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The Effect of Endurance Training with Garlic and Stevia Supplementation on AgRP and α-MSH in Brain Tissue of Obese Wistar Rats

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ABSTRACT

Background: The aim of this study is to investigate the effect of endurance training with garlic and stevia supplementation on AgRP and α-MSH in the brain tissue of obese Wistar rats. **Methods:** 36 obese male Wistar rats fed with a high-fat diet (24 g fat, 24 g protein, and 41 g carbohydrate/100 g) were divided into six groups, including: 1) sham; 2) stevia supplementation; 3) garlic supplementation; 4) endurance training; 5) stevia supplementation plus endurance training, and 6) garlic supplementation plus endurance training. Moreover, 6 rats were included in the healthy control group to investigate the effects of obesity on the research variable. Training groups performed incremental endurance training for 15-50 minutes at a speed of 15-25 m/min, for eight weeks. Garlic and stevia supplements were added to the diet of the supplementation groups at a dose of 250 mg/kg. **Results:** Levels of AgRP in stevia supplementation group were significantly higher than endurance training (P=0.019) and endurance training plus stevia supplementation (P=0.018) groups. Levels of α-MSH in the garlic supplementation (P=0.001), endurance training (P=0.002), stevia supplementation plus endurance training (P=0.001), and garlic supplementation plus endurance training (P=0.001) groups were significantly higher than the sham group. In addition, in the endurance training plus stevia supplementation group, the levels were significantly higher than the stevia supplementation group (P=0.002). **Conclusion:** Endurance training plus garlic supplementation as well as endurance training plus stevia supplementation seem to play a synergistic role in appetite control protein; however, more studies on AgRP changes, following training and garlic and stevia supplementation are needed.

Keywords: *Training; Garlic; Stevia; Agouti-related protein; α-MSH; Obesity*

Introduction

Last year, 39% of adults have been reported as overweight (with a body mass index of more than 25 kg/m^2) and 13% as obese. This trend continues to increase (Labban *et al.*, 2020). Increased fat accumulation and metabolic disorders lead to many diseases (Davari *et al.*, 2022, Yeung and Tadi, 2020). Following obesity, a disorder occurs in appetite-dependent hormones, which are divided into anorectic (appetite suppressant) and orexinergic (appetite stimulant), leading to

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impaired appetite and a feeling of hunger and satiety (Zouhal *et al.*, 2020). Dysfunction of hormones and increase in reactive oxygen species (ROS) and N-methyl-D-aspartate (NMDA-R) leads to neuronal damage (Labban *et al.*, 2020). Furthermore, increased circulating levels of leptin and insulin hormones in satiety state binds to pro-opiolanocortin (POMC) neurons and increases expression and maturation of α-melanocyte-stimulating hormone (α-MSH). Via binding to melanocortin 4 receptor, α-MSH reduces energy intake and appetite; conversely, in the state of starvation, activation of agouti-related neuropeptide (AgRP) leads to increased expression of neuropeptide Y (NPY), decreased insulin , and leptin, increased orexigenic and ghrelin, and ultimately increased appetite. However, following obesity, increased oxidative stress and glutamate causes damage and disruption in this pathway, causing damage and disruption to the appetite-related parts of hypothalamus (Baldini and Phelan, 2019).

On the other hand, researchers believe that weight loss following exercise depends on many factors such as energy expenditure, circulating levels of hormones, intestinal NPY, and intensity of exercise (Zouhal *et al.*, 2020). In addition, by increasing tissue sensitivity to insulin and improving lean body mass, it seems that physical activity is affective on weight loss; regular physical activity increases fat metabolism, modulates hormone leptin at the primary level, and helps regulate appetite (Zouhal *et al.*, 2020). The researchers showed that eight weeks of aerobic training increased plasma ghrelin levels in middle-aged men, but did not increase ghrelin gene expression in lymphoid cells (Ahmadi *et al.*, 2019); one session of high intensity interval training also increased NPY and decreased appetite for 24 hours after exercise in obese women (Sepideh and Bahman, 2020). However, eight weeks of resistance training had no significant effect on acyl ghrelin and NPY in inactive men, and orexin levels and appetite increased after resistance training (Jafari *et al.*, 2017).

Due to inconsistent findings concerning the effects of exercise training, molecular cell pathways that are dependent on appetite, and the potential use of dietary supplements and herbs in weight loss and reduction of body fat mass, as well as the impact on calorie and food intake in rats with type 2 diabetes, there is a need for further research in this area (Hosseini *et al.*, 2018). Among medicinal plants, garlic is an effective for maintaining health due to its phenolic active elements and antioxidant properties. Garlic consumption with its antioxidant mechanism improves fat metabolism, increases nitric oxide, reduces amyloid beta levels, amyloid precursor protein (APP), and protein hyperphosphorylation by inhibiting glycogen synthase kinase (GSK)-3β, improves synaptic function, and reduces neuroinflammation, leading to beneficial effects on brain tissue (Song *et al.*, 2020). Garlic is a stimulant for optimal change in ATP/ADP ratio, and by activating mitogen-activated protein kinase (MAPK), it activates the synthesis of proteins responsible for appetite control in the brain (Huynh *et al.*, 2016). Although previous studies have examined the role of garlic supplementation on metabolic function in nervous system, no specific study has been performed to investigate the effect of garlic supplementation on AgRP and α-MSH protein levels.

In addition to garlic, stevia is another medicinal plant which has long been used in traditional medicine because of its glycosides. Consuming stevia reduces calorie intake and improves glucose metabolism (Anton *et al.*, 2010). In addition, due to its vitamins, minerals, and phenolic acids, stevia has the ability to neutralize high oxidative stress, which increases the efficiency of enzymatic and non-enzymatic antioxidant systems (Jahangir Chughtai *et al.*, 2020). In a study, the beneficial effects of stevia consumption at doses of 25, 250, 500, and 1000 mg/kg improved liver enzymes and reduced fat mass and weight of obese female rats (Elnaga *et al.*, 2016). Studies have shown that aerobic training and the use of herbs have beneficial effects on metabolism; however, due to the importance of recognizing appropriate interventions to control appetite and its effect on obesity, the effect of garlic and stevia supplementation along with exercise training on

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the path of physiological control of appetite is not known. Therefore, the aim of this study is to investigate the effect of endurance training with garlic and stevia supplementation on AgRP and α-MSH on brain tissue of obese Wistar rats.

Materials and Methods

S*tudy setting:* In this experimental study, 42 male Wistar rats with an average weight of 200±20 g were purchased from Breeding Center of Shahrood University of Medical Sciences and kept in physical education laboratory for seven days for adaptation.

Inclusion criteria for animals in the current research were physical health, the ability to perform exercises, being at the age of eight weeks, and a weight of 180-220 g. Exclusion criteria for the animals were the observation of any disease during the research process and inability to perform exercises.

During the study, rats were kept in standard conditions in terms of temperature (22 to 24 °C), relative humidity of 55 to 60%, and light-dark cycle of 12-12 hours in polycarbonate cages with autoclave capability and *ad libitum* access to water and food. Following the adaptation period, 36 rats were exposed to a high-fat diet containing 45% of total energy from fat (derived from animal oil), comprising 24 g of fat, 24 g of protein, and 41 g of carbohydrates per 100 g to reach a weight of over 310 grams. It is noteworthy that according to scientific sources, weighing over 310 grams in rats is considered obesity (Hosseini *et al.*, 2018).

Thirty-five obese male Wistar rats fed with a high-fat diet were divided into six groups, including: 1) sham; 2) stevia supplementation; 3) garlic supplementation; 4) endurance training; 5) stevia supplementation plus endurance training, and 6) garlic supplementation plus endurance training. Moreover, 5 rats were included in healthy control group (group 7) to investigate the effects of obesity on research variables. Training groups performed incremental endurance training for 15-50 minutes at a speed of 15-25 m/min, five sessions per week, for eight weeks (Cho *et al.*, 2017, Haghshenas *et al.*, 2014). Garlic and stevia supplements were added to the diet of the supplementation groups at a dose of 250 mg/kg (Cho *et al.*, 2017). In this vein, garlic supplement with a dose of 250 mg/kg was added to the diet, and stevia supplement was added to the diet of rats with a dose of 250 mg/kg.

Endurance training protocol: Training protocol consisted of five days of familiarizing the high-fat diet rats with the environment and running on a treadmill, which was performed on a zero-degree slope treadmill for 30 minutes at a speed of 8 m/min. Then, training intensity was designed based on Cho research (Cho *et al.*, 2017), which was performed on obese and elderly rats. Based on the training protocol, in each training session, the rats ran the first 5 minutes of the training at a speed of 8 meters per minute; then, they ran the second 5 minutes at a speed of 11 meters per minute, then 20 minutes at a speed of 15 meters per minute, and eventually, the last 10 minutes at a speed of 10 meters per minute on a zero-degree slope. It is noteworthy that in this study, the rats worked out according to their enduring power; in other words, they were returned to their cages whenever they could not work out anymore (Cho *et al.*, 2017).

Dissection and sampling procedures: 48 hours after the last training session and following 12 hours of fasting, rats were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (15 mg/kg). After ensuring complete anesthesia and analgesia, the brain tissue was perfused using 10 cc of sodium chloride 9%. In order to extract brain tissue, first, the rats were completely handled; after cutting the upper part of the skull, brain tissues were carefully separated, and after weighing and washing, they were placed in special tissue storage cryotubes, and were immediately transferred to be kep at -80 °C.

Measuring AgRP, α-MSH in brain tissue: To measure the levels of AgRP, POMC (α-MSH) gene expression in brain tissue, qReal Time PCR was used. RNA extraction from tissues in all groups was performed according to the protocol of the manufacturer (Kiagen, Germany).

For this purpose, about 50 g of brain tissue was well lysed in a microtube free of RNase enzyme, and then, one ml of Trizol solution was added to it. The solution was mixed with the help of a

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micropipette and incubated at room temperature for 5 minutes. After that, the mentioned solution was homogenized with 200 microliters of chloroform. Next, the microtubes containing the solution were centrifuged at a speed of 12,000 rpm for 15 minutes at a temperature of 8-2 °C until supernatant phase containing RNA was transferred to the microtubes free of RNase enzyme.

After adding isopropanol, the solution was incubated again for 20 minutes at 15-30 °C, and then, centrifuged. Following centrifugation, the supernatant phase was drained, 500 microliters of 75% ethanol (prepared with DEPC-treated water) was added, and the mixture was vortexed until sediment was removed from the bottom of the microtube. Finally, the pure RNA precipitate was dissolved in DEPC-treated water and stored in a freezer at -70 °C.

To remove RNA contamination, RNase-free DNase enzyme was used. For one microgram of extracted RNA, one microliter (Fermentase, 1 µl) of DNase and one microliter of buffer x 10 were added, and the volume of the solution with DEPC-treated water was increased to 10 microliters. The resulting solution was incubated for 15 minutes at 37 °C, and then, it was placed at 65 °C for 15 minutes to inactivate the enzyme.

To ensure the quality of RNA, agarose gel electrophoresis and light absorption at 260 nm were used with Sigma PicoDrop device (made in USA). In addition, the formula (C (µg/µl) = A260 × ε × d / 1000) was used to evaluate RNA quality. After cDNA synthesis, reverse transcription reaction was performed using the protocol of the manufacturer in the Fermentase (40 U/µl) kit (K1621), the primers designed (**Table 1**) based on the guide of AgRP, and α-MSH genes at the PUBMED site. To determine the efficacy and specificity of primers, pre-primers were evaluated using the software available at the NCBI site.

After designing primer (**Table 2**) using the protocol recommended in kit catalog and SYBR Green Master mix, the mixed primers of 10 pmol/µl, sterile water, cDNA template, and RNase and DNase free H2O solution were placed in Bioneer Real-Time PCR device. The device functions in such a way that a specific temperature was given to the device every hour, so that in the first cycle, the temperature started from 35 °C, and in the final stage, it reached 80 °C; as a result, the enzymes could perform maximum transcription at the appropriate temperature and reach a point where the fluorescent light could be seen on the monitor of the device. It is worth mentioning that at temperatures higher than 72 °C, the duration of the cycles reaches 20-30 seconds in order to prevent damage to the structure of the enzymes. Therefore, this phase is called threshold cycle (CT).

Table 1. Rats' training protocol in one session regarding eight weeks of training.

Intensity	Time	Grade (%)	Frequency (day)
8 m/min	5 (min)	0	5
11 m/min	5 (min)		
15 m/min	20 (min)		
8 m/min	10 (min)		

Furthermore, to measure gene expression levels of the variables, internal control gene of beta 2 myoglobulin (B2m) was used. After confirming the completion of the qReal Time PCR and reaching the cycle threshold, first, internal control gene CT and target gene CT were subtracted from the control group. Then, they were subtracted from each other in the target group. Next, the results of these two subtractions were subtracted again, and the total ΔΔCT was obtained. To finalize data, the formula $2^{-\Delta\Delta CT}$ was used.

Given the examination of changes reaching the threshold cycle which were to be measured with respect to the internal control gene, data criteria were reported based on fold change.

Ethical considerations: It is noteworthy that all the ethical principles of working with laboratory animals in this study were carried out according to Helsinki agreement and under the supervision of the Ethical Committee for Working with Laboratory Animals, Islamic Azad University, Aliabad Katoul Branch, with the approved code IR.IAU.AK.REC.1399.024.

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Data analysis: Shapiro-Wilk test was used to examine the normality of data distribution. For inferential analysis and determination of inter-group differences, one-way analysis of variance (AVOVA), and to investigate the location of inter-group differences, Tukey's *post hoc* test were used in SPSS19 software at a significance level of ≤0.05.

Table 2. Primer sequence of the research variables.

Gene's name	Primer sequence	bp
B2m	*Forward: 5'- CGTGCTTGCCATTCAGAAA -3'* *Reverse: 5'- ATATACATCGGTCTCGGTGG -3'*	244
AgRP	*Forward: 5'- CGTGTGGGCCCTTTATTAGA -3'* *Reverse: 5'- CAGACCTTCTGATGCCCTTC -3'*	191
POMC (α-MSH)	*Forward: 5'- GAAGGTGTACCCCAATGTCG -3'* *Reverse: 5'- CTTCTCGGAGGTCATGAAGC -3'*	225

Results

The results of Shapiro-Wilk test showed that data distribution was normal. **Figures 1** and **2** demonstrated mean and standard deviation of research variables. The results showed a significant difference in AgRP (*P*=0.012) and α-MSH (*P*=0.001) in the research groups. According to Tukey's *post hoc* test, there was no significant difference in AgRP gene expression between healthy control and sham groups (*P*=0.88); In addition, there was no significant difference between stevia (*P*=0.20), garlic (*P*=0.99), endurance training (*P*=0.91), endurance training plus stevia (*P*=0.91), and endurance training plus garlic (*P*=0.99) groups compared to the sham group. However, AgRP gene expression in the stevia group was significantly higher than the endurance training (*P*=0.01) and stevia plus endurance training (*P*=0.01) groups (**Figure 1**).

There was no significant difference between the healthy control and sham groups (*P*=0.44) regarding α-MSH gene expression; no significant difference was also observed in the sham and stevia groups (*P*=0.05); however, in garlic (*P*=0.001), endurance training (*P*=0.002), stevia plus endurance training (*P*=0.001), and garlic plus endurance training (*P*=0.001) groups, the levels were significantly higher than the sham group. Also, in endurance training group plus stevia, the levels were significantly higher than stevia group (*P*=0.002, **Figure 2**).

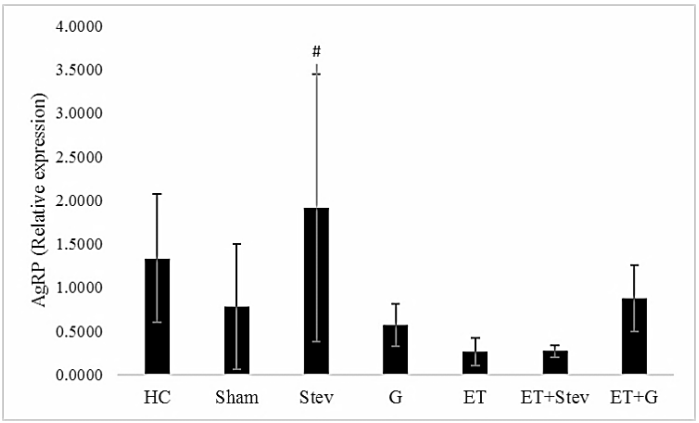

Figure 1. Levels of AgRP gene expression in the research groups

HC: healthy control; Sham: Obese control; Stev: Stevia; G: Garlic; ET: Endurance training; ET + Stev: Endurance training + Stevia; ET + G: Endurance training + Garlic.
: Significant increase compared to the training and training + stevia groups

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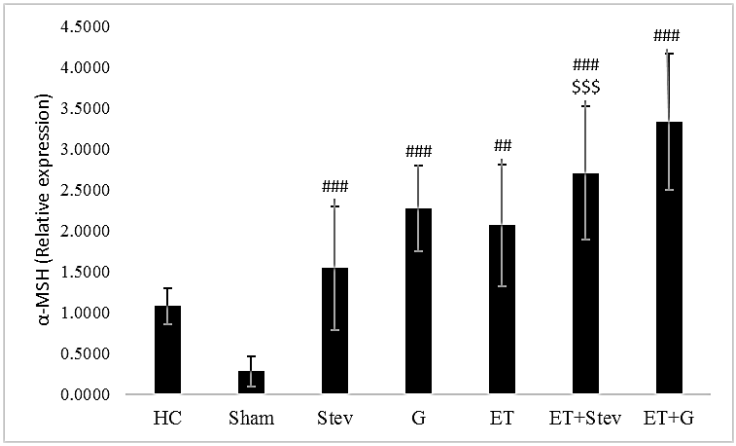

Figure 2. Levels of α-MSH gene expression in the research groups

HC: healthy control; Sham: Obese Control; Stev: Stevia; G: Garlic; ET: Endurance Training; ET + Stev: Endurance Training + Stevia; ET + G: Endurance Training + Garlic.

##(P = 0.01), ### (P = 0.001) Significant increase compared to the sham group; $$$ (P = 0.001) Significant increase compared to the stevia group.

Discussion

The results of the present study showed that exercise training increased α-MSH gene expression in brain tissue of obese rats; however, no significant change in AgRP was observed after training. Exercise training can improve homeostasis in various parts of brain, including the hypothalamus (Vehapoğlu *et al.*, 2016). Impaired hypothalamic homeostasis control and weight gain, known as hypothalamic obesity, is affected by leptin-melanocortin mechanism and affects neuropeptides AgRP and α-MSH. The relationship between increased α-MSH gene expression and weight loss has been reported in previous studies, but in relation to AgRP, this protein seems to be less dependent on weight and body mass index due to the degree of hypothalamic impairment (Emet *et al.*, 2021).

Nonetheless, exercise training improves neurogenesis, orexigenic, and anorexic functions in animal studies (Carnier *et al.*, 2013). However, improvement in the function of neuropeptides which affects metabolism depends on the type of exercise training. In this regard, various results have been reported. A study showed that aerobic training had a better effect on AgRP in obese adolescents (Carnier *et al.*, 2013). In another study, researchers found that obesity was associated with inflammation in hypothalamus. However, exercise training with the mechanisms of leptin modulation, AgRP activation, and increased expression of uncoupled protein-1 (UCP1) resulted in increased brown adipose tissue (Rodrigues *et al.*, 2018). In justifying the lack of change in AgRP levels, it can be said that, in the state of starvation, AgRP and NPY besides decreasing insulin and leptin levels as well as increasing orexigenic and ghrelin, lead to an increased desire to receive food (Baldini and Phelan, 2019).

The results of the present study showed that AgRP and α-MSH levels increased significantly after stevia consumption, while garlic supplementation only increased α-MSH levels in brain tissue of obese rats. Consumption of garlic can reduce adipokines such as leptin by mechanisms of reducing inflammatory factors and oxidative stress and increasing total antioxidant capacity. In response to this, there is a decrease in leptin receptor expression at the level of nerve cells, resulting in decreased protein levels of AgRP. Activation of melanocortin 4 can lead to activation of NPY, and ultimately increase in α-MSH (Donma and Donma, 2020). Another point is that improving metabolism of nerve cells following the consumption of garlic modulates orexinergic and anorexigenic hormones and plays a role in

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improving the function of hypothalamus (Donma and Donma, 2020). In this regard, researchers found that consumption of garlic improved fat profile and glycemic indices, increased expression of POMC and hormone-sensitive lipase, decreased leptin, and improved beta-adrenergic receptor in high-fat diet obese rats (Amor *et al.*, 2019).

Additionally, consumption of stevia improved serum antioxidants, reduced the desire to eat (Farhat *et al.*, 2019), and reduced food intake (Anton *et al.*, 2010). In another study, researchers showed that stevia consumption reduced inflammatory factors, improved insulin and glucose metabolism, and reduced food intake in mice (Rosales-Gómez *et al.*, 2018). Limited studies have been performed on the effect of stevia on proteins, which affect appetite; however, researchers have shown that stevia reduces the level of reactive oxygen species by its antioxidant mechanism; Furthermore, by reducing inflammatory factors such as IL-1β, IL-6 and TNF-α, and by increasing insulin secretion, it improves glucose metabolism and ATP levels (Rosales-Gómez *et al.*, 2018). It seems that the simultaneous increase of AgRP and α-MSH levels in this study increased after reducing inflammatory factors and improving glucose metabolism and neurotrophin effects in hypothalamus; this increase can also be obtained in modulation of appetite.

The results of the present study showed that exercise regarding both stevia consumption and garlic consumption increased α-MSH gene expression levels in brain tissue of obese rats. Based on the studies conducted, aerobic training with garlic supplementation seems to activate hormone-sensitive lipase, improve fat and sugar metabolism, and increase the expression of metabolic proteins in hypothalamus by mechanism of activating AMPK (Amor *et al.*, 2019, Baldini and Phelan, 2019, Carnier *et al.*, 2013, Donma and Donma, 2020). Therefore, it seems that garlic consumption and aerobic training with synergistic effects play a role in modulating AgRP and increasing α-MSH in the brain tissue of rats exposed to high-fat food. Researchers have reported that both training and garlic consumption increase AgRP and contribute to negative energy balance. But in response to it, AGRP is secreted from hypothalamus (and possibly muscle) to stimulate food intake and energy needs, and this may be one of the extra compensatory mechanisms of glycogen (Hosseini-Khakhak *et al.*, 2009). It also appears that exercise and consumption of stevia with similar mechanisms, such as increasing antioxidant capacity, improving inflammatory factors, and reducing leptin, play a role in reducing appetite (Baldini and Phelan, 2019, Farhat *et al.*, 2019, Rodrigues *et al.*, 2018, Rosales-Gómez *et al.*, 2018). Regarding the simultaneous effect of training and stevia supplementation, a study showed that aerobic training with stevia supplementation synergistically reduced liver enzymes (Akbari *et al.*, 2020), decreased serum omentin levels, and improved fat profile (Akbari *et al.*, 2019) in diabetic rats. In another study, resistance training and stevia supplementation increased mitochondrial biogenesis in rats (Lima *et al.*, 2019).

Due to the role of NPY, leptin, ghrelin, and other proteins in regulating appetite, the lack of measurement of variables is a limitation of the present study. Therefore, it is suggested that more related adipokines and neuropeptides be measured in future studies. Due to different roles exercising plays in oxidative stress and inflammation, it seems that lack of measurement for these variables is another limitation of the present study, so it is suggested that these factors be measured in future studies as well .

Conclusion

Endurance training with garlic supplementation as well as endurance training with stevia supplementation seem to be synergistically involved in appetite control protein; however, further studies are needed on changes in AgRP levels, following training and supplements used in this study.

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Authors' contributions

All authors involved to design and organizing the research. The first author involved to implement of the project and statistical analysis of data. all authors involved to write and editing of the manuscript.

Conflict of interest

The authors declared no conflict of interest.

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