.78 kcal/g), totalizing 56.70 kcal; 10 g of toasted peanuts (5.95 kcal/g), totalizing 59.50 kcal; 10 g of milky chocolate (6.11 kcal/g), totalizing 61.10 kcal and 0.5 g of maize biscuit (3.55 kcal/g), totalizing 17.75 kcal, being offered as pellets[12].

Before training, the rats were submitted to an adaptation program during five days, starting with 15 minutes and water level of 20 cm, being progressively increased to 30 minutes and water level of 50 cm.

After this stage, the animals were submitted to training with duration of 10 weeks in glass tanks of 100 x 50 x 60 cm and water temperature of 30° ± 1°C. Each group was submitted to 60-min sessions. The two days/week and five days/week training sessions in consecutive days were based on Duarte protocol[12].

Twenty-four hours after the last training session, the animals were sacrificed by means of intraperitoneal injection with thiopental (20 mg/100g of body weight). Later, the animals were weighted (g), followed by the removal of the anterior tibial muscle.

Fragments of the median portion of this muscle were immersed in n-hexane –70°C[9] during two minutes. Histological cuts (8 µm) were submitted to HE stain and to NADH-TR, m-ATPase (pH 9.4) after pre-incubation in acid medium (pH 4.4) and Sudan Black[15] histochemical methods.

The hypertrophy degree of the muscular fibers was evaluated based on the HE-stained blades through the lesser fiber diameter method[15], using a computerized image analysis system. For each animal, 100 fibers were measured (µm). Besides the use of the optical microscope *Nikon Alphaphot – 2 YS2*, the images were collected with the aid of the camera *CV-730 PDC 12 VDC Fuse*: 800 mA and visualized through the *Personal Computer 300 GL* system, with 14 inches *Philips* monitor and *ImageLab* 2000 program.

Following, the morphological description and analysis of fibers and the histochemical analysis were performed. Based on the NADH-TR reaction, the fibers were classified and designated as SO (slow oxidative), FOG (fast oxidative glycolytic) and FG (fast glycolytic). The fast and slow contractile ability was obtained through the m-ATPase reaction after pre-incubation in acid medium and the lipids content was evaluated through the Sudan Black reaction. The analysis of the fibers frequency in function of their diameters was also performed (intervals of 20 µm).

The data regarding the initial and final body weight of the animals studied as well as the fibers diameter were submitted to one-way ANOVA statistical test. The significance level adopted was of 5%.

RESULTS

As revealed through the HE stained preparations, in the untrained group fed with normal diet and in the untrained group fed with hypercaloric diet, the morphology of the anterior tibial muscle revealed fibers with different diameters, polygonal shapes and some presented hypertrophy (figures 1A and 2A). In group T2XN, the fibers revealed to be smaller, with rounded shape and higher diameter variations (figure 1C).
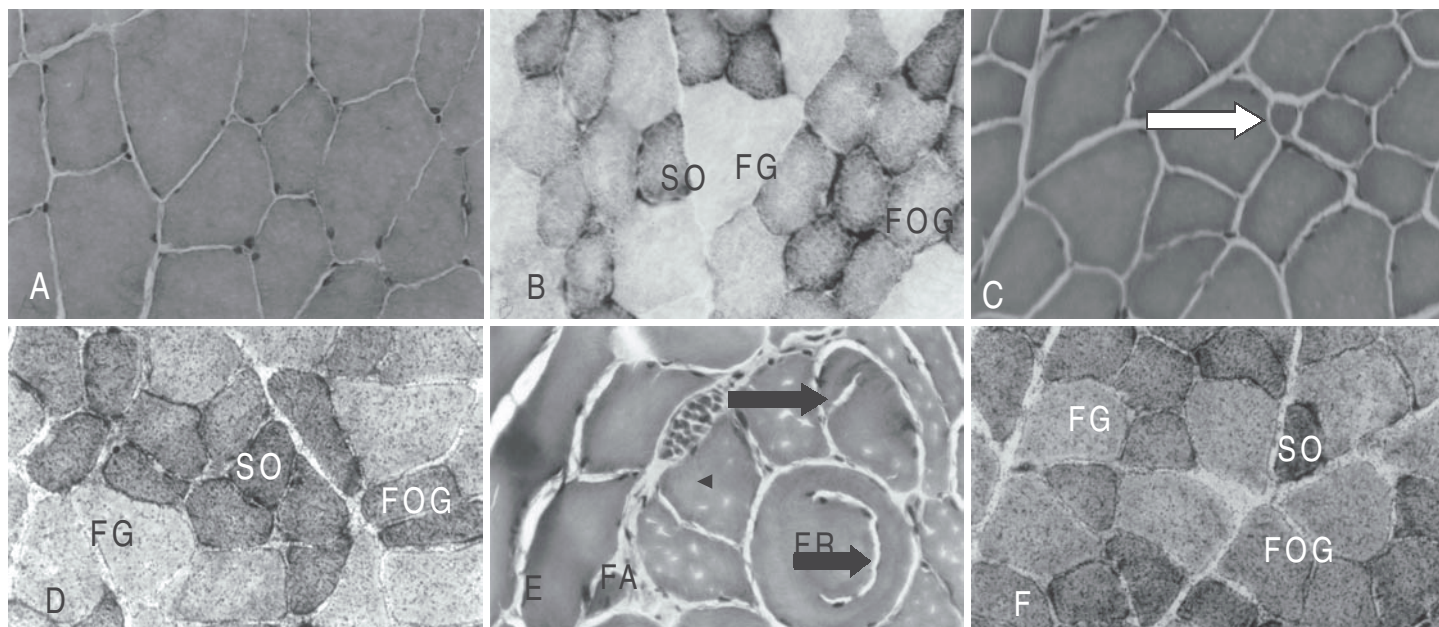

Fig. 1 – *Transversal sections of the anterior tibial muscle. **A)** normal untrained group (NU) HE. 128x; **B)** normal untrained group (NU), types of fibers (SO, FOG, FG) NADH-TR. 128x; **C)** normal group trained 2 days/week (T2XN) small fiber (arrow) HE. 128x; **D)** normal group trained 2 days/week (T2XN), types of fibers (SO, FOG, FG) Sudan Black. 128x; **E)** normal group trained 5 days/week (T5XN), rounded fiber (RF), atrophic fiber (AF), splitting (arrows), HE. 128x; **F)** normal group trained 5 days/week (T5XN), Sudan Black, 128x.*
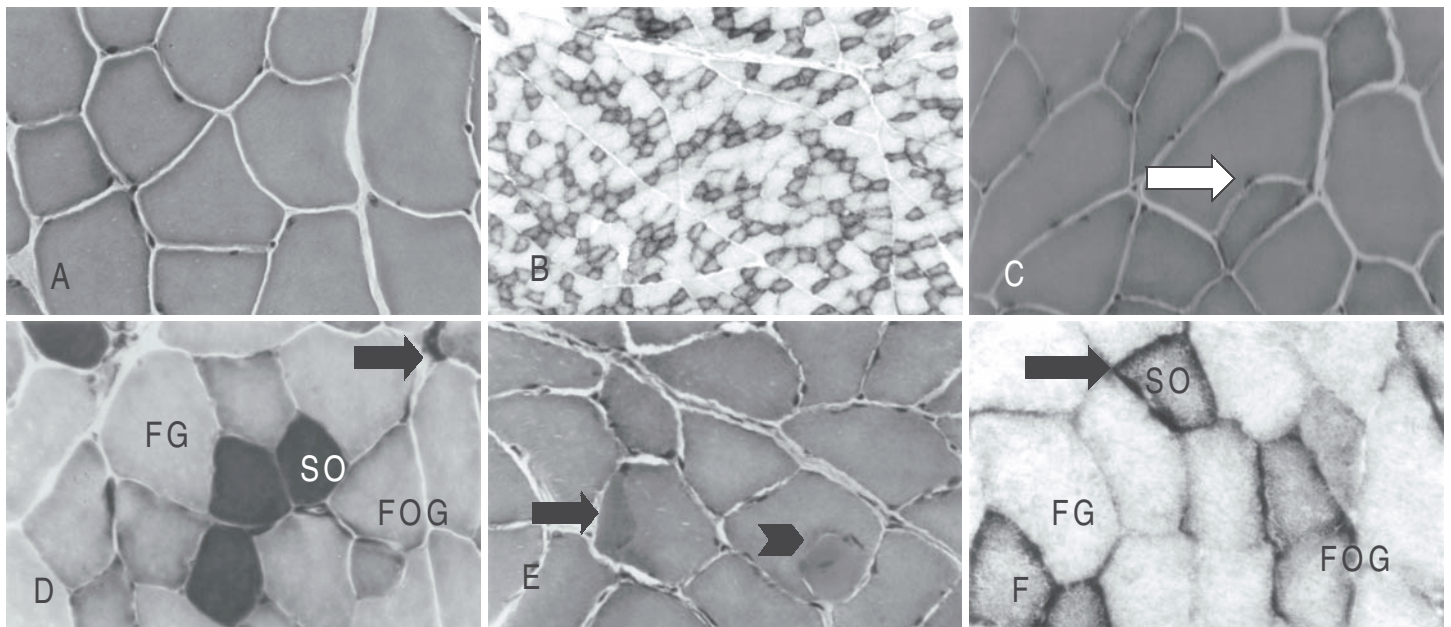
*Fig. 2 – Transversal sections of the anterior tibial muscle. **A)** hypercaloric untrained group (HU) HE. 128x; **B)** hypercaloric untrained group (HU) NADH-TR. 34x; **C)** hypercaloric group trained 2 days/week (T2XH), splitting (arrow) HE. 128x; **D)** hypercaloric group trained 2 days/week (T2XH), types of fibers (SO, FOG, FG) m-ATPase. pH4,4. 128x; **E)** hypercaloric group trained 5 days/week (T5XH), splitting (arrow head), hypercontraction area (arrow). HE. 128x; **F)** hypercaloric group trained 5 days/week formazan aggregate (arrow). NADH-TR, 128x.*

In group T2XH, some angular and atrophic and some hypertrophic fibers were observed and some of them presented segmented appearance (splitting), fact also observed in groups T5XN and T5XH (figures 1E; 2C and 2E). In group T5XN, besides the splitting with internal myonuclei, the presence of atrophic fibers occurred (figure 1E).

In group T5XH, besides the splitting (arrow head), cytoplasm areas with hypercontraction were also observed (arrow) (figure 2E).

For both trained and untrained groups, the presence of three types of fibers was observed in the NADH-TR reaction, characterizing the mosaic distribution pattern (figures 1B and 2B). In trained groups, the SO and FOG fibers showed more intense reactivity pattern with shapeless formazan aggregates in the subsarcolemmal region (figure 1F).

The myofibrillar ATPase reaction showed to be intense in SO fibers, moderate in FOG fibers and weak in FG fibers in all groups. In trained groups, the FOG fibers presented diameters a little smaller and more rounded, and the FG fibers, hypertrophy. In group T2XH, some SO fibers presented atrophic appearance (arrow) with accentuated polymorphism (figure 2D).

In normal and hypercaloric untrained groups, the Sudan Black method revealed high lipids concentration in SO fibers, moderated in FOG fibers and very weak in FG fibers. On the other hand, in trained groups, the lipids content in SO and FOG fibers was more intense. In FG fibers, the lipids concentration showed to be higher in normal groups and slightly lower in hypercaloric groups (figures 1D, 1F, and 2F).

The statistical treatment of the variable initial and final body weight of the rats studied is shown in table 1.

TABLE 1
Comparison of initial body weight (IW) and final body weight (FW) of groups NU, NH, T2XN, T2XH, T5XN and T5XH. The significance of differences is indicated by the value of *P*

Groups	IW	FW	P
NU	437.5 ± 59.5	497.7 ± 56.2	0.19152
NH	437.5 ± 59.5	523.4 ± 60.5	0.0602
T2XN	431.2 ± 32.46	518.1 ± 14.8	0.00245
T2XH	443.7 ± 37.50	551 ± 50.7	0.0145
T5XN	368.7 ± 12.5	469.6 ± 14.1	0.000038
T5XH	418.7 ± 37.5	539.1 ± 40.2	0.00467

The statistical treatment of the muscular fibers average diameters of groups SN, SH, T2XN, T2XH, T5XN, T5XH is shown in table 2.

TABLE 2
Statistical treatment of the average diameter (µm) of fibers of rats from groups NU, NH, T2XN, T2XH, T5XN, T5XH

Groups	NU	NH	T2XN	T2XH	T5XN
NH	0.48_A				
T2XN	$2 \times 10^{-5}{}_B$	$5 \times 10^{-4}{}_B$			
T2XH	0.06_A	0.2_A	0.007_B		
T5XN	0.03_B	0.1_A	0.02_B	0.7_A	
T5XH	$7 \times 10^{-9}{}_B$	$1 \times 10^{-6}{}_B$	0.32_A	$5 \times 10^{-5}{}_B$	$4 \times 10^{-4}{}_B$

A: averages of column with letter A are not statistically significant.
B: averages of column with letter B are statistically significant.
(P < 0.05)

The number and the frequencies (%) of fibers in function of diameters in intervals of 20 µm of the groups of rats studied are shown in table 3.

TABLE 3
Number and percentage of fibers in function of the diameter of the anterior tibial muscle (intervals of 20 µm) of animals from groups NU, NH, TX2N, TX2H, T5XN and T5XH

Diameter/fibers Groups/rats	< 20	21-40	41-60	> 61	N
NU		174 (40.7%)	214 (50.1%)	39 (9.1%)	400
NH	3 (0.7%)	146 (35.7%)	204 (49.8%)	57 (13.9%)	400
T2XN		151 (37.5%)	150 (37.5%)	99 (24.7%)	400
T2XH	1 (0.2%)	139 (34.7%)	217 (54.2%)	43 (10.7%)	400
T5XN		118 (29.5%)	221 (55.2%)	60 (15%)	400
T5XH	1 (0.2%)	116 (29%)	189 (47.2%)	95 (23.7%)	400

DISCUSSION

In the six groups studied here, the anterior tibial muscle presented fibers with different diameters, ranging from 45.1 µm in group SN to 53.3 µm. Between groups SN and T2XN, SN and T5XN,

SN and T5XH, SH and T2XN, SH and T5XH, T2XN and T2XH, T2XH and T5XN, T5XN and T5XH, significant hypertrophy of fibers was observed.

The short pre-training period seems to have contributed, at least in part, for the fibers hypertrophy. Furthermore, the training itself seems to have been intense and the duration of 10 weeks, not long enough. These facts may have contributed for the hypertrophy of some fibers.

Another aspect to be considered, according to Dall Pai et al.[16,17], is the post-birth growth pattern of muscular fibers of rats, being linear from 12 to 45 days. In the subsequent ages, the growth is differential between the types of fibers, being more intense and continuous in the white fibers.

Besides the hypertrophy, the trained rats, especially from groups T2XH, T5XN and T5XH, presented cytoplasm longitudinal segmentation (splitting), rounded hypertrophic fibers, and fibers with smaller diameters suggestive of denervation and under phagocytosis process. These alterations showed to be more intense in groups trained five days/week. The internal or lateral segmentation process of fibers composes a morphophysiological adaptation aiming at reducing the distance between the capillary bed and the center of the cytoplasm, thus favoring metabolic changes and the occurrence of lesions.

On the other hand, the presence of 0.2 to 0.7% of fibers with diameter below 20 μm in both trained and untrained groups seems to show that the protocol used presented no significant effect on the number of muscular fibers, once its occurrence may be a result of atrophic process and/or formation of new fibers.

The presence of different lesion degrees in the muscular fibers was also observed by Camargo et al.[18], who exercised rats during 60 days in treadmill, in daily sessions of 60 minutes, five days/week. Elapsed 30, 45 and 60 days, all active groups revealed atrophic fibers under phagocytosis process and other fibers with rounded shape and centralized nucleuses. The lesions showed to be more severe in animals belonging to active group of 60 days.

With regard to the oxidative metabolism, the exercised rats of this research showed reactivity slightly increased in SO and FOG fibers, especially in groups trained five days/week, demonstrating increase on the oxidative capacity. This aspect presented under the form of higher formazan aggregates in relation to the other animals studied.

Despite not being quantified, this result is in agreement with results obtained by Fitts et al.[19], when they submitted university students to swimming training during 20 days with one daily sessions of 90 minutes in the first 10 days and two daily sessions of 90 minutes in the last 10 days. According to the authors, the oxidative activity almost doubled in the first phase, with no significant alterations in the second phase.

Melichna et al.[9] also observed elevation on the oxidative capacity of fibers of the long extensor muscles of fingers and soleus in a dynamic training (treadmill) in young rats after four weeks in sessions of five days/week with duration from 20 minutes to two hours a session. Similar results were also obtained by Misumi et al.[11] training horses in running and swimming. The animals were divided into three groups: running, swimming plus progressive running, and running plus progressive swimming. Five months later, the authors observed an increase on the FOG fibers and a decrease on the FG fibers in groups that associated running and swimming training.

A significant increase on the percentage of oxidative fibers and a decrease on the glycolytic fibers was also observed by Lopez-Rivero et al.[10] in horses submitted to a four-week training with different velocities and session time variations.

In the same way, Saad et al.[20] observed significant increase on the aerobic capacity in the straight muscles of the abdomen and in the paraexternal intercostalis muscles of rats submitted to swimming exercise as result of the increase on the percentile of SO fibers and decrease on the FOG fibers in rats that swam 60 days, five days/week in sessions of 60 minutes. On the other hand, the FG fibers increased in rats that swam 15 days, five days/week in sessions of 60 minutes. A significant increase on the SO fibers in the paraexternal intercostalis muscles was observed in rats that swam 60 days, increase on the FOG fibers in rats that swam 45 and 60 days and decrease on the FG fibers in rats that swam during 30, 45 and 60 days.

Likewise, the results of the present research revealed intense lipids concentration in SO fibers, moderate in FOG fibers, and weak in FG fibers in group NU, being slightly higher in SO and FG fibers of group NH. With regard to the trained groups, in group T2XN, the lipids concentration was higher in SO and FOG fibers and lower in FG fibers when compared to group T2XH, where the lipids concentration was lower in SO and FOG fibers and higher in FG fibers. In group T5XN, the lipid content showed to be very intense in SO fibers and more intense in the other fibers when compared with previous groups. In group T5XH, a higher lipids accumulation was observed in SO and FOG fibers and lower in the FG fibers, when compared with T5XN.

One may conclude that diets presented significant effect on the body weight of the trained groups. However, no statistically significant difference was observed between the different diets (normal and hypercaloric). Furthermore, fibers distribution presented no alterations in both untrained and trained groups, keeping a mosaic distribution pattern. The reactivity pattern for slow and fast contraction was similar in the different groups of rats studied. The swimming exercise, according to the protocol used here, promoted cytoplasm longitudinal segmentation (splitting), and the formation of denervated fibers, and rounded and hypertrophic fibers, and this hypertrophy was statistically significant in some groups.

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