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DHEA and non-alcoholic fat liver disease: increased gene expression of peroxisome proliferation-activated receptor γ (PPARγ) and fatty acid synthase (FAS)

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ABSTRACT. Dehydroespiandrosterone (DHEA) is associated with improvements in chronic degenerative diseases, including obesity, insulin resistance, and cardiovascular diseases. Nevertheless, it is observed an increase in its concentration in individuals with liver lipid infiltration, but it is not precise if this condition emerges as a cause or a consequence. In this way, we aimed to identify gene expression alterations in lipid and glucose liver metabolism markers, as well as oxidative stress markers. For this purpose, male Wistar rats, 12-14 months old were treated with subcutaneous injections of DHEA (only dose of 10 mg kg^{-1}); and after 7 days, hepatic gene expression by PCR real time were performed for the following genes: G6Pase, PEPCK, FAS, PPARγ, malic enzyme, ChREBP, LXR, catalase, GPx, iNOS, NADPH oxidase subunits and PCNA. We observed a tendency of reduction in G6Pase gene expression in treated group ($p = 0.08$). In addition, it was identified an increase in liver PPARγ and FAS gene expressions, two markers of increased activity of lipogenic pathway. We also observed an increase in iNOS gene expression, a known inductor of systemic and hepatic insulin resistance. In conclusion, our data indicates that the treatment with DHEA can be associated with the development of liver lipid infiltration and hepatic insulin resistance.

Keywords: dehydroespiandrosterone, hepatic steatosis, de novo lipogenesis, hepatic insulin resistance.

DHEA e doença gordurosa hepática não-alcoólica: aumento na expressão gênica do *peroxisome proliferation-activated receptor* γ (PPARγ) e ácido graxo sintase (FAS)

RESUMO. A deidroepiandrosterona (DHEA) encontra-se associada a melhorias em quadros de obesidade, resistência à insulina e doenças cardiovasculares. Porém, observa-se um aumento na sua concentração em indivíduos portadores de infiltração lipídica hepática, mas sem saber precisar se o mesmo surge como causa ou consequência. Assim, objetivamos identificar alterações na expressão gênica hepática de marcadores relacionados ao metabolismo lipídico e glicídico e de estresse oxidativo. Para tanto, ratos machos com 12-14 meses de idade foram tratados com injeção subcutânea de DHEA (dose única 10 mg kg^{-1}), e após 7 dias foram feitas análises da expressão gênica hepática por PCR em tempo real das seguintes proteínas: G6Pase, PEPCK, FAS, PPARγ, enzima málica, ChREBP, LXR, catalase, GPx, iNOS, subunidades da NADPHoxidase e PCNA. Observamos uma tendência à redução da expressão gênica da G6Pase no grupo tratado ($p = 0,08$). Também identificamos um aumento na expressão gênica hepática do PPARγ e FAS, dois indicadores de aumento da atividade das vias de lipogênese. Observamos um aumento na expressão gênica da iNOS, um conhecido agente indutor de resistência à insulina sistêmica e hepática. Em conclusão, nossos dados indicam que o tratamento com DHEA pode estar associado com o desenvolvimento de um quadro de infiltração lipídica hepática e resistência à insulina hepática.

Palavras-chave: deidroepiandrosterona, esteatose hepática, lipogênese de novo, resistência à insulina hepática.

Introduction

Dehydroepiandrosterone (DHEA) (and its sulfated form - DHEA-S) is the most abundant steroid hormone in the circulation. It presents secretion peaks around the third decade of life, however from fifth decade on; their plasma levels begin to decline (RAINEY; NAKAMURA, 2008; RAINEY et al. 2002). Parallel to this decline in DHEA plasma levels, it is observed an increased incidence of chronic degenerative diseases like obesity, insulin resistance, diabetes mellitus type 2, liver lipid infiltration, dyslipidemias, and cardiovascular diseases, among others.

According to this, several researchers sought to study this relationship and find the beneficial effects

of DHEA administration in a diversity of diseases related to aging. Studies from our laboratory demonstrated benefic effects of DHEA treatment on the secretory capacity of insulin in rats with 12-14 months old (MEDINA et al., 2006), on muscular and hepatic insulin sensitivity in rats aged 6 months old (CAMPBELL et al., 2004) and on the improvement of aortic function in ovariectomized rats (CAMPOREZ et al., 2011). Additionally, DHEA treatment has been associated with adiposity reduction (TAGAWA et al., 2011).

On the other hand, some studies show no effects or even an adverse action of DHEA treatment in several body systems. Focusing on hepatic tissue, the main tissue of the present study, Hayashi et al. (1994) observed presence of hepatic carcinogenesis in F-344 rats treated with DHEA. In human beings, it was observed an increase in DHEA-S levels in male patients presenting liver lipid infiltration (KOGA et al., 2011). Saruç et al. (2003) obtained similar data, observing increased DHEA and DHEA-S levels in post-menopausal women with liver lipid infiltration. However, in both studies, it was not possible to confirm if this increased in DHEA/DHEA-S levels would be a cause or a consequence, since the switch from non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH), and DHEA/DHEA-S were found reduced (CHARLTON et al., 2008).

Studies show that several beneficial effects associated with DHEA treatment are provided from an improvement in body oxidative balance (ARAGNO et al., 2000; CAMPOREZ et al., 2011; HUERTA-GARCÍA et al., 2012; KHALIL et al., 2000). On the other hand, an imbalance among body oxidative stress and antioxidants defenses is a characteristic of NAFLD progression to NASH. Once the presence of hepatic steatosis is established, mitochondria constantly act on fat oxidation, releasing a great quantity of oxygen reactive species that should be in equilibrium with antioxidants defenses. When an imbalance occurs, the excess of oxygen reactive species increases lipid peroxidation, the presence of cytokines and the activation of Fas/FasL pathway, resulting in NASH (ROLO et al., 2012). Additionally, some studies indicate pro-oxidant action related to DHEA (HAYASHI et al., 1994; ZHOU; WAXMAN, 1998).

In this way, we aimed to identify gene expression alterations in lipid and glucose liver metabolism markers, as well as oxidative stress markers in adults male rats treated with dehydroepiandrosterone.

Material and Methods

Animals

Male Wistar rats, 12-14 months old from Animal Resource of the Institute of Biomedical Sciences of the University of São Paulo were kept in cages (3 animals per cage), in a 12h light: 12h darkness cycle (lights on at 7h) and with full access to food (Nuvilab balanced chow pellets, Nuvital SA, Columbo, Brazil) and water. After this period, animals were divided at random in control group (subcutaneous injection of canola oil) and treated group (subcutaneous injection of DHEA 10 mg kg^{-1} diluted in mineral oil). After 7 days from DHEA administration, animals were anesthetized with sodium pentobarbital (Hypnol, Cristalia, Itapira, Brazil; 4.0 mg 100 g^{-1} of body weight intraperitoneally) and sacrificed for liver collection and preparation for further analysis. All procedures and protocols were in accordance with the Guidelines for Ethical Care of Experimental Animals and were approved by of the Ethical Committee for Animal Research (CEEA) of the Institute of Biomedical Sciences (32/2008 - addendum 051/2005).

Real Time RT-PCR

Total RNA was extracted from hepatic tissue using Trizol solution, according to the manufacturer's specifications. Isolated RNA (2 μg) was reverse-transcribed to cDNA and the gene expression markers of glucose and lipid metabolism (G6Pase, PEPCK, PPARγ, FAS, malic enzyme, LXR, SREBP-1c, and ChREBP), oxidative stress (catalase, GPx, iNOS and NADPH oxidase subunits) and cell proliferation (PCNA) were identified by RT-PCR using ROTOR GENE 3000 (Corbett Research, Mortlake, Australia), and SYBER GREEN as fluorescent agent. Gene expression was calculated using 2-deltaC(T) method, using HPRT gene expression as internal control. Primers sequences are listed in Table 1.

Statistical Analysis

Data are presented as mean ± SEM and the Student "t" test was performed, after data were tested for normality. The analysis was performed by the statistical software package Graph Pad Prism 5.0. The significance was set at $p < 0.05$.

Table 1. Primers sequences.

Gene	Forward	Reverse
SREBP-1c	5'-GGAGCCATGGATTGCACATT-3'	5'-AGGAAGGCTTCCAGAGAGGA-3'
faz	5'-CACAGCATTCAGTCCTATCCACAGA-3'	5'-CACAGCCAACCAGATGCTTCA-3'
Malic Enzyme	5'-GCCCTGAATATGATGCGTTT-3'	5'-CACAGACGCTGTTCCTTGAA-3'
ChREBP	5'-GTCCGATATCTCCGACACACTCTT-3'	5'-CATTGCCAACATAAGCATCTTCTG-3'
PPARγ	5'-ATGCCAAAAATATCCCTGGTTTC-3'	5'-GGAGGCCAGCATGGTGTAGA-3'
G6Pase	5'-GGTCACTGCATGATCACAGG-3'	5'-CCTTGGAATCCAGAATGCTC-3'
LXR	5'-GGCTTCACTGGTTGATCCAT-3'	5'-AGGGGGTTGATTCTTGAGGT-3'
PEPCK	5'-CAGAGCTGAATTCCCTTC-3'	5'-AGCTGTGAGGTGTCAC-3
Catalase	5'-CTATTGCCGTCCGATTCTC-3'	5'-GTCCCAGTTACCATCTTCAGTGT-3'
GPx	5'-TCCACCGTGTATGCC-3'	5'-TGTCCGAACTGATTGC-3'
iNOS	5'-AGGCAAGCCCTCACCTACTT-3'	5'-GTGGGGTTGTTGCTGAACTT-3'
$p22^{phox}$	5'-GAGGTCCGCAAGAAGCCAAG-3'	5'-GAAACTCAAGCAGGAGCCACTG-3'
$gp91^{phox}$	5'-TGCCCAGTACCAAAGTTTGCC-3'	5'-GACCCACGATCCATTTCCAAG-3'
$p47^{phox}$	5'-CTTCATTCGCCACATCGC-3'	5'-TTTCTGTAGACCACCTTCTCCG-3'
$p40^{phox}$	5'-GAGAGAGGACATTGCCCTTA-3'	5'-AAGTAGAATCCTGTCCAGTG-3'
$p67^{phox}$	5'-GAAAGCATGAAGGATGCCTGG-3'	5'-ATAGCACCAAGATCACATCTCC-3'
PCNA	5'-TTTGAGGCACGCCTGATCC-3'	5'-GGAGACGTGAGACGAGTCCAT-3'
HPRT	5'-GCTGAAGATTTGGAAAAGGTGT-3'	5'-ACAGAGGGCCACAATGTGAT-3'

Results and discussion

Our study aimed to identify alterations in liver gene expression of rats with 12-14 months old (because in this age, rats are obese and present glucose intolerance, and probably NAFLD), induced by only one dose of DHEA. As identified in the literature, DHEA presents antihyperglycaemic action, but its effects were not completely elucidated.

Liver plays a major role in energetic homeostasis, which guarantees the maintenance of glycaemia between meals, inhibiting glucose release and promoting its storage during post-absorptive periods. In this way, we sought to investigate gene expression of two key enzymes in hepatic glucose release; G6Pase, involved in the last step of glucose release on bloodstream, and PEPCK, a limitant step on gluconeogenesis activity (Figure 1).

Thus, although some previous work demonstrated the effects of DHEA treatments on gluconeogenic pathway (AOKI et al., 2004; YAMASHITA et al., 2005), we did not observe any alterations in PEPCK gene expression. However, we observed a strong tendency of reduction on G6Pase gene expression ($p = 0.08$), what could be one of the responsible factors for reduction in circulating glucose. Aoki et al. (1999) related a reduced activity in G6Pase and Frutose 1,6-bisphosphatase in the liver from db/db rats treated with DHEA for 15 days, and it presented a linear relationship with glycaemia. Despite the reduction in the enzyme activities, Aoki et al. (2000) demonstrated that only G6Pase had reduced gene expression. Additionally, in human hepatoma cells (HepG2) it was identified reduced activity, gene expression and protein concentration of G6Pase after incubation with DHEA (YAMASHITA et al., 2005). Confronting our data with those from literature, we can assume that DHEA should present a role on hepatic glucose control.

On Figure 1, we also show data from gene expression analysis of proteins related to lipid metabolism (LXR, PPARγ, FAS, SREBP-1c, ChREBP, and malic enzyme). As observed, DHEA administration promoted an increase in PPARγ and FAS gene expressions ($p < 0.05$), with no interference in other analyzed mRNA. So, besides its apparent beneficial effect in hepatic glucose output, it is possible to suggest an increased de novo lipogenesis, since there was an increase in PPARγ and FAS gene expression in rats treated with DHEA. The increase of PPARγ induced by DHEA treatment was once related, but in rat epididymal fat pad (KARBOWSKA; KOCHAN, 2005). PPARs present an important role in the regulation of genes involved in lipid utilization and storage, lipoprotein metabolism, adipocytes differentiation, and insulin action (ROGUE et al., 2010). However, cellular effects of PPAR seem to be specific. While in adipose tissue PPARγ acts increasing insulin sensibility due to its ability in uptaking fatty acids from circulation (ROGUE et al., 2010); in the liver, the increased in PPARγ gene expression presents negative results. Some studies point out that increased PPARγ causes the presence of lipid microvesicles, which is associated with a steatotic condition (YU et al., 2003).

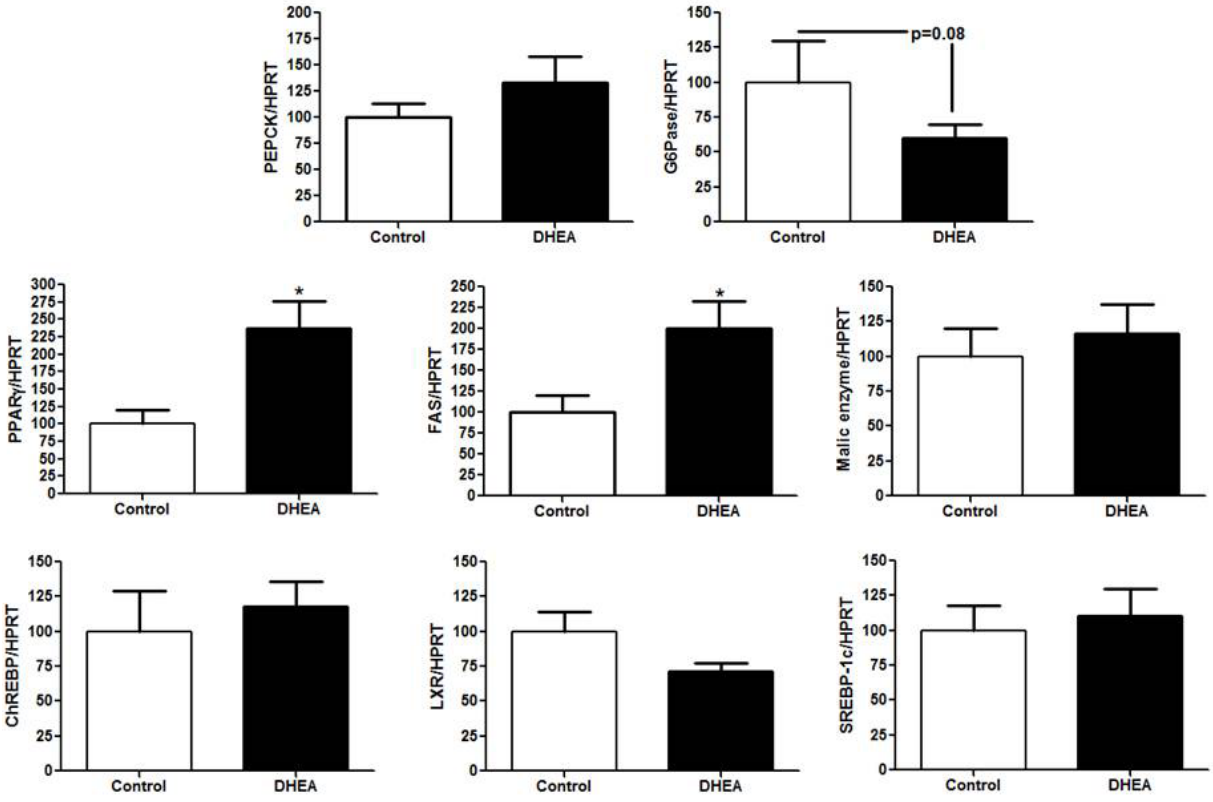

Figure 1. Liver gene expression of markers of carbohydrate and fat metabolism in response to DHEA administration. *$p < 0.05$ ($n = 4$).

In contrast, the treatment with a PPARγ activator, thiazolinediones, reduces hepatic steatosis. However, when administered in animals with overexpression of hepatic PPARγ, KKA^y and ob/ob mice, thiazolinediones accentuated the steatotic condition (BEDOUCHA et al., 2001; MATSUSUE et al., 2003). It indicates that an increase in hepatic PPARγ gene expression can be detrimental for this tissue. Additionally, there are experimental evidences that relate the presence of an increased level of DHEA/DHEA-S with a condition of liver lipid infiltration (KOGA et al., 2011; SARUÇ et al., 2003).

The concomitant increase in FAS gene expression can be related to increased liver lipid inclusions, since this enzyme catalyzes the last step in lipid biosynthesis (de novo lipogenesis) (DORN et al., 2010). This increase in FAS strengthens our assumption that DHEA is acting as a stimulating agent in liver lipid inclusions.

DHEA is also known as an influential agent of oxidative stress pathway. In this way, due to the relationship among oxidative stress and carbohydrate and fat metabolism, we sought to investigate gene expression of catalase, GPx and iNOS (Figure 2), in addition to NADPH oxidase subunits (Figure 3). The administration of DHEA raised iNOS gene expression ($p < 0.05$), but it did not influence catalase and GPx. Besides, DHEA administration did not change gene expression of all components of NADPH oxidase.

Previous data lead to dual effects of DHEA treatment: pro-oxidant and antioxidant properties, depending on the dose used. Pharmacological concentrations of DHEA are associated to pro-oxidant actions (MASTROCOLA et al., 2003). However, the change in iNOS gene expression was observed in a non-pharmacological dose. Elevated hepatic iNOS is directly associated with hepatic and systemic damage. Shinozaki et al. (2011) demonstrated hyperglycemia, hyperinsulinemia and hepatic insulin resistance in mice with high iNOS gene expression.

On the other hand, we did not observe any change in gene expression of catalase, glutathione peroxidase, and NADPH oxidase subunit. However, Jacob et al. (2011), verified that 5-week DHEA treatment was able to increase glutathione peroxidase protein levels in liver of young and aged rats. Moreover, DHEA was an efficient antioxidant agent in the aorta of ovariectomized rats (CAMPOREZ et al., 2011).

Finally, DHEA administration is associated with an increased cellular proliferation. There are data indicating a positive effect on tumor development induced by PPARγ agonists in different tissues (ROGUE et al., 2010), as well as high doses of

DHEA, which present mitogenic characteristics (KOPPLOW et al., 2005). In this way, we sought to identify gene expression of a cellular proliferation marker, the PCNA. As one can observe in Figure 4, DHEA administration did not increase gene expression of this marker.

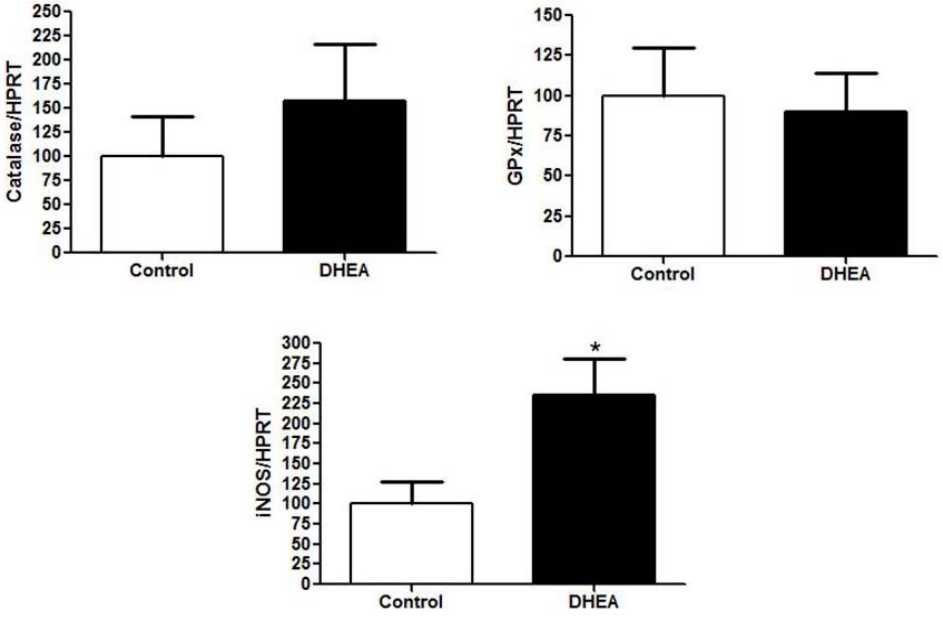

Figure 2. Liver gene expression of catalase, GPx, and iNOS in response to DHEA administration. *p < 0.05 (n = 4).

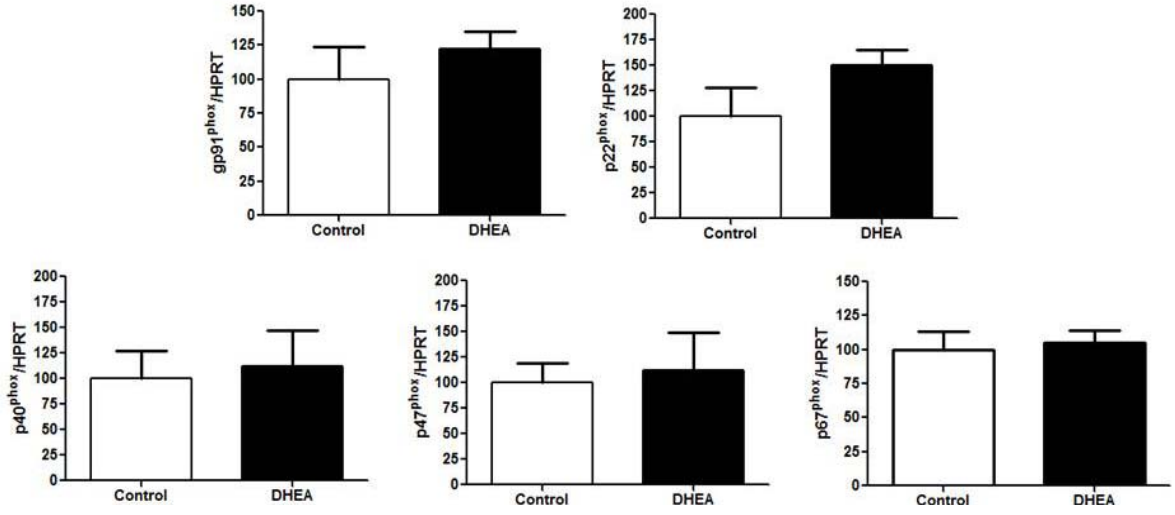

Figure 3. Liver gene expression of NADPH oxidase subunits in response to DHEA administration. (n = 4).

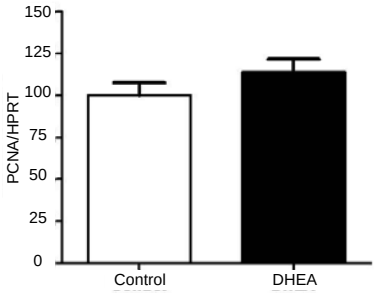

Figure 4. Liver gene expression of PCNA in response to DHEA administration. (n = 4).

Conclusion

In conclusion, despite several beneficial changes associated with DHEA treatment in several tissues, our data indicate reservations about the effectiveness of its administration on liver functionality in 12-14 months old rats. Our data indicate an important possible beneficial action involving G6Pase gene expression, and consequently a reduction in hepatic glucose release. However, apparently, the accumulation and/or excess of glucose in the liver would be directed to de novo lipogenesis pathway, due to increased PPARγ and FAS gene expressions, what could lead to the accumulation of hepatic fat. In this way, the increase in DHEA/DHEA-S levels could be the cause and not the consequence of liver lipid infiltration. In association with this, we found an increase in iNOS, which also favors hepatic insulin resistance. In addition, it is important to stress that the present study was based only in gene expression analysis, and further studies about DHEA administration are necessary to more effective conclusions (because mRNA could generate elevated or reduced protein concentration, and change the hypothesis of this study); like its role in cell mechanisms and the response to different time of exposure to this steroid.

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