EFFECTS OF P-CHLOROPHENYLALANINE (pCPA) ON ETHANOL WITHDRAWAL SYNDROME IN RATS

P-Klorfenilalaninin Sıçanlarda Etanol Yoksunluk Sendromu Üzerine Etkileri

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ABSTRACT

Objective: In the present study, the effects of p-chlorophenylalanine (pCPA), a serotonin depleting agent, treatment on ethanol withdrawal syndrome have been investigated in rats. Thus, contributions of central serotonergic system to various signs of ethanol withdrawal syndrome have been examined and clarified.

Methods: Ethanol (7.2% v/v) was given to adult male Wistar rats by a liquid diet for 21 days. pCPA (100 mg/kg) and saline were injected to rats intraperitoneally 24 h before ethanol withdrawal testing. After 2nd, 4th and 6th h of ethanol withdrawal, rats were observed for 5 min, and withdrawal signs, including locomotor hyperactivity, agitation, stereotyped behavior, wet dog shakes, tremor and audiogenic seizures were recorded or rated. pCPA (100 mg/kg) were also administered to no ethanol-dependent control rats and locomotor activity was measured and evaluated like in ethanol-dependent group.

Results: pCPA treatment produced some significant increases in locomotor activity of both control and ethanol-dependent rats during the observation period. pCPA caused some increases the severity of agitation and intensity of wet dog shakes at 2nd and 6th hrs of ethanol withdrawal, respectively. It was no effective on the other signs of ethanol withdrawal except audiogenic seizures. pCPA pretreatment inhibited significantly the incidence of audiogenic seizures.

Conclusion: Our findings suggest that pCPA intensified locomotor hyperactivity, agitation and wet dog shakes but attenuated audiogenic seizures during ethanol withdrawal in ethanol-dependent rats. These signs of ethanol withdrawal may be related to serotonergic mechanisms in central nervous system.

Key words: P-chlorophenylalanine (pCPA), ethanol dependence, ethanol withdrawal syndrome, rat(s).

ÖZET

Amaç: Sunulan çalışmada, serotonin depolarını boşaltan bir ajan olan p-klorfenilalaninin (pCPA) sıçanlarda etanol yoksunluk sendromu üzerine etkileri incelenmiştir. Böylece etanol yoksunluk sendromunun çeşitli belirtilerine santral serotonerjik sistemin katkısı incelenmiş ve açıklığa kavuşturulmuştur.

Yşntem: Etanol (% 7.2 v/v) erişkin Wistar türü erkek sıçanlara sıvı diyet tekniği ile 21 gün süre ile verildi. pCPA (100 mg/kg) sıçanlara etanol yoksunluğunun test edilmesinden 24 saat önce intraperitoneal yoldan enjekte edildi. Etanol yoksunluğun 2, 4 ve 6. saatlerinde sıçanlarda 5'er dakika süre ile lokomotor hiperaktivite, ajitasyon, stereotipik davranışlar, ıslak köpek silkinmesi, tremor ve odiyojenik nöbetler gibi yoksunluk belirtileri değerlendirilerek bunların sıklık ve şiddeti kaydedildi veya skorlandı. pCPA (100 mg/kg) ayrıca etanole bağımlı olmayan kontrol sıçanlara da enjekte edilerek lokomotor aktivite üzerine etkileri değerlendirildi.

Bulgular: pCPA uygulaması gözlem süreci boyunca hem etanole bağımlı hem de bağımlı olmayan sıçanların lokomotor aktivitelerinde anlamlı düzeyde artışlara neden oldu. pCPA etanol yoksunluğun 2. saatinde ajitasyonun şiddetinde, 6. saatinde ise ıslak köpek silkinmesi davranışının sıklığında anlamlı ölçüde artışlara neden oldu. Odiyojenik nöbetler hariç diğer yoksunluk belirtileri üzerine etkisizdi. pCPA verilmesi odiyojenik nöbetlerin sıklığını anlamlı ölçüde inhibe etti.

Sonuç: Bulgularımız pCPA'nın etanole bağımlı sıçanlarda lokomotor hiperaktivite, ajitasyon ve ıslak köpek silkinmesi gibi yoksunluk belirtilerini daha da şiddetlendirirken, odiyojenik konvulsiyonların sıklığını azalttığına işaret etmektedir. Etanol yoksunluğunun bu belirtileri santral serotonerjik mekanizmalar ile ilişkili olabilir.

Anahtar kelimeler: P-klorfenilalanin (pCPA), etanol bağımlılığı, etanol yoksunluk sendromu, sıçan.

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INTRODUCTION

Serotonergic drugs are of particular interest in that point, especially because of the hypothesized links between mood disorders and ethanol consumption. As a 5-HT deficiency seems to be involved in ethanol dependence as well as maintenance of ethanol consumption (1-4), it could be expected to influence the occurrence of mood symptoms in ethanol dependents, especially during withdrawal. This hypothesis was tested in ethanol dependent rats, suggesting that deficits in accumbal 5-HT release may contribute to the negative affective consequences of ethanol withdrawal (5).

A couple of previous study from our laboratory indicated some marked reductions in striatal serotonin (5-hydroxytriptamine, 5-HT) levels of rats during early ethanol withdrawal (6) and chronic ethanol consumption (7). Uzbay et al. (6) also sowed that the signs of ethanol withdrawal such as increased locomotor activity and stereotyped behaviors, tremors, wet dog shakes, agitation and audiogenic seizures appeared in a parallel group of ethanol-dependent rats. These observations imply that there might be an association between decreasing serotonergic activity and ethanol dependence. This thought has also been supported by attenuating effects of agents such as fluoxetine that increase serotonergic activity in synaptic cleft on the some signs of ethanol withdrawal syndrome in rats (8).

P-chlorophenylalanine (pCPA) reduces brain 5-hydroxytryptamine (5-HT) without altering the dopamine and norepinephrine content. It depletes central 5-HT by inhibiting tryptophan hydroxylase, an enzyme necessary for the synthesis of 5-HT (9). Experimental studies have been shown that pCPA reduced frequency of withdrawal jumping (10) and wet dog shakes (11) in morphine dependent rats. Fuchs and Coper (12) also showed that p-CPA treatment reduced the sensitivity to the naloxone-precipitated withdrawal reaction, but did not affect the development of physical dependence. In contrast to the results of these studies, it has been found that pCPA increased the severity of diazepam withdrawal signs in rats (13). Studies involved in effects of pCPA on ethanol withdrawal syndrome have limited yet. A few old studies which were performed on mice did not put forward any clear results explaining the effects of pCPA on ethanol withdrawal or the role of serotonin in the ethanol withdrawal signs (14-17). No study has also been reported in ethanol dependent rats.

The main objective of the present study was to investigate the effects of pCPA on the signs of ethanol withdrawal syndrome. This was done by observing several signs of ethanol withdrawal syndrome such as locomotor hyperactivity, agitation, stereotyped behavior, wet dog shakes, tremor and audiogenic seizures in ethanol dependent rats after depletion of 5-HT by pCPA pretreatment. Thus, we wanted to get a further support to the hypothesis suggest that there might be a relationship decreased serotonergic activity and ethanol dependence.

METHODS

Animals and laboratory: All procedures in this study are in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (USA). Adult male Wistar rats (210-330 g weight at the beginning of the experiments) were subjects. They were housed in a quiet and temperature and humidity-controlled room (22 ± 3 °C and 65 ± 5%, respectively) in which a 12-h light/dark cycle was maintained (07:00-19:00 h light). Exposure to ethanol and all behavioral experiments involved in ethanol withdrawal syndrome were carried out in the separate and isolated laboratories, which have the same environmental conditions with the colony room.

Chronic ethanol administration to rats: For chronic ethanol exposure, the rats were housed individually and ethanol was given in the modified liquid diet as previously described (18). At the beginning of the study, rats were given the modified liquid diet without ethanol for 7 days. Then liquid diet with 2.4% ethanol was administered for 3 days. The ethanol concentration was increased to 4.8% for the following 4 days and finally to 7.2% for 21 days. Liquid diet was freshly prepared daily and presented at the same time of the day (10:00 h). The weight of the rats was recorded every day, and daily ethanol intake was measured and expressed as g per kg per day. Control rats (n= 8) were pair fed an isocaloric liquid diet containing sucrose as a caloric substitute to ethanol.

Drugs used in the study: pCPA was purchased from Sigma Chemical (USA). The drug was dissolved in saline. pCPA or saline were injected to rats intraperitoneally at a volume of 1 ml / 200g body weight. Drug solutions were prepared freshly in the morning of each experiment.

Evaluation of ethanol withdrawal syndrome: At the end of the exposure to 7.2% ethanol-containing liquid diet, ethanol was withdrawn from the diet by replacing the diet with one that did not contain ethanol at 10:00 h. Ethanol-dependent rats were then assigned into several groups (n= 8 for each group). pCPA (100 mg/kg) and saline were injected to the ethanol groups. The same dose of pCPA and saline were applied to the next independent two groups of the rats for 21 days isocaloric liquid diet intake. pCPA (100 mg/kg) was injected to the rats 24 h before saline injections. Saline injections were done 30 min before locomotor activity measurements and/or ethanol withdrawal testing.

The rats were then observed for 5 min at the 2nd, 4th and 6th h of the withdrawal period. At each observation time, rats were assessed simultaneously for the following behavioral conditions: agitation, tremor, stereotyped behavior and wet dog shakes. Locomotor activities of the rats were also recorded (Opto Varimex Minor, Columbus, OH, USA) as a total of horizontal, vertical and ambulatory activities of the rats and expressed as mean ± SEM. Wet dog

shakes, tremors and audiogenic seizures were assessed as incidence. Wet dog shakes behavior was considered positive if they occurred at least three times during the observation period. Tremor was determined after lifting rats vertically by the tail; positive was assigned to rats showing clearly distinct forelimb tremor when they were rotated 180° around axis of the tail. Grooming, sniffing, head weaving, gnawing and chewing were observed as major stereotyped behaviors during the ethanol withdrawal in the study. Stereotypic behaviors and agitation were scored using a rating scale as previously described (19) (Table 1).

Table 1: Rating scale for agitation and stereotyped behavior signs induced by ethanol withdrawal in rats (19)

Signs	Scoring
Agitation	0: no irritability or aggressive behavior 1: rats showing mild or moderate irritability 2: very irritable 3: handling vocalization and moderately aggressive 4: handling vocalization and very aggressive 5: spontaneous vocalization and very aggressive
Stereotyped behavior	0: no stereotyped behavior 1: rats showing only one stereotyped behavior 2: two stereotyped behavior 3: three stereotyped behavior 4: four stereotyped behavior 5: all of stereotyped behavior

After 6 h of withdrawal testing, rats were exposed to an audiogenic stimulus (100 dB) for 60 sec in a separate and soundproof place in the laboratory. The incidence and latency of the audiogenic seizures was recorded.

All experiments were carried out during the light period. All ratings were done by a naive observer who was unaware of which treatment the rats received.

Statistical analysis

Changes in locomotor activities and latency of audiogenic seizures of ethanol-dependent rats as compared with ethanol non-dependent control rats and the effects of pCPA on locomotor activity were analyzed by unpaired (between groups) Student's t-test. The intensities of the agitation and stereotyped behavior in different groups were compared by Mann-Whitney-U test. Comparison of the incidences of the audiogenic seizures, wet dog shakes behavior and tremors were done by Chi-square test. The level of significance was set at $p< 0.05$ levels.

RESULTS

Daily ethanol consumption of the rats: Daily ethanol consumption of the rats was in a range of 11 to 15 g/kg during the exposure to ethanol (7.2%, v/v). No significant difference between the ethanol-ingesting groups was observed (data not shown). Rats feeding ethanol contained liquid diet did not have a serious body weight loss. Even they gained their body weights slightly. No rats died during the study.

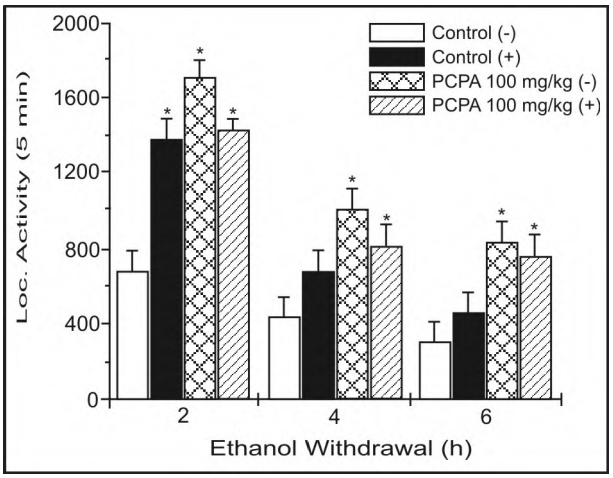
Behavioral changes during ethanol withdrawal: A significant effect on locomotor hyperactivity was observed in the ethanol-dependent groups at the 2nd and 6th hrs of the withdrawal-testing period as compared with the ethanol non-dependent saline groups (Student's t test; $ps< 0.05$) (Fig. 1). Other behavioral signs of ethanol withdrawal syndrome such as agitation, tremor, stereotyped behaviors and wet dog shakes appeared at the 2nd h and lasted during the whole observation period (Fig. 2 A-D dark bars). Audiogenic seizures occurred at 6th h of ethanol withdrawal with an incidence of 62.5 % and latency of 13 sec in ethanol-dependent control group (Table 2). No ethanol withdrawal signs were observed in the ethanol no dependent rats.

Effects of pCPA on ethanol withdrawal syndrome: pCPA administration produced some significant increases in the locomotor activities of both ethanol dependent and no ethanol dependent rats according to saline treated controls. However, there were no significant differences between pCPA treated ethanol dependent and no dependent groups (Fig. 1).

While pCPA treatment intensified agitation and wet dog shakes at the 2nd and 6th hrs of ethanol withdrawal, respectively, it was not effective on tremors and stereotyped behaviors (Fig 2).

pCPA inhibited significantly the incidence of audiogenic seizures in ethanol withdrawn rats (Table 2).

Figure 1: Effects of pCPA on the locomotor activities of the rats



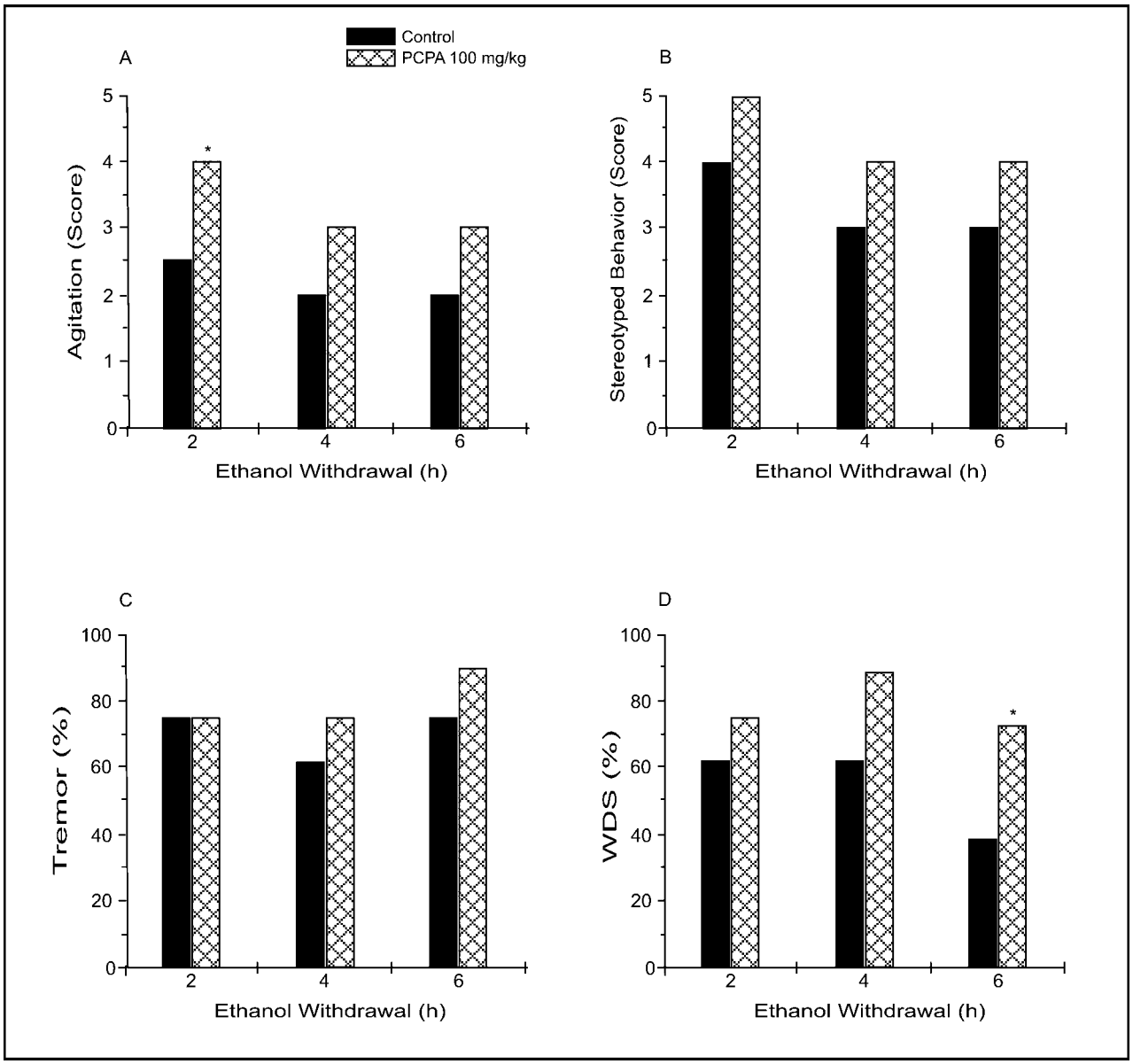
n= 8 for each group; h= hour; * $p< 0.05$ significantly different from Control (-), Student's t test; -: ethanol non-dependent; +: ethanol-dependent

Table 2: Effects of pCPA on the audiogenic seizures in ethanol-dependent rats.

Treatment	Incidence (%)	Latency (Mean ± SEM)
Saline	67.5	13.2 ± 0.9 (5)
pCPA	25.0*	23.5 (2)

n= 8 for each group; * $p<0.05$ significantly different from control (chi-square test); ** $p<0.05$ significantly different from control (Student's t test); Figures in the parenthesis represent the number of animals that have seizure activity after audiogenic stimulus. Statistical analysis did not perform in the groups that have less than three subjects appearing seizure activity.

Figure 2: Effects pCPA on the signs of ethanol withdrawal syndrome



n= 8 for each group; h= hour; * p< 0.05 significantly different from Control, Mann-Whitney-U test for score and Chi-Square test for %

DISCUSSION

The main finding of the present study is that pCPA affected some of the signs of withdrawal syndrome in ethanol-dependent rats. While it intensified locomotor hyperactivity, agitation and wet dog shakes, it inhibited the incidence of audiogenic seizures. The augmenting effects of pCPA on locomotor hyperactivity were shown in all observation period and more prominent than those shown in agitation and wet dog shakes. Because pCPA is a strong depleting agent of central 5-HT, our results suggest that locomotor hyperactivity, agitation and wet dog shakes which are the symptoms appearing during ethanol withdrawal in rats may be related to reducing central 5-HT levels. These findings are also in line with our previous results indicating reduced 5-HT levels during ethanol withdrawal in rats (6,8).

Some authors reported that pCPA was ineffective on handling-induced seizures during ethanol withdrawal in mice (14,16,17). In contrast to these authors, we found that pCPA was very effective on audiogenic seizures in ethanol withdrawn rats. It inhibited significantly intensity of the audiogenic seizures. Discrepancies between the studies may be related to different species of animals or methodological applications. On the other hand, it has been suggested that pCPA itself had also anticonvulsant effects in mice (20). Thus, the own anticonvulsant activity of pCPA may also be responsible to its inhibitory effect on audiogenic seizures in the present study. Further detailed studies need for understanding if there is a role of 5-HT in ethanol-withdrawal-induced audiogenic seizures in rats.

Consistent with our previous findings (6,19,21,22) the present data demonstrated that daily ethanol consumption ranged from 11 to 16 g/kg more than 15 consecutive days produced physical dependence in rats. Majchrowicz (23) also showed that dependence and signs of ethanol withdrawal could

be produced in rats with 4-day intragastric administration of 9-15 g/kg of ethanol per day. Thus, we observed several signs of ethanol withdrawal such as locomotor hyperactivity, agitation, tremor, stereotyped behavior, wet dog shakes and audiogenic seizures after withdrawn ethanol from liquid diet.

Serotonergic system seems to be involved in ethanol dependence (1-4). It could be expected to influence the occurrence of mood symptoms in ethanol dependents, especially during withdrawal. Thus, in the present study, increases especially in locomotor activity were observed in rats after depleting 5-HT. In our study, pCPA did not only increase locomotor activity in ethanol dependent rats, but also in naïve animals. On the other hand, there is no statistically significant difference between dependent and non- dependent groups for pCPA-induced increases in locomotor activity. Reason for not showing a more extensive increases in locomotor activities of ethanol-dependent rats may be provided of maximal depletion of central 5-HT after pCPA treatment. However, our results show that there is a clear relationship between 5-HT depletion and locomotor hyperactivity in rats. Extensive locomotor activity may also provide some contributions to increased agitation and wet dog shakes. Dringenberg et al. (24) suggested that pCPA treatment caused some suppression in locomotor activity of rats. Our findings are not in line the results of Dringenberg's study. Using higher doses of pCPA (150-1000 mg/kg) by Dringenberg et al. may be responsible for the discrepancy.

pCPA pretreatment did not affect tremor and stereotyped behavior that appear during ethanol withdrawal. Although this finding implies that these signs of ethanol withdrawal may not be related to 5-HT depletion, we used only one dose of pCPA in our study. More experiments with different doses of pCPA seem to be necessary. However, in the preliminary studies, we observed some toxic effects by higher doses of pCPA especially in ethanol given groups. Thus, we could not use higher doses of pCPA.

The dose of pCPA and its injection protocol were selected according to previous studies. We found that a single dose pretreatment of pCPA (100 mg/kg) blocked significantly tianeptine-induced analgesic effect in mice (25). Some authors observed an increase of irritability and aggression in rodents treated with the higher doses such as 300 mg/kg of pCPA (26,27). We also observed increased agitation, wet dog shakes and locomotor hyperactivity in the ethanol-dependent control rats. These observations imply that pCPA (100 mg/kg) is capable to inhibit the serotonergic activity in rats. Koe and Weissman (9) also found that intraperitoneal injection of pCPA (100 mg/kg) produced marked depletion of brain 5-HT in adult rats.

However, there are some limitations in the present study. First, we tested single dose of pCPA during ethanol withdrawal. Although used dose of pCPA (100 mg/kg) was suitable, testing different doses would help to understand better the role of serotonergic system in ethanol withdrawal syndrome. Secondly, we evaluated early withdrawal period of first six hrs in our study. Additionally investigation of later ethanol withdrawal period after acute or chronic pCPA treatment could give us more detailed information on relationship between serotonergic system and ethanol dependence. Finally, determination of brain serotonin levels before and after pCPA treatment in both ethanol-dependent and non-dependent animals would provide certain evidence to our hypothesis suggesting in the present study.

In conclusion, our results suggest that pCPA intensified locomotor hyperactivity, agitation and wet dog shakes but attenuated audiogenic seizures during ethanol withdrawal in ethanol-dependent rats. These signs of ethanol withdrawal may be related to central serotonergic mechanisms.

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