

NITRIC OXIDE METABOLITES PLASMA LEVEL IN ALCOHOL DEPENDENT MALE PATIENTS DURING SIX-MONTH ABSTINENCE

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ABSTRACT – BACKGROUND: It is suggested, that nitric oxide (NO) may be involved in pathomechanism of alcohol dependence and withdrawal symptoms. THE AIM of this study was to determine the NO metabolites level and its relation to demographic and clinical factors in alcohol dependent patients during six months long abstinence period. **PATIENTS AND METHODS:** We studied 47 alcohol dependent male patients who drank alcohol 14 days at the latest, and 20 non-alcoholic males, who haven't drank alcohol for the last month. In all patients twice, at the study start, and four weeks and six months long abstinence period the plasma NO metabolites level was determined. At the six month visit turned up 18 males, in who NO metabolites determination was made. **RESULTS:** During first four weeks of controlled abstinence alcoholics had lower NO metabolites plasma level than control group. In abstinent alcoholics (n=18) we observed the significant decrease of mean nitrites concentration between 4th week and 6th month of observation period. At each determination NO plasma level in multiple regression correlated significantly mainly with factors defining alcohol drinking and dependence to it. **CONCLUSION:** In alcohol dependent male patients during 6 months long abstinence period the NO metabolites level lower than in control group, what may suggest decrease in NO synthesis or accelerated consumption in this patients group. NO metabolites level in studied alcoholics was related to intensity of alcohol drinking and severity of dependence to it.

Key words: nitric oxide, alcohol dependence, alcohol drinking relapse.

STĘŻENIE METABOLITÓW TLENKU AZOTU U MĘŻCZYŹN UZALEŻNIONYCH OD ALKOHOLU W OKRESIE SZEŚCIOMIESIĘCZNEJ ABSTYNENCJI

STRESZCZENIE – Z danych z piśmiennictwa wynika, że donory tlenku azotu mogą odgrywać rolę w patomechanizmie uzależnienia od alkoholu i przebiegu zespołu abstynencyjnego. Celem pracy była ocena stężenia metabolitów tlenku azotu (NO) w osoczu pacjentów z zespołem zależności alkoholowej (zza) w okresie 6 miesięcy abstynencji. **MATERIAŁ I METODY:** Do badania włączono 47 pacjentów z zza, którzy pili alkohol nie dawniej niż 14 dni przed badaniem oraz 20 niezależnych mężczyzn, którzy nie pili alkoholu w ciągu ostatniego miesiąca i zadeklarowali utrzymanie abstynencji alkoholowej przez następne 4 tygodnie. U każdego trzykrotnie, na początku badania, po 4 tygodniach i 6 miesiącach abstynencji oznaczono m.in. stężenie metabolitów NO. **WYNIKI:** Pacjenci z zza mieli mniejsze stężenia metabolitów NO niż osoby z grupy kontrolnej. U pacjentów z zza, którzy utrzymali abstynencję w ciągu półrocznego okresu obserwacji (n=18) wykazano znamienne zmniejszenie stężenia metabolitów NO między 4 tygodniem i 6 miesiącem obserwacji. W analizie wieloczynnikowej, przeprowadzonej przy wykorzystaniu krokowo postępującej regresji wielokrotnej, wykazano, że stężenie metabolitów NO w każdym z oznaczeń korelowało dodatnio z ilością wypitego uprzednio alkoholu i ujemnie ze wskaźnikami głębokości uzależnienia. **WNIOSEK:** U pacjentów z zespołem zależności alkoholowej w okresie abstynencji stężenie metabolitów tlenku azotu wykazywało związek z intensywnością picia alkoholu w okresie jego nadużywania oraz głębokością uzależnienia, było jednak mniejsze niż u osób zdrowych, z czego można wnioskować o jego mniejszej syntezie lub zwiększonym zużyciu w tej grupie chorych.

INTRODUCTION

Nitric oxide (NO) is produced from the precursor L-arginine in all human body cells, and the greatest concentration is observed in the lung, liver, brain, heart, endothelium, macrophages and platelets. There are known constitutive and inductive forms of nitric oxide synthase (NOS). Among constitutive forms are distinguished neuronal NOS (type I, nNOS) and endothelial NOS (type III, eNOS). Inductive NOS (iNOS) isn't normally present in the cells and requires transcriptional activation by cytokines, bacterial cell wall products or other inflammatory stimuli [41]. Alcohol drinking may also influence the nitric oxide synthesis [2, 14, 19, 47], both via changes in constitutive and inductive NOS activities, as well as both after acute and chronic administration [14, 41], but the investigations results are ambiguous and show both suppressive and stimulating alcohol effect on NO synthesis [41]. Nitric oxide plays a great role in circulatory, digestive, neural and immunological systems function regulation. It takes part in blood pressure control, inhibits mast cells degranulation, posses antioxidant and antiaggregant properties, regulates vascular tone and inhibits both proliferation of smooth muscle cells and adhesion of leukocytes and platelets [32]. It also controls activity and morphological state of digestive tract, respiratory

and urinary systems. In neural system NO acts as neurotransmitter and regulates many functions, both in its central and in peripheral part. Some reports showed, that NO may be also involved in molecular mechanisms for substances abuse and dependence to opioids, ethanol, and psychostimulants, as cocaine, marihuana and nicotine [6, 12, 31, 34, 35, 38, 42, 43] as well as psychotropic drugs [28]. Moreover, nNOS-derived NO participates in the development of rapid tolerance to ethanol [36] and inhibitors of NOS modulate withdrawal from opioids, nicotine and ethanol, diminishing many signs of withdrawal syndrome [42, 50]. That is why modulation of NO systems may be a potential therapeutic target for treatment of substance abuse, and therefore estimation of NO metabolism in alcohol dependent patients seems to be very important. Although changes in NO synthesis after alcohol drinking, both in animals and in human were previously studied, the abstinence effect on this process is poorly understood. Therefore we decided to study NO metabolites plasma level changes in alcohol dependent patients during six month long abstinence and preventing relapse treatment period.

PATIENTS AND METHODS

The investigation was performed in a group of 47 alcohol dependent male patients, diagnosed according to ICD-10 (International Classification of Diseases Tenth Revision) criteria, hospitalized in Addiction Treatment Unit, Department of Psychiatry, The Ludwik Rydygier Medical University in Bydgoszcz (Poland) in 1999 and 2001. Eighty non-alcoholic males, in mean age of $40,7 \pm 7,0$ acted as a control group, who did not drink alcohol at least at least one month before the study. The inclusion criteria of the patients group were: male sex, age between 30-50 years, performance of alcohol dependence ICD-10 criteria, abstinence keeping motivation and alcohol abuse period not formerly than 14 days before the study start. The exclusion criteria were: acute and chronic inflammatory processes symptoms, the presence of the other diseases, which could have an influence on the NO metabolism, psychoses, dementia, addiction to other substances than alcohol and nicotine, and any drugs taking. In all alcohol dependent patients the demographic and clinical data were assessed. Quantity of alcohol drunk during 90 and 30 days before the study start was determined using WHO Timeline/IDS study and in standard drinks counted (1 standard drink = 13,6g=1oz of pure ethanol). The severity of alcohol dependence using Short Alcohol Dependence Data (SADD) [24] and Polish version of Michigan Alcohol Screening Test (MAST) [15], as well as presence of delirium tremens and withdrawal epilepsy in anamnesis was assessed. The mean age of patients was $40,8 \pm 8$ years, mean duration of alcohol dependence was $17,7 \pm 7$ years, mean age of alcohol dependence onset was $22,2 \pm 6,4$ years, mean score in Michigan Alcoholism Screening Test (MAST) was $44,9 \pm 21,5$ and mean score in Short Alcohol Dependence Data (SADD) was $26,2 \pm 7,6$. For 90 days before admission to the hospital patients have drunk on the average for $50,9 \pm 25,6$ days, 952 ± 670 standard drinks (1 drink=1 oz of pure ethanol, on average 142,8 of pure ethanol per day), and

for 30 days before the study start – $259 \pm 176,6$ standard drinks (116,6g of pure ethanol per day). All patients were smokers both before and during the study. During observation period studied persons didn't take any drugs. They were treated with training to cope with alcohol craving.

Blood sampling for all determinations was made at the study beginning and after four weeks and 6 months of the observation. Because of ethical cause, in control group blood samples were taken only at the study start and after four weeks of abstinence. Biochemical markers were determined using standard laboratory methods. The blood samples for detailed measurements were refrigerated in -80 Celsius grads until determination was made. The nitric oxide production was estimated on the basis of averaged, twice plasma nitrite concentration determination using colorimetric method and Nitric Oxide Colorimetric Assay manufactured by Boehringer Mannheim, according to instructions of a set producer. Using this assay the total nitrite level as an indicator of nitric oxide production in biological samples is determined. Because nitric oxide in human body is rapidly converted to nitrite and nitrate, this assay provides for enzymatic reduction of nitrate by nitrate reductase. Next, using Greiss reagent, in acid solution, nitrite is converted to nitrous acid with diazotizes sulfanilamide. This sulfanilamide-diazonium salt is then reacted with N-ethylenediamide to produce a chromofore which is measured spectrophotometric at 540 nm. Normal range of plasma nitrites level according to set producer was 0,4-100mcmol/l.

Abstinence keeping was controlled during four weeks long hospitalization period on the basis of physical examination as well as alcohol presence in exhaled air and the below mentioned biochemical markers of alcohol abuse level. After discharge from the Addiction Treatment Unit alcohol drinking was diagnosed on the basis of interview, the level of biochemical markers of alcohol abuse (mean corpuscular volume, HDL cholesterol concentration, gamma-glutamyltransferase – GGT, aspartate aminotransferase – AST, alanine aminotransferase – ALT) determined during control visits, objective familial interview, and medical documentation analysis (from outpatients clinic). For the second visit, after four weeks of observation turned up all studied subjects, and for the third visit, after 6 months came 27 (54%) alcoholics, but only 18 (38%) remained abstinent during this period.

All subjects gave their informed consent to participate in this study, which was approved by the Local Ethics Committee of The Ludwik Rydygier Medical University in Bydgoszcz. The investigation was in compliance with the Declaration of Helsinki for medical research.

The results were presented as the mean \pm standard deviation (SD). Normal distribution of variables using W Shapiro-Wilk test was assessed. Statistical significance of differences was determined using respectively paired and unpaired t-Student test, one- and two-factorial ANOVA with two and three repetitions and least significantly difference Turkey post hoc test as well as logistic regression, and stepwise progressing method of multiple regression in statistical software STATISTICA PL 5.0.

RESULTS

At the study start (during first two weeks of abstinence duration) alcohol dependent patients had borderline lower NO metabolites plasma level than control group, and this trend was observed also after four weeks of abstinence period (Table 1). However, the significant main effect ($F=6.55$, $p<0.013$) of alcohol abuse (in comparison to the control group) on the changes in NO metabolites levels during first four weeks of the observation we found using ANOVA method with two repetitions. The mean nitrites level from two determinations during the first four weeks of observation was lower in alcoholics than in control group (31.9 ± 13.5 vs. 44.8 ± 22.4 mcmol/l; $p=0.013$). After the next observation period, between fourth week and sixth month, in abstinent alcoholics we observed the significant decrease of mean nitrites concentration ($p=0.01$), which caused significantly lower NO metabolites concentration in abstinent patients after six months abstinence than in control group at the study start ($p=0.036$) and after four weeks observation ($p=0.035$) (Table 1).

TABLE 1

Nitric oxide metabolites concentration (mcmol/l) in abstinent alcoholics and in control group in respective determinations. In (/ /) number of subjects in determinations at the study start/ after 4 weeks / after six months of abstinence was presented.

	Mean±SD			p=		
	Baseline (0)	After 4 weeks (4)	After 6 months (6)	0 : 4	0 : 6	4 : 6
Alcoholics (47/47/18)	30.7±17.6	33.1±17.3	26.2±11.8	0.48	0.10	0.01
Control group (18/18)	41.9±28.5#	44.1±32.4*	** - in relation to 0 and 4 level	0.83		

ANOVA, Turkey post hoc test, statistical significance of differences between alcoholics and control group- # – $p=0.07$, * - $p=0.11$; ** – $p<0.05$

Observed gradual decrease of mean nitrites level in alcoholics during abstinence period (Table 1), suggested ethanol influence on NO synthesis. Because of this, using step wise, progressing method of multiple regression, we tested whether alcohol drinking is the only one factor affecting NO synthesis. As independent variables we taken factors potentially affecting endothelial function, as well as NO synthesis and metabolism: age, daily nicotine dose (enumerated as the product of number of daily smoked cigarettes and mean nicotine content in one cigarette), daily tar dose (enumerated as the product of number of daily smoked cigarettes and mean tar content in one cigarette), SADD score, MAST score, number of drinking days during 90 days before the study start, number of standard drinks drunk during 90 and 30 days before the study start, family history of alcoholism, age of alcohol dependence onset, length of alcohol dependence, decrease in alcohol tolerance, history of delirium tremens, withdrawal epilepsy in anamnesis, body mass index (BMI), waist to hip ratio (WHR), tissue type plasminogen activator antigen (t-PA:Ag) to plasminogen activator inhibitor type 1 antigen (PAI-1:Ag) concentrations ratio value (t-PA:Ag/PAI-1:Ag, as a marker of endothelial function), LDL cholesterol

TABLE 2
Demographic and clinical features of studied alcohol dependent patients and control group at the study start.

Feature	Alcoholics (n=47)	Control group (n=18)	p=
Age (years)	40.8±8.0	40.78±7.0	0.95
SADD (score)	26.2±7.6	0.9±0.3	0.0001
MAST (score)	44.9±21.5	0.8±0.4	0.001
Age of alcohol dependence onset (years)	22.2±6.4	0	0.0001
Length of alcohol dependence (years)	17.7±7.0	0	0.0001
Number of drinking days during 90 days before the study start	50.9±25.6	9.1±6.8	0.0001
Number of standard drinks drunk during 90 days before the study start (drinks)	952.0±670.5 142.8g of pure ethanol	17.3±12.9 2.6g of pure ethanol	0.0001
Number of standard drinks drunk during 30 days before the study start	259.1±176.6 116.6g of pure ethanol	0	0.0001
Family history of alcoholism (n. %)	34 (64%)	1 (5%)	0.01
Delirium tremens in anamnesis (n. %)	5 (9%)	0	0.19
Withdrawal epilepsy in anamnesis (n. %)	5 (9%)	0	0.19
Decrease in alcohol tolerance (n. %)	11 (23%)	0	0.029
Smoking (n. %)	45 (96%)	5 (25%)	0.0001
Mean daily nicotine dose (mg/d)-in smokers	28.6±13.1	15.6±9.2	0.039
Mean daily tar dose (mg/d)- in smokers	337.0±144.0	184.0±113.5	0.03
Systolic blood pressure (mmHg)	114.3±14.0	129.7±18.7	0.001
Diastolic blood pressure (mmHg)	75.3±8.9	82.0±8.4	0.012
BMI (kg/m ²)	25.0±3.0	27.7±3.7	0.002
WHR	0.97±0.05	0.96±0.07	0.72

Abbreviations: SADD- Short Alcohol Dependence data, MAST- Michigan Alcoholism Screening Test, BMI- body mass index, WHR- waist to hip ratio, unpaired Student t-test.

TABLE 3a
Parameters of multiple regression equations (corrected determination and b coefficients, and p values) and variables, which entered into equation, for NO metabolites level determinations at the study start. (corrected R² = 0.97; p=0.006).

Variable	Beta coefficient	p=
BMI	-0.79	0.003
Withdrawal seizure	-0.16	0.001
GGT activity	0.89	0.002
Age of dependence onset	-0.18	0.002
LDL cholesterol	-0.9	0.002
SADD	-0.38	0.004
Family history of alcoholism	-0.26	0.005
Decrease in alcohol tolerance	-0.47	0.003
t-PA/PAI-1	-0.58	0.004
Number of drinks consumed during 30 days before the study start	0.28	0.007
Number of drinks consumed during 90 days before the study start	0.26	0.008
WHR	-0.37	0.007
MAST	-0.17	0.009
Length of alcohol dependence	-0.16	0.012

TABLE 3b

Parameters of multiple regression equations (corrected determination and b coefficients, and p values) and variables, which entered into equation, for NO metabolites level determinations after four weeks abstinence period. (corrected R² = 0.96; p=0.0021).

Variable	Beta coefficient	p=
WHR	-1.4	0.001
Number of drinking days during 90 days before the study start	1.1	0.001
Abstinence maintenance for 6 months	0.43	0.008
Family history of alcoholism	-0.56	0.006
SADD score	-1.2	0.001
LDL cholesterol concentration	-0.88	0.003
Age of alcohol dependence onset	0.023	0.07
t-PA/PAI-1	0.32	0.03
Withdrawal epilepsy	-0.5	0.002
Decrease in alcohol tolerance	-0.35	0.058

Abbreviations used in table 3ac: BMI – body mass index, GGT – gamma glutamyltransferase, LDL – LDL cholesterol, SADD – short alcohol dependence data; t-PA – tissue type plasminogen activator; PAI-1 – plasminogen activator inhibitor type 1; WHR – waist to hip ratio

TABLE 3c

Parameters of multiple regression equations (corrected determination and b coefficients, and p values) and variables, which entered into equation, for NO metabolites level determinations at six months visit. (corrected R² = 0.88; p=0.0004).

Variable	Beta coefficient	P=
abstinence maintenance for 6 months	-0.87	0.001
Length of alcohol dependence	-0.85	0.0003
Delirium tremens in anamnesis	0.43	0.008
Family history of alcoholism	-0.8	0.004
Daily tar dose	0.49	0.004
Decrease in alcohol tolerance	0.15	0.24
Onset of alcohol dependence	-0.15	0.32

concentration, GTP activity. Drinking relapse before the 6 month visit was also included as an independent variable in NO metabolites determinations analysis.

We found significant regression equations for all three NO metabolites measurements (Tab. 3). All these equations had high determination coefficients value, what showed, that almost all NO metabolites concentration variance could be accounted for included independent variables influence. Detailed data are presented in the table 2a-c. Nitrites plasma level was related to the quantity of alcohol drunk only at the study start, but at the each determination was related to the parameters of dependence severity.

DISCUSSION

In this investigation we studied changes in NO metabolites plasma level in alcohol dependent males during six months long abstinence period. We found that alcoholics during first four weeks of abstinence had lower NO metabolites plasma level than control group (Tab.1, ANOVA). Moreover we observed, that in alcoholics NO

level at respective determinations within six months long abstinence period, besides individual features and factors potentially affecting endothelium function, was in multi- factorial analysis positive related to values of parameters defining alcohol drinking intensity and negative to alcohol dependence severity, as withdrawal epilepsy, decrease in alcohol tolerance, SADD score, length of alcohol dependence, age of onset of alcohol dependence and so one (Tab. 3a-c). These observations have some clinical implications.

Firstly, gradual decrease of NO metabolites level with prolongation of abstinence period as well as positive correlations between NO metabolites concentration after alcohol abuse period and number of standard drinks consumed during 30 and 90 days before the study start suggested stimulating effect of alcohol drinking on NO synthesis. Stimulatory ethanol effect on NO synthesis is consistent with some other reports results [2, 41, 47]. Banan et al. [7] showed stimulatory ethanol effect on inducible NOS activity and NO synthesis. Enomoto et al. [14] in rats model found, that alcohol drinking may influence bacterial lipopolysaccharide (LPS)- induced NO synthesis by isolated Kupffer cells, and observed inhibitory effect two hours after ethanol application and stimulatory effect, when ethanol was given 24 hours before the measurement. Nevertheless the other authors obtained opposite results [2, 41]. Potentially endothelial origin of this NO can be indirectly suggested by simultaneous with NO metabolites level increase, the rise of t-PA:Ag and PAI-1:Ag (endothelial function markers) plasma concentration in relapsed patients (data not presented), and by the results of multiple regression analysis, which showed, that t-PA/PAI-1 ratio independently correlated with NO metabolites plasma level in the first two determinations (Tab.3). It is known, that NO, prostacyclin and t-PA synthesis are mutually related [37].

Secondly, it may be too, that early alcohol withdrawal period also may stimulate the NO synthesis. It was observed, that alcohol drinking cessation may stimulate nitric oxide production in brain sites of action involved in the expression of withdrawal syndrome signs, although not all its symptoms occurrence results from nitric oxide action [2] Treatment with a nitric oxide donor (L-arginine) inhibited the anaesthetic effect of alcohol, blocked the effect of the NOS inhibitor on alcohol induced anaesthesia and enhanced the severity of some alcohol withdrawal signs [1, 2, 30, 50]. On the other hand administration of NOS inhibitors alone, or in the combination with clonidine decreased intensity of opioid withdrawal syndrome signs, which have similar pathomechanism to alcohol withdrawal syndrome [2, 23, 31]. While, the results of Uzbay and Erden [44] study showed, that L-arginine (NO precursor) at high doses alleviates the signs of ethanol withdrawal syndrome in rats.

Thirdly, multi- factorial analysis results (Tab.3a-c) suggested involvement of NO in alcohol dependence pathogenesis. Spanagel's et al. [39] study also showed that nonselective NOS inhibitors decreased alcohol consumption in mice, although their effect was not mediated by neuronal NOS, but by peripheral NOS isoforms. This suggested, that nNOS gene is critically involved in the regulation of alcohol drinking behaviour.

Fourthly, lower nitrites level in alcoholics than in control group might result from endothelium dysfunction, or decreased NOS reaction on stimuli [14], or NO consumption in free radicals reactions with peroxynitrites (ONOO-) overproduction [7], or limitations in NO synthesis substrates (L-arginine) availability. Endothelial dysfunction in our patients potentially might be induced by inflammatory factors (cytokines, endotoxins) [26], by oxidized LDL [21, 52], or by peroxynitrites [4, 52], potentially formed during NO reaction with free radicals overproduced during ethanol metabolism [52] or smoking [24]. Endothelial dysfunction induced by alcohol misuse was reported by Sun and Mayhan [40], who found significant impairment in reactivity of arterioles to acetylcholine and ADP, but not nitroglycerin, in rats fed the alcohol diet for longer than three weeks period. Because in this study therapy with antioxidants significantly improved the impaired nitric oxide synthase-dependent dilatation of pial arterioles, authors concluded that impaired NOS-dependent cerebral vasodilatation during chronic alcohol consumption may be related, in part, to enhanced release of oxygen-derived free radicals, reported by other author too [52]. Endothelial dysfunction, expressed in our study by decreased NO availability, may be a potential factor responsible for increased cardiovascular events risk in heavy drinkers, known from epidemiological study results (the right arm of J-shaped curve illustrating the relationships between coronary artery disease prevalence and quantity of alcohol drinking) [3, 13]. This suggestion arose from hypothesis, that NO production stimulation is a potential factor mediating favourable effect of regular moderate alcohol drinking [13, 49].

Above considerations suggest, that NO synthesis blockade may become a promising treatment method of alcohol withdrawal syndrome and probably could prevent alcohol drinking relapse. But on the other hand, this therapy direction may have potentially harmful effects, because NO plays an important role in the human body function. Among the other things, NO regulates endothelium and platelets function, blood pressure and in this way is involved in atherosclerosis pathogenesis and protective effects of moderate alcohol drinking [3, 13, 18, 37, 49]. Because of this, it was planned the interventional investigations estimating the effect of NO-donors (L-arginine) on endothelium function and cardiovascular events risk [9, 11]. Whereas, the recent prospective cohort study in eastern Finland middle-aged men, who were free of prior coronary artery disease didn't show significant associations between dietary arginine intake and the risk of acute coronary events [46]. Even then, theoretically it seems, that selective nNOS inhibitors would be more safe.

Our study has some limitations. Nitric oxide is a very labile substance, which quickly transforms into metabolites, such as nitrites and nitrates. Using modern methods, the nitric oxide synthesis may be assayed via (1) direct measurement of nitric oxide concentration or NOS protein expression, for example in respective parts of rats brain [17, 35, 36], in exhaled air [51], in isolated human placental villous tissue [25]; (2) determination of its metabolites from plasma and urine, and (3) functional measurement of vascular NO-dependent responses [45]. In our study we estimated NO synthesis only via its metabolites serum level determinations, however this source seems to us to be better in even-

tually clinical application. Moreover, in our study we didn't estimate carbohydrate deficient transferin (CDT) concentration as a marker of abstinence keeping, because in Poland this parameter determination availability is very limited.

In conclusion, (1) during first four weeks of abstinence period alcohol dependent males had lower mean NO metabolites level than control group and their level decreased within 6 months long abstinence period; (2) the NO metabolites level in studied alcohol dependent male patients, among other things, was positive related to quantity of alcohol drinking during abuse period and negative related to values of parameters showing alcohol dependence severity.

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