Myocardial Stereological Adaptations in Wistar Rats Fed with Different High-Fat Diets during 18 Months

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Summary This study has the purpose of investigating the influence of different high-fat experimental diets on myocardial structure in rats. Twenty-seven male rats were fed from 21 d old (postnatal age) until 18 mo old with one of the following supplemented diets: soybean oil (S) (n=6), canola oil (CA) (n=8), or lard and egg yolk (LE) (n=6) or canola oil+lard and egg yolk (CA+LE) (n=7). The blood pressure (BP) was measured, and after the sacrifice the cardiac biometry and the myocardial stereology were determined: cross-sectional area of cardiomyocyte (A), volume density (Vv), surface density (Sv), and length density (Lv) in relation to the cardiomyocytes (cm), connective tissue (ct), and blood vessels (v). The CA group rats had lower BP, A[cm], and Vv[ct]; they had greater Vv[cm], Sv[cm], Vv[v], Lv[v], and Sv[v] than the other groups. The S rats had intermediary values for the myocardium and blood vessel parameters between the CA and LE group rats. These results support the notion that the long-term use of canola oil in the diet is better to preserve the myocardium structure, including microvascularization, than soybean oil or lard and egg yolk.

Key Words stereology, myocardium, canola oil, diet, rat

Canola oil contains about 10% α-linolenic acid (ALA; 18:3n-3) and is a rich source of monounsaturated fatty acid (MUFA) (1). Vertebrates are able to de novo synthesize only saturated and MUFA chains and must obtain their PUFA from their diet or intestinal biota. ALA is the precursor of the marine long chain n-3 fatty acids eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). ALA competes with its n-6 counterpart linoleic acid (LA; 18:2n-6) for desaturation and chain elongation. The major component of soybean oil is linoleic acid, and LA is in this pathway converted to arachidonic acid (AA; 20:4n-6) (2). Dietary fats influence the lipid composition of body membranes, depending on their unsaturated and saturated fatty acid contents (3). The composition of dietary fat affects cell membrane composition, thus cell membrane function and metabolic rates (4, 5). The n-3 fatty acids are rapidly incorporated into cell membranes after their ingestion from the diet and profoundly influence biological responses. These lipids influence membrane stability, membrane fluidity, cell mobility and formation of eicosanoids (6, 7). The fatty acid composition of cardiac membrane phospholipids is profoundly altered by the changes in dietary lipid intake (8). Heart muscle lipids are in a dynamic state, continuously modified by external and internal conditions such as dietary fat or hormones (9).

With increasing body size in mammals, there was a relative decrease in polyunsaturation and an increase in monounsaturation of phospholipids (the exception is the brain, which retains high polyunsaturation/low monounsaturation irrespective of body size). This trend was especially manifest in the n-3 PUFA (10).

The general similarity of the cardiovascular system among mammals, including man, elects the rat as an experimental model widely used (11). Kajstura and co-workers (12) observed that programmed myocyte cell death is more frequent in the left ventricular free wall of old rats and that this form of myocyte cell death increases at subsequent age intervals in rats. The cardiac hypertrophy and consequent ischemia caused by nitric oxide synthesis inhibition do not significantly increase the cardiomyocyte apoptosis in the left ventricular wall (13).

In a previous study we analyzed some quantitative aspects of the influence of canola oil on the myocardial structure of younger rats (14-18).

The scope of the stereology is to determine three-dimensional quantitative parameters of morphological structures from bidimensional counts. For that, stereology uses the geometry and the probabilistic statistics. The volume density (Vv) measures the relative occupation of the test area for the area of a certain structure. The surface density (Sv) evaluates the relationship between the area and the volume of morphologic structures allowing discrimination of membrane-bounded
structures (cellular and intracellular structures). Length density \((L_v)\) should be used to evaluate tubular structures or structures similar to filaments when \(S_v\) is not indicated. The study of blood vessels is a good indication to calculate \(L_v\) (19).

The aim of this study is to investigate the possible influence of the long-term administration of the high-fat different experimental diets (soybean oil, canola oil, lard and egg yolk) on myocardial structure in 18 mo old rats, using stereological methods.

**MATERIALS AND METHODS**

**Animals and diets.** We have studied male rats (Wistar strain) obtained from colonies maintained in the State University of Rio de Janeiro from birth to 18 mo old. The investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Rats were housed in a polypropylene cage in groups of five animals. They were kept in a temperature-controlled \((21 \pm 1^\circ C)\) and humidity-controlled \((60 \pm 10\%)\) room and submitted to a 12-h light and dark cycle (artificial lights, 7 a.m.-7 p.m.) and to an air exhaustion cycle (15 min/h). All groups received experimental diets in which the basal diet included cornstarch, casein, wheat flour, egg white, and mineral and vitamin mixtures.

The diet groups are soybean oil \((S)\) \((n=6)\), canola oil \((CA)\) \((n=8)\), lard and egg yolk \((LE)\) \((n=6)\), and CA+LE \((n=7)\). The diets contained, as a percentage of energy, 47% carbohydrate, 29% fat, and 24% protein. The content of cholesterol in the LE and CA+LE diets were 3.0 g/kg and 1.5 g/kg of diet, respectively. The calculated energy values of all diets were not significantly different. Diets were prepared weekly in our laboratory and stored at +4°C. The diet composition (20, 21) is summarized in Tables 1 and 2.

**Methods.** From 21 d old (postnatal age) until 18 mo old the animal groups received different diets and had free access to water. All rats were fed once a day during 18 mo, and diet intake was daily monitored.

The blood pressure \((BP)\) and the body mass \((BM)\) were verified monthly with animals superficially anesthetized by using diethyl ether. The BP was verified by using the noninvasive method of the tail-cuff plethysmography (RTBP1007, Kent Scientific Co., Litchfield, CT, USA). At the end of the experiment, the animals were anesthetized by using diethyl ether inhalation, and the amount of 3 mL of 10% KCl was injected into the left ventricle until diastolic cardiac arrest.

The hearts were taken out by excising the vessels at the base, immediately above the aortic and pulmonary valves. The heart volume was determined according to the submersion method of Scherle (22), in which the water displacement because of organ volume is recorded by weighing. In the day of the sacrifice, the

**Table 1. Composition of the experimental diets (g/kg).**

| Food*** | Group | S     | CA    | LE    | CA+LE |
|---------|-------|-------|-------|-------|-------|
| Casein  | S     | 305.0 | 305.0 | 305.0 | 305.0 |
| Casein  | CA    | 120.0 | 120.0 | 0     | 60.0  |
| Corn starch | LE  | 150.0 | 150.0 | 150.0 | 150.0 |
| Wheat flour | S   | 245.0 | 245.0 | 245.0 | 245.0 |
| Soybean oil | CA | 180.0 | 0     | 0     | 0     |
| Canola oil | LE  | 0     | 180.0 | 0     | 90.0  |
| Egg yolk | S     | 0     | 0     | 180.0 | 90.0  |
| Lard    | CA    | 0     | 0     | 120.0 | 60.0  |
| Total   | CA+LE | 1,000.0 | 1,000.0 | 1,000.0 | 1,000.0 |
| Vitamin mixture* | S   | 50.0  | 50.0  | 50.0  | 50.0  |
| Mineral mixture** | CA | 30.0  | 30.0  | 30.0  | 30.0  |
| Cholesterol | LE  | 0     | 0     | 3.0   | 1.5   |

\(S\) is the soybean oil group, \(CA\) is the canola oil group, \(LE\) is the lard and egg yolk group, and \(CA+LE\) is the canola oil plus lard and egg yolk group.

* Vitamin mixture: 50.0 mg. ** Mineral mixture: 30.0 mg. * Vitamins (per kg of diet): thiamin 6.5 mg, riboflavin 5.3 mg, pyridoxine 6.3 mg, niacin 7.5 mg, folic acid 1.3 mg, biotin 0.5 mg, cyanocobalamin 0.19 mg, retinyl palmitate 1.562 UL, cholecalciferol 1.250 UL, tocopherol acetate 1.83 mg, ascorbic acid 185 mg.

**Table 2. Fatty acids composition (g/100 of the experimental diets.**

| Fatty acid (FA) | Group |
|-----------------|-------|
|                 | S     | CA    | LE    | CA+LE |
| 8:0             | ---   | 0.09  | 0.06  |
| 10:0            | ---   | 1.00  | 0.67  |
| 12:0            | ---   | 0.85  | 0.57  |
| 14:0            | ---   | 0.86  | 0.57  |
| 16:0            | 10.35 | 5.40  | 23.50 | 17.4 |
| 16:1            | ---   | 0.30  | 0.67  | 0.55 |
| 18:0            | 3.55  | 1.67  | 7.62  | 5.60 |
| 18:1            | 25.60 | 56.30 | 49.35 | 51.70|
| 18:2ω6          | 55.06 | 25.00 | 13.08 | 17.00|
| 18:3ω3          | 5.44  | 8.40  | 1.53  | 3.83 |
| 20:0            | ---   | 0.60  | 0.20  |
| 20:1            | ---   | 1.30  | 0.43  |
| 22:0            | ---   | 0.40  | 0.13  |
| 22:1            | ---   | 0.50  | 0.17  |
| 24:0            | ---   | 0.20  | 1.50  | 1.90 |
| FA saturated    | 13.90 | 8.20  | 35.40 | 26.30|
| FA monounsaturated | 25.60 | 58.40 | 50.00 | 52.87|
| FA polyunsaturated | 60.50 | 33.40 | 14.60 | 20.83|
| P/S/M           | 4:1:2 | 4:1:2 | 1:2:3 | 1:1:2|
| n-6FA:n-3FA     | 10:1  | 3:1   | 8:5:1 | 4:5:1|
| P+M/S           | 6.2   | 11.2  | 1.8   | 2.8  |

See abbreviations in Table 1 (20).
heart mass/body mass ratio (HBR) was determined as
HBR = (HM/BM) \times 100 (%).

Fragments of the left ventricular myocardium were
obtained by using the orientator method (23), then
fixed for 48 h in buffered 4% formaldehyde pH 7.2 and
embedded in Paraplast Plus\textsuperscript{®}, sectioned at 3 μm thick.
The sections were stained with trichrome methods
(Masson and Sirius red) that stain connective tissue and
nuclei. The analysis used a video-microscopic system
and the M\textsubscript{42} test system (composed of 21 straight seg-
ments, d, 42 test points, and test areas, A\textsubscript{T}, equal to
36.36 d\textsuperscript{2}) (Fig. 1) (24).

We analyzed the myocardium by considering the
cardiomyocyte and the cardiac interstitium (this was sub-
divided into blood vessels and connective tissue with
nerves). Several myocardial fragments per animal (up
to ten fragments) were embedded together in a block.
These fragments were about 2 mm long and were faced
to be cut by lottery. From each block several not serial
slices were cut (up to 10) and five microscopic fields
were randomly analyzed by blindly moving the stage of
the microscope.

We analyzed the stereological parameters by consid-
ering only well-preserved structures and not crossing
the forbidden line (19, 24–27):

- Volume density (cardiomyocyte and cardiac intersti-
tium)
  \[ V_v = \frac{P_p}{P_T} \times 100 \% \]
  (P\textsubscript{p} is where the points hit the myo-
cardium; P\textsubscript{T} indicates the total test points)

- Length density (intramyocardial vessels)
  \[ L_v = \frac{2Q_A}{d} \times (\text{mm}/\text{mm}^3) \]
  (Q\textsubscript{A} is the density per area)

- Surface density (cardiomyocyte and intramyocardial
  vessels)
  \[ S_v = \frac{2I}{L_T} \times (\text{mm}^2/\text{mm}^3) \]
  (I is the intersection number
  of the myocardium components with the test lattice,
  L\textsubscript{T} is the test line length)

- Cross-sectional area (cardiomyocyte)
  \[ A = \frac{V_v}{L_v} \text{ (μm}^2) \]

Data analysis. The Mantel-Haenszel test (T) was
used to compare the survival curves of the animals in
the different groups (28). If the null hypothesis is true,
T should be distributed approximately as \( \chi^2 \)
random variable (chi-square with one degree of freedom).
The differences of the biometrical parameters among the
groups were tested by the analysis of variance and the
multiple comparison Newman-Keuls test. The differ-
ences of the stereology were tested by use of the non-
parametric Kruskal-Wallis analysis of variance and the
Mann-Whitney test. In all instances the significant level
of 0.05 was considered for significant statistics (29).

RESULTS

The study had begun with 60 rats, but this sample
was reduced because of natural death after 18 mo of ex-
perimentation (Fig. 2). After 18 mo of experimentation,
the groups had different samples of survival rats: 6 an-
imals in the S group, 8 in the CA group, 6 in the LE
group, and 7 in the CA+LE group. However, these dif-
fferences in survival were not significant (T=0.5,
p=0.48). Table 3 summarizes the results.

Body mass and HBR
The BM and HBR had no significant differences
among groups in the 18 mo old rats fed the different
diets.

Blood pressure
After 18 mo, the highest BP was found in the LE
group, and the lowest BP was found in the CA group.
Among the groups, significant differences were ob-
served between S vs. LE (+45%), S vs. CA+LE (+15%),
CA vs. LE (+47%), CA vs. CA+LE (+17%), and LE vs.
CA+LE (-20%).

The cardiomyocyte stereology
The volume density of the cardiomyocytes (Vv[cm])
was greater in the CA group and smaller in the S group.
However, the Vv[cm] was not different between S vs. LE,
CA vs. LE, CA vs. CA+LE, and LE vs. CA+LE. The
Vv[cm] was different between S vs. CA (+10%) and S
vs. CA+LE (+8%). The surface density of the cardio-
mioocytes (Sv[cm]) was greater in the CA group and
smaller in the LE group. The Sv[cm] was different be-
tween S vs. CA (+35%), CA vs. LE (-38%), CA vs.
CA+LE (-27%), and LE vs. CA+LE (+17%). The cross-
sectional area of the cardiomyocytes (A[cm]) was not
different between S vs. LE. It was greater in the LE
group and smaller in the CA group. The A[cm] was dif-
f erent between S vs. CA (-35%), S vs. CA+LE (-20%),
CA vs. LE (+81%), CA vs. CA+LE (+36%), and LE vs.
CA+LE (-25%).

The cardiac interstitium stereology
a) The connective tissue. The volume density of the
connective tissue (Vv[ct]) was not different between S vs. LE. It was smaller in the CA group and greater in the LE group. The Vv[ct] was different between S vs. CA (-32%), S vs. CA+LE (-13%), CA vs. LE (+52%), CA vs. CA+LE (+29%), and LE vs. CA+LE (-16%).

b) The intramyocardial vessels. The volume density of the vessels (Vv[v]) was not different between S vs. CA, S vs. CA+LE, and LE vs. CA+LE. It was greater in the CA group and smaller in the LE group. The Vv[v] was different between S vs. LE (-29%), CA vs. LE (-55%), and CA vs. CA+LE (-45%). The Lv[v] was not different between S vs. LE and S vs. CA+LE. It was greater in the CA group and smaller in the LE group. The length density of the vessels (Lv[v]) was different between S vs. CA (+73%), CA vs. LE (-46%), and CA vs. CA+LE (-29%). The surface density of the vessels (Sv[v]) was not different between S vs. LE, S vs. CA+LE, and LE vs. CA+LE. It was greater in the CA group and smaller in the LE group. The Sv[v] was different between S vs. CA (+59%), CA vs. LE (-54%), and CA vs. CA+LE (-40%).

DISCUSSION
In the present study, the high-fat diets were made to be isocaloric and to provide 29% of their total energy as fat that was more than the total fat recommended to rats (21). The experimental diets contained comparable amounts of soybean oil, canola oil, lard, and egg yolk or canola+lard and egg yolk. These diets have different P+M/S ratios, and their Σn-6/n-3 PUFA distribution ranges from 10:1 (in the S group) to 3:1 (in the CA group), to 8.5:1 (in the LE group), and to 4.5:1 (in the

Fig. 2. Survival of rats fed different high-fat diets. Groups: CA, canola oil; CA+LE, canola+lard and egg yolk; LE, lard and egg yolk; S, soybean oil.

Table 3. Statistics of the quantitative results (BM and BP are mean ± standard deviation; the other variables are median ± confidence interval 95%).

| Groups      | BM (g)  | BP (mmHg) | Vv[cm] (%) | Sv[cm] (mm²/mm³) | A[cm] (µm²) | Vv[ct] (%) | Vv[v] (%) | Lv[v] (mm²/mm³) | Sv[v] (mm²/mm³) |
|-------------|---------|-----------|------------|-----------------|-----------|-----------|-----------|----------------|----------------|
| S           | 583±74  | 130±9     | 62±2       | 161±14          | 152±10    | 31±2      | 7±2       | 5,482±706      | 63±9           |
| CA          | 389±132 | 128±6     | 68±3       | 217±6           | 90±5      | 21±2      | 11±2      | 9,480±1,427    | 100±16         |
| LE          | 495±109 | 188±6     | 63±2       | 135±12          | 163±9     | 32±2      | 5±1       | 5,073±931      | 46±7           |
| CA+LE       | 560±73  | 150±5     | 67±2       | 158±12          | 122±11    | 27±2      | 6±2       | 6,747±1,393    | 60±21          |

Probability

| Probability | S vs. CA | S vs. LE | S vs. CA+LE | S vs. LE | CA vs. LE | CA vs. CA+LE | CA vs. LE | LE vs. CA+LE |
|-------------|----------|----------|-------------|----------|-----------|--------------|----------|--------------|
| NS          | NS       | 0.01     | <0.001      | <0.001   | NS        | <0.001       | NS       | NS           |
| NS          | NS       | 0.002    | NS          | NS       | NS        | NS           | NS       | NS           |
| NS          | NS       | 0.05     | 0.005       | NS       | NS        | NS           | NS       | NS           |
| NS          | NS       | <0.001   | <0.001      | <0.001   | NS        | <0.001       | NS       | NS           |
| NS          | NS       | <0.001   | <0.001      | <0.001   | 0.001     | 0.001        | 0.01     | 0.02         |
| NS          | NS       | 0.001    | 0.02        | 0.004    | 0.01      | 0.01         | NS       | NS           |

BM is body mass, BP is blood pressure, CA is canola oil group, [ct] is connective tissue, LE is lard and egg yolk group, Lv is length density, [cm] is cardiomyocyte, NS is not significant, S is soybean oil group, Sv is surface density, [v] is blood vessels, Vv is volume density.
CA + LE group). This quantitative study demonstrated that rats fed canola oil a diet supplemented with canola oil had the best values concerning myocardial structure, and the rats fed a lard and egg yolk diet had the worst values (i.e., an increased BP and a decreased number of cardiac myocytes with concomitant hypertrophy of the remaining myocytes). The myocardial stereological parameters to the microvessels significantly decreased in this group.

The addition of egg yolk to the LE and CA + LE diets did not affect the serum total cholesterol level among the groups probably because the rat is different from humans in its serum lipid and lipoprotein constitution, and producing sustained hyperlipidemia in this species is very difficult (30).

The composition of proteins of all diets was not significantly different. All diets contained the same amount of casein, and to compensate for the addition of egg yolk, the amount of egg white was reduced in LE and CA + LE groups. Thus the amount of protein was the same in all diets, differing only in the lipid composition.

The canola oil has appeared in retail food outlets in the past decade. Of particular interest is its high monounsaturated content, which is comparable to that of olive oil (31). Canola oil differs from olive oil primarily by the inclusion of the n-3 fatty acid α-linolenic.

The present experiment was performed with old rats, which have a significant loss of cardiomyocytes and reactive hypertrophy of the remaining cells that characterize the aging process (16, 18, 32). Although canola oil seemed to preserve the cardiac structure and blood pressure more than the other lipids in the diets did, the aging process can explain that the differences in the survival of the animals were not significant.

The hypotensive effect of ALA may be mediated by influencing the dietary LA/ALA ratio. The endogenous synthesis of vasoactive eicosanoids may alter when the balance between n-3 and n-6 fatty acid intake is changed (2). We have observed a moderate hypertension in the LE group animals. Epidemiological evidence has suggested an inverse relationship between the consumption of diets high in vegetable oil and BP, and it is known that dietary fat affects the BP (33). A diet with saturated fatty acids increases the BP (34), and the diet with PUFA and MUFA decreases the BP (35).

Several theories exist regarding the BP modulating indirect effect of dietary fats. Experimental studies indicate that high-fat diets clearly impair insulin action both in the liver and the skeletal muscle. Although n-3 fatty acids appear to offer protection, saturated fatty acids worsen insulin resistance caused by high-fat feeding (5, 36), and the insulin resistance is often associated with hypertension in human patients (37). The incorporation of unsaturated fat into lipid membranes increases membrane permeability, thereby stimulating the sodium and cation transport. Another explanation is that polyunsaturated fat converts to prostaglandins, which reduces BP via effects on arterial vasodilatation, electrolyte balance, renal renin release, and/or pressor hormones (38). The EPA incorporated into the plasma membrane competes with arachidonic acid (AA), thereby suppressing the production of thromboxane A₂, and EPA is converted to vasodilative thromboxane A₂ and prostaglandin I₃ (39). The long-term administration of canola oil reduced BP in the present study, probably avoiding the cardiac abnormalities associated with hypertension that include left ventricular hypertrophy and vascular changes. The latter may affect the cardiac microvasculature and predispose to myocardial ischemia (40).

The addition of canola oil into the diet greatly improved the myocardial vascularization after 18 mo, as demonstrated by the Vv[V], Sv[v], and Lv[v] that were highest in the CA group. However, when the canola oil (which contains α-linolenic acid) was mixed with lard and egg yolk (a cholesterol-rich diet), it did not give the effect observed in the CA group. The formation of a vessel from pre-existing vessels is of critical importance not only during normal growth, but also in pathological situations. Some diseases are enhanced by excessive vascular growth (e.g., tumors), whereas inadequate vascular growth contributes to morbidity and mortality (e.g., ischemic heart disease) (41).

The myocardial healing process includes changes in extracellular matrix composition associated with the phenotypic modulation of fibroblasts. Early and later lesion areas showed a population of spindle-shaped cells expressing α-smooth muscle actin content. These cells are apparently associated with type III collagen and fibronectin accumulation in the ischemic lesion areas, which contributes to maintenance of the mechanical performance of the heart throughout the healing process (42).

Regarding the modulating direct effect of dietary fats, the experimental studies showed that dietary lipid intake alters the fatty acid composition of cardiac membrane phospholipids (8), and the lipid composition of cell membranes plays an important role in defining various membrane properties (43). The dynamic properties and organization of membranes depend on the membrane lipid composition (44). Yechiel and Barenholz (45) demonstrated in cultured rat heart myocytes that lipid composition undergoes changes during aging, including alterations in the content of cholesterol and degree of phospholipid acyl-chain saturation (45). In this study four stereological parameters analyzed the cardiomyocyte: The CA group rats had the greater Vv[cm] and Sv[cm] and the smaller A[cm], thus indicating better preservation of the number and size of the cardiomyocytes in this group than in the other groups. The CA group rats had the smallest Vv[ct], suggesting a small interstitial fibrosis with maintained myocardial microvasculatization in these animals.

The myocardium in rats with nitric oxide synthesis blockade and concomitant antihypertensive treatment showed hypertrophied cardiomyocytes, capillary rarefaction, and the tunica media and tunica intima of small arteries were thickened and had an increase in collagen fibrils (46). This description is similar to the morphology found in the LE group of the present study.
The S group rats had intermediary values for the myocardium and blood vessels stereology; they presented the worst results to the Vv[cm] and l[v[cm]. The soybean oil has a great n-6 fatty acid percentage in this composition (47). It is well established that n-6 fatty acid deficiency causes growth retardation; therefore n-6 deficiency during gestation could have caused irreversible damage to brain (48). However, the n-3 fatty acids, could change fatty acid composition in rat’s heart and then modify the permeability of cell membrane receptor activities, thereby influencing heart function (49). These influences are apparently not caused by n-6 fatty acids (47). McLennan and Dellimore (50) suggested that high levels of LA in soybean oil can reduce the effectiveness of ALA and that it is the ratio of LA : ALA that can determine the effectiveness of conversion of ALA into EPA.

The CA+LE group rats presented the second best value for the Vv[cm], suggesting that the intake of canola oil in the mixture with lard and egg yolk contributes to maintain a good density of cardiomyocytes preventing the adverse effect of the long-term saturated fatty acid intake. These results support the notion that the long-term use of canola oil in the diet is better to preserve the myocardium structure, including microvascularization, than soybean oil or lard and egg yolk.

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