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Exposure to alcohol and tobacco smoke causes oxidative stress in rats

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Abstract:
Background: Tobacco smoking and alcohol abuse causes oxidative stress in humans and underlay numerous chronic degenerative diseases. Liver is the main organ exposed to alcohol toxic metabolites, whereas tobacco smoke is chiefly harmful to the lungs.
Methods: The aim of the current study was the assessment and comparison of selected oxidative stress markers, reduced glutatione (GSH), glutathione S-transferase (GST), superoxide dismutase (SOD), catalase, nitrites and protein nitrosylation and DNA damage in the livers and in the lungs of alcohol-addicted rats exposed to tobacco smoke alone or in combination with a single dose of ethanol.
Results: The highest levels of GSH were measured in the liver of smoke only exposed animals and in the lungs of rats exposed to smoke and alcohol. In the liver of animals treated with a single dose of alcohol or with smoke and alcohol, GST was significantly higher than in the group exposed to smoke only. SOD and catalase showed the highest activities in the livers of rats receiving a single dose of alcohol. High concentration of nitrites was observed in the lungs of animals treated with smoke and alcohol in combination, which corresponded to elevated protein nitrosylation in this group, whereas in the livers of these animals relatively low level of nitrites was accompanied with the lowest concentration of nitrosylated proteins. In the liver of alcohol only treated rats the highest nitrites corresponded to the highest protein nitrosylation. In the lungs of all treatment groups the range of DNA damage was higher, than the respective values in the livers. Although alcohol is not considered a specific toxicant to the lungs it was found to cause oxidative stress in this organ.
Conclusions: The obtained results suggest that in the ethanol-addicted rats combined exposure to smoke and alcohol differentially modulate endogenous antioxidant defense system and reactions to oxidative stress.

Key words:
tobacco smoke, alcohol, oxidative stress, rat

Abbreviations: CYP 2E1 – cytochrome P450 izozyme 2E1, GSH – reduced glutathione, GST – glutathione S-transferase, NAD – nicotinamide adenine dinucleotide, NADH – nicotinamide adenine dinucleotide – reduced form, ROS – reactive oxygen species, RNS – reactive nitrogen species, SOD – superoxide dismutase

Introduction

Tobacco smoking and alcohol abuse causes a serious threat to human health. Most often individuals addicted to alcohol are also tobacco smokers. Addiction to alcohol and tobacco may develop through similar mechanisms, moreover, stress, increased tolerance related with the adaptation of the central nervous system to the presence of alcohol [8] and sensitivity to these xenobiotics may enhance addiction [16, 24]. Alcohol is metabolized mainly in the liver, where it undergoes oxidation through the alcohol dehydrogenase [28] and aldehyde dehydrogenase activities. These enzymes require nicotinamide adenine dinucleotide (NAD) as a cofactor and as a result of the reaction a cellular pool of the reduced form, nicotinamide adenine dinucleotide-reduced form (NADH), becomes elevated, which impairs the redox balance. Products of these reactions, acetaldehyde and acetic acid, respectively, are directly responsible for the alcohol toxicity. Ethanol oxidation occurs also in the subcellular fraction of peroxisomes and microsomes, chiefly due to activity of CYP 2E1 isoform of cytochrome P450. Chronic alcohol consumption induces strongly this izoform, which results in generation of reactive oxygen species (ROS) and alcohol-derived radicals, moreover, an increase in CYP 2E1 activity enhances metabolic transformation of numerous xenobiotics into more toxic products [6, 7, 20]. Additionally, chronic alcohol abuse changes morphology of mitochondria membranes, which, together with the NADH increase, raises the electron flow through the complex 1 of respiratory chain and ROS formation [46]. Finally, in the liver, alcohol activates Kupffer cells, capable of pro-inflammatory cytokines induction and ROS and reactive nitrogen species (RNS) generation [44]. In the liver, a common consequence of chronic alcohol abuse is inflammation, cirrhosis or cancer. Despite numerous social drawbacks, alcohol abuse has impact on drinker's individual health and/or life, whereas tobacco smoke is equally toxic to active and passive smokers. Tobacco smoking induces strong addiction and smoke components affect circulation in all tissues and organs causing elevated risk of atherosclerosis, hypertension, chronic obturative pulmonary disease and certain forms of cancer [41, 47]. Tobacco smoke itself is a source of free radicals, moreover, inhalation of particles activates the lung epithelium and fibroblasts and enhances recruitment of inflammatory cells, macrophages and neutrophils to the lung tissue. These cells undergo activation resulting in ROS and RNS synthesis and release and in upregulation of factors involved in inflammation and fibrosis [27]. The deleterious effects of tobacco smoke is observed also in non-smokers, yet exposed to smoke as a contaminant of environment and thus, to protect community health, numerous countries introduce a ban on smoking in public space [29].

A common effect of joint exposure to tobacco smoke and alcohol is oxidative stress, resulting in damage to proteins, polysaccharides, lipids and DNA, leading to the enhancement of pro-inflammatory reactions, accelerated ageing, immunity disturbances and disease development. Smokers abusing alcohol are more prone to develop various cancers [35]. Endogenous antioxidant defense system tends to overcome these unfavorable changes through the enhancement of enzymatic (e.g., catalase, superoxide dismutase (SOD), glutathione peroxidase) and nonezymatic (e.g., albumin, uric acid, reduced glutathione) mechanisms. In the case of insufficiency or depletion of these factors an oxidative stress occurs which forms the basis for numerous pathologies development [45].

The aim of the current study was the assessment and comparison of selected oxidative stress markers, reduced glutathione (GSH), glutathione S-transferase (GST), SOD, catalase, nitrites and protein nitrosylation and DNA damage in the livers and in the lungs of alcohol-addicted rats exposed to tobacco smoke alone or in combination with a single dose of ethanol.

Materials and Methods

All the experiments were conducted according to the Regional Ethics Committee guidelines for animal experimentation, No. of agreement 2/2008, 18th January 2008.

Animals

Male Wistar rats (240 ± 20 g) from the Department of Toxicology, University of Medical Sciences, Poznań, Poland were housed in polycarbonate cages with hardwood chip bedding. A standard laboratory rat chow and water were available with no limitations and a 12/12 h light/dark cycle was maintained over the study period.

Addiction of rats to ethanol was performed in 9 weeks according to the previously established protocol [30]. Rats were considered addicted if their daily ethanol consumption reached 10–37% of the total liquid uptake (mean 10.5 g of ethanol/kg/day) [3, 39]. Alcohol-addicted animals were divided into groups containing 6 rats:

I – rats exposed to tobacco smoke;

II – rats which obtained a single *po* dose of 10% ethanol (2 g/kg b.w.);

III – rats exposed to tobacco smoke which then obtained a single *po* dose of 10% ethanol (2 g/kg b.w.) immediately after exposition to tobacco smoke.

Tobacco smoke exposure

Rats were placed in a dynamic toxicological chamber immediately after being pointed as addicted [10] and exposed to the tobacco smoke generated from a Polish brand of cigarettes without a filter tip. The concentration of carbon monoxide in the chamber reflected the smoke content in the inhaled air and was continuously monitored to maintain 1,500 mg CO/m^3 of air, during 5 days, 6 hours a day. Oxygen level was maintained at 20 ± 0.5% of the air volume. The air in the chamber was exchanged 10 times per hour. In the chamber temperature and humidity were, respectively, 20–22°C and 50–60%.

Sample collection

Five hours after exposition to tobacco smoke has completed and 5 h after ethanol administration rats were killed (xylazine and ketamine, 40 mg/kg b.w. and 5 mg/kg b.w., respectively). Livers and lungs were dissected, homogenized and frozen in –80°C. In a portion of a fresh tissue homogenate comet assay for DNA damage evaluation was performed.

Chemicals

Bovine serum albumin, GSH, 1-chloro-2,4-dinitrobenzene, 5,5-dithio-bis-(2-nitrobenzoic acid), H_2O_2, sulfanilamide, TRIS, *N*-(1-naphthyl)ethylenediamine dihydrochloride, ammonium sulfamate, mercury chloride, 2-(morpholino)ethanesulfonic acid, low melting point agarose, ethidium bromide and Folin-Ciocalteu reagent were purchased from Sigma (St. Louis, USA). Normal melting point agarose was from Prona, Spain. Triton X-100 was purchased from Park Scientific, (Northampton, UK). All other chemicals were commercial products of the highest purity available from local suppliers.

Biochemical determinations

Cotinine Direct ELISA kit (BioQuant, San Diego, USA) was applied for cotinine in rat urine measurement. Protein concentrations in tissue homogenates were determined by the method of Lowry et al. [25] using bovine serum albumin as standard. GSH was assayed colorimetrically with 5,5-dithio-bis-2-nitrobenzoic acid according to the method described by Ellman [9]. RANSOD test (Randox Laboratories Ltd., Crumlin, UK) was applied for SOD activity measurement. Catalase activity was determined by spectrophotometrically followed decomposition of H_2O_2, as described by Beers and Sizer [2]. GST activity was measured in the reaction of glutathione with 1-chloro-2,4-dinitrobenzene, according to the protocol published by Habig et al. [12]. Protein S-nitrosylation was measured colorimetrically with *N*-(1-naphthyl)-ethylenediamine and sulfanilamide according to the procedure described by Bonina et al. [4]. Concentration of nitrites was determined by the method of Griess modified by Kleinbongard et al. [22]. Single cell gel electrophoresis (comet assay) in alkaline conditions (pH > 13) was performed in liver and lung homogenates according to the method presented by Hartmann et al. [13]. Samples were embedded in the LMP agarose and then submitted to the procedures of cell lysis, DNA unwinding, electrophoresis and neutralization and then were dehydrated in the absolute ethanol, dried and stored at room temperature, protected from light. Just before microscopic evaluation, the slides were rehydrated and stained with ethidium bromide (0.05 mg/ml). Images of comets were captured with a digital camera. The comets were divided into 5 groups according to the degree of the DNA damage [5]. A total damage score for each sample on

the slide was calculated by multiplying the number of cells classified to each grade of damage by the numeric value of the grade and summing over all grades. The results obtained in the arbitrary point units were expressed as the percentage of the values obtained in the control group.

Statistical analysis

The statistical analysis was performed by one-way ANOVA. The statistical significance between the experimental groups was assessed by Student *t*-test with $p < 0.05$.

Results

Cotinine (nicotine metabolite) was found in urine of tobacco smoke-exposed animals, 351.1 ± 76.76 ng/ml (group I) and 313.17 ± 31.9 ng/ml (group III), respectively. In alcohol-exposed rats only trace amounts of cotinine were determined.

Table 1 shows results of GSH concentration and GST activity measurements. In the liver of alcohol-exposed animals (group II) GSH was significantly lower than in tobacco smoke-exposed (group I) and alcohol and tobacco smoke-exposed (group III) animals. In the lung of tobacco smoke-exposed rats, GSH reached its lowest level, exposure to alcohol or tobacco smoke and alcohol gave significantly higher values. In the liver of animals in group I, GST was markedly diminished when compared to activities measured in this tissue of animals from groups II and III. The highest level of GST activity was found in rats exposed to alcohol only, however, the differences from results obtained in other groups was not significant.

Activities of SOD and catalase are presented in Table 2. SOD in the liver of the tobacco smoke only exposed rats was significantly lower than the results obtained from alcohol only-treated animals or tobacco smoke and then alcohol-treated rats. The liver catalase

Tab. 1. Results of GSH concentration and GST activity measurements

Experimental group (n = 6 [d])	Reduced glutathione (GSH)[a]			Glutathione S-transferase (GST)[b]		
	I	II	III	I	II	III
Liver	25.08 ± 4.612[c]	7.36 ± 0.713[e]	11.76 ± 2.660[e]	85.00 ± 16.484	135.20 ± 59.906	136.71 ± 48.259
Lungs	10.04 ± 3.777	19.58 ± 4.355[f, g]	39.85 ± 12.34[f]	38.32 ± 14.151	54.99 ± 23.761	41.15 ± 24.880

I – rats exposed to tobacco smoke for 5 days; II – rats which obtained a single *po* dose of 10% ethanol (2 g/kg b.w.); III – rats exposed to tobacco smoke for 5 days which then obtained a single *po* dose of 10% ethanol (2 g/kg b.w.); [a] concentration, mmol/mg protein; [b] activity, U/mg protein; [c] values are the means ± SD of measurements run in duplicate; [d] sample size; [e] significantly different from group I – $p < 0.001$ (ANOVA – $p < 0.0001$; degrees of freedom = 2.15; F values = 53.106); [f] significantly different from group I – $p < 0.001$ (ANOVA – $p < 0.0001$; degrees of freedom = 2.15; F values = 22.487); [g] significantly different from group III – $p < 0.01$ (ANOVA – $p < 0.0001$; degrees of freedom = 2.15; F values = 22.487)

Tab. 2. Activities of SOD and catalase

Experimental group (n = 6 [c])	Superoxide dismutase (SOD)[a]			Catalase[a]		
	I	II	III	I	II	III
Liver	11.15 ± 1.663[b]	18.35 ± 1.472[d]	17.06 ± 1.859[d]	33.84 ± 9.945	96.04 ± 19.955[e, f]	20.20 ± 9.611
Lungs	10.37 ± 4.801	8.29 ± 1.835	6.94 ± 0.703	29.51 ± 8.140	32.60 ± 12.769	41.15 ± 24.880

I – rats exposed to tobacco smoke for 5 days; II – rats which obtained a single *po* dose of 10% ethanol (2 g/kg b.w.); III – rats exposed to tobacco smoke for 5 days which then obtained a single *po* dose of 10% ethanol (2 g/kg b.w.); [a] SOD and catalase activities: U/mg protein; [b] Values are the means ± SD of measurements run in duplicate; [c] sample size; [d] significantly different from group I – $p < 0.001$ (ANOVA – $p < 0.0001$; degrees of freedom = 2.15; F values = 31.627); [e] significantly different from group I – $p < 0.001$ (ANOVA – $p < 0.0001$; degrees of freedom = 2.15; F values = 49.908); [f] significantly different from group III – $p < 0.001$ (ANOVA – $p < 0.0001$; degrees of freedom = 2.15; F values = 49.908)

Tab. 3. Levels of nitrite and nitrosylated proteins

Experimental group (n = 6[c])	Nitrites[a]			Nitrosylated proteins[a]		
	I	II	III	I	II	III
Liver	1.61 ± 0.268[b]	2.320 ± 0.413[d]	1.998 ± 0.332	643.2 ± 220.45	734.8 ± 128.73[g]	257.0 ± 60.12[f]
Lungs	1.21 ± 0.217	2.85 ± 0.654[e]	2.82 ± 0.706[e]	369.5 ± 70.85	314.3 ± 45.79[i]	588.0 ± 93.77[h]

I – rats exposed to tobacco smoke for 5 days; II – rats which obtained a single *po* dose of 10% ethanol (2 g/kg b.w.); III – rats exposed to tobacco smoke for 5 days which then obtained a single *po* dose of 10% ethanol (2 g/kg b.w.); [a] nitrites and nitrosylated proteins concentrations: nmol/mg protein; [b] values are the means ± SD; [c] sample size; [d] significantly different from group I – $p < 0.01$ (ANOVA – $p < 0.0095$; degrees of freedom = 2.15; F values = 6.452); [e] significantly different from group I – $p < 0.001$ (ANOVA – $p < 0.0002$; degrees of freedom = 2.15; F values = 16.284); [f] significantly different from group I – $p < 0.01$ (ANOVA – $p < 0.0001$; degrees of freedom = 2.15; F values = 16.828); [g] significantly different from group III – $p < 0.001$ (ANOVA – $p < 0.0001$; degrees of freedom = 2.15; F values = 16.828); [h] significantly different from group I – $p < 0.001$ (ANOVA – $p < 0.0095$; degrees of freedom = 2.15; F values = 23.703); [i] significantly different from group III – $p < 0.001$ (ANOVA – $p < 0.0002$; degrees of freedom = 2.15; F values = 23.703)

Tab. 4. Results from comet assay

Experimental group (n = 6[c])	DNA damage[a]		
	I	II	III
Liver	92.5 ± 6.950[b]	88.3 ± 4.633[e]	97.2 ± 2.787
Lungs	99.3 ± 4.179	96.3 ± 1.862[f, g]	101.8 ± 1.941[g]

I – rats exposed to tobacco smoke for 5 days; II – rats which obtained a single *po* dose of 10% ethanol (2 g/kg b.w.); III – rats exposed to tobacco smoke for 5 days which then obtained a single *po* dose of 10% ethanol (2 g/kg b.w.); [a] arbitrary points; [b] values are means ± SD of measurements run in duplicate; [d] sample size; [e] significantly different from group III – $p < 0.05$ (ANOVA – $p < 0.0276$; degrees of freedom = 2.15; F values = 4.602); [f] significantly different from group III – $p < 0.05$ (ANOVA – $p < 0.0159$; degrees of freedom = 2.15; F values = 5.527); [g] significantly different from values in the liver ($p < 0.05$)

was markedly elevated in rats receiving only alcohol, in comparison with the mean values from other groups. In the lungs, there were no differences in the tissue SOD and catalase activities in all 3 experimental groups. In the liver and lung of animals exposed to alcohol, only the highest levels of nitrite were found (Tab. 3), whereas in the tissues of animals inhaling tobacco smoke only the respective nitrite levels were significantly lower. Nitrosylated proteins were markedly diminished in the liver of rats exposed to tobacco smoke and then treated with alcohol, whereas in the lung this parameter was significantly elevated. Results from comet assay are presented in Table 4. In the liver and as well in the lung, joint exposure to alcohol and tobacco smoke gave higher rates of DNA damage than those achieved upon exposure to single toxicants. Comparison of values obtained in both tissues brought about significantly greater DNA damage response from lungs in rats exposed to alcohol ($p = 0.0076$) or alcohol and tobacco smoke ($p = 0.0078$).

Discussion

Alcohol combined exposure to tobacco smoke and alcohol raises difficulties in elucidation of their interaction. Nicotine delays gastric emptying, moreover, decomposition of alcohol begins in the stomach upon action of the alcohol dehydrogenase, thus diminishing alcohol level in blood [36]. addiction affects metabolism of a range of centrally acting drugs and toxins, which may be responsible for diverse changes in response oxidative insult [31].

The observed alterations of antioxidant defense elements (GSH, GST, SOD and catalase) as well as parameters representing result of xenobiotic insult in tissues showed diverse organ reactivity to alcohol or tobacco smoke alone or in combination. The effects of a single toxicant overlap when tobacco smoke and alcohol exposure were commonly applied.

Reduced glutathione in the liver was markedly decreased in animal groups treated with alcohol (group II) and jointly treated with tobacco smoke and alcohol (group III) in comparison to the value measured in rats exposed to tobacco smoke only (group I). As suggested by Nordmann, this strong depletion may result from decreased hepatic GSH synthesis [34]. Diminishment of hepatic and lung GSH content upon chronic alcohol ingestion was reported by Husain et

al. [18]. GSH participates in reaction catalyzed by glutathione S-transferase and in our study, in both alcohol treated groups of rats, the enzyme activity was significantly elevated, which may also add to the GSH decrease in the liver. However, in the lung the lowest GSH level was found in the tobacco smoke only exposed animals. Lung tissue is rich in antioxidants which form effective antioxidant barrier against environmental ROS and those generated endogenously by inhaled toxicants. In our previously published report on antioxidant status in rats chronically exposed to tobacco smoke, we found increased Trolox Equivalent Antioxidant Capacity in lungs with concomitant decrease in the liver, kidney and brain. This parameter depends on GSH and other small molecular antioxidants and the observed level alterations may reflect the lung adaptation to tobacco smoke-induced stress [11]. Rahman and MacNee stated that a decrease in GSH in lungs occurs upon acute inhalation of tobacco smoke, whereas chronic exposure to tobacco smoke results in GSH elevation [37]. In a different model, when rats were exposed to tobacco smoke only for 15 min twice daily in a period of 30 days, Baskaran et al. measured increased GST activities in the liver and in the lung and decreased concentrations of GSH in these tissues, when compared to the non-exposed controls [1]. In our current study, treatment with alcohol significantly increased GSH concentration in the lung and joint exposure to tobacco smoke and alcohol caused even greater GSH augmentation. Alterations of GSH levels may reflect the lung adaptation to stress induced by tobacco smoke, however, alcohol strongly enhances this response.

SOD and catalase are considered important elements of the antioxidant defense systems and react diversely to oxidative challenge. In our current experiment, SOD was elevated in the liver of animals treated with alcohol, no matter if rats were previously exposed to tobacco smoke, or not and catalase was increased only in alcohol-exposed animals. Usually these enzymes enhance their activities upon oxidative stress, which was described in various reports based on diverse experimental models. Husain et al. [18] observed that ethanol, nicotine, or a combination of ethanol plus nicotine significantly increased SOD activity in the liver, however, the effect of nicotine must be carefully analyzed as it was administered subcutaneously. In the same report, Husain described decreased activity of the liver catalase upon ethanol, nicotine, or a combination of ethanol plus nicotine. It may be then postulated that metabolism of the alkaloid nicotine, not only tobacco smoke inhalation, has a strong impact on antioxidant enzymes activities. In the experiment described by Baskaran et al., increased activities of the liver and the lung SOD and catalase were reported [1]. It may be then postulated that the applied relatively limited exposure of rats to tobacco smoke resulted in the induction of these antioxidant enzymes as adaptation to the oxidative insult. Opposite direction of SOD and catalase alteration in rat lungs upon exposure to tobacco smoke was reported by Luchese et al., who applied a sub-chronic exposition protocol. After 30 days of tobacco smoke inhalation, 5 times per week, at least for 15 min, 17% diminishment of SOD activity and 28% increase in catalase activity in lungs were measured [26].

Beside ROS, tobacco smoke is also an abundant source of nitric oxide and other RNS which contribute to oxidation reactions and modify macromolecules. Nitrosative/oxidative insult to proteins results in protein nitrosylation, moreover, it was found that RNS are capable of reducing endogenous antioxidants [42]. Ethanol metabolism also results in RNS elevation and protein nitrosylation, due to the induction of the immune system [48]. Nitrosylation caused by ethanol exposure significantly decreased cytosolic aldehyde dehydrogenase that may lead to cellular accumulation of acetaldehyde and increased toxicity [33]. In our current study we found no difference in the level of nitrosylated proteins in the liver and in the lung of animals exposed to tobacco smoke or given a single dose of ethanol, however, in rats treated with both toxicants a significant drop was measured in the liver, whereas a significant increase was found in the lung. Tissue nitrite concentration is a derivative of RNS appearance. In our experiment, the highest nitrite level was found either in the liver or in the lung of rats treated with a single dose of alcohol, which indirectly confirms the finding of Zima et al. [48] and Wang et al. [43].

Comet assay is a common method applied to the genotoxicity assessment, showing DNA single and double strand breaks appearing upon xenobiotic, especially oxidative, insult *in vivo* or *in vitro* and demonstrating efficiency of the DNA repair systems.

Inhalation of tobacco smoke causes DNA damage, namely DNA-adducts formation due to metabolic activation of tobacco-specific N-nitrosoamines and polycyclic aromatic hydrocarbons [14, 19]. Moreover, ROS and RNS in tobacco smoke may induce oxidative/nitrosative stress which results in DNA damage

(e.g., nitrosative deamination or nitrosylation of nucleic acid bases, subsequent transitions and/or transversions and DNA strand breaks) and inactivation of DNA repair enzymes [17, 33]. However, when comet assay was applied to the assessment of smoke effect on DNA, the reported range of nucleic damage did not clearly indicate that tobacco smoke was a damaging factor [15, 23, 40]. Ethanol consumption causes direct DNA damage which results from acetaldehyde, capable of the formation of carcinogenic exocyclic DNA etheno adducts and inhibition of DNA repair systems [38]. In our current experiment, combined exposure to tobacco smoke and alcohol caused greater hepatic and pulmonary DNA damage than measured after a single dose of alcohol or exposure to tobacco smoke only. Values obtained in the lungs of rats treated with alcohol or tobacco smoke with alcohol were significantly greater than respective values in liver, suggesting that the lungs are more sensitive to exogenous oxidants. Alcohol is not considered a specific toxicant to the lung tissue, however, it was found to cause oxidative stress in this organ, as after four-week application in rats an increased levels of hydrogen peroxide and of lipid peroxidation products were measured [21]. Thus, although the liver is a site of ethanol metabolism and conversion to toxic acetaldehyde, other tissues may be also involved in direct or indirect effects of this xenobiotic.

The obtained results suggest that in the ethanol-addicted rats combined exposure to smoke and alcohol differentially modulate endogenous antioxidant defense system and reactions to oxidative stress.

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