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Anti-Apoptotic Effects of Aminoguanidine Against Liver Damage on Experimental Diabetes in Rats

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Abstract
Objective: This study was designed to investigate the antiapoptotic and improving effects of aminoguanidine on the histological alterations in liver in streptozotocin (STZ)-induced diabetic rat model.
Material and Methods: 32 male Sprague Dawley rats were divided into the following 4 groups: Control, Aminoguanidine (AMG), Streptozotocin (STZ), Streptozotocin + Aminoguanidine (STZ+AMG). The animals in group STZ and STZ+AMG were made diabetic by intraperitoneal injection of streptozotocin 45 mg/kg. Histochemical and immunohistochemical staining methods were applied to sections obtained from paraffin blocks and preparations were examined by using Leica DFC-280 light microscope.
Results: The sections taken from the control and AMG groups were normal in histological appearance. However, multifocal nodules containing inflammatory cells were observed in the diabetic group. Moreover necrotic hepatocytes and hemorrhagic areas were observed among intact hepatocyte of lobul in the STZ group. Glycogen loss was observed in the hepatocytes localized at the periphery of lobule. In addition, the numbers of cells with positive staining by caspase-3 were significantly increased. On the other hand, it was seen that, in the groups administered with STZ, histological injury in the liver was attenuated by the administration of AMG. Moreover, it was found that the number of caspase-3 positive cells was significantly decreased in the STZ+AMG group.
Conclusion: We concluded that chronic aminoguanidine administration reduced liver injury in STZ- induced diabetic rats. Thus, we suggest that aminoguanidine may be used to prevent the development of diabetic liver damage.
Key Words: Aminoguanidine; Diabetes mellitus; Streptozotocin; Rat.

Deneysel Diyabet Oluşturulan Sıçanlarda Meydana Gelen Karaciğer Hasarına Karşı Aminoguanidinin Antiapoptotik Etkileri

Özet
Amaç: Bu çalışma, streptozotosin (STZ) ile oluşturulan diyabetik sıçan modelinde, karaciğerde ortaya çıkan histolojik değişiklikler üzerine aminoguanidinin antiapoptotik ve iyileştirici etkilerinin araştırılması amacıyla planlandı.
Gereç ve Yöntemler: Çalışmada 32 adet Sprague Dawley erkek sıçan kullanıldı. Sıçanlar her biri 8 adetten oluşan 4 gruba ayrıldı. Kontrol, aminoguanidin (AMG), Streptozosin (STZ), Streptozosin+AMG (STZ+AMG). Deneysel diyabet STZ ve STZ+AMG gruplarında tek doz STZ'nin (45 mg/kg) intraperitoneal (i.p.) uygulanması ile oluşturuldu. Alınan dokular, rutin doku takip işlemlerinden geçirilerek parafine gömüldü. Parafin bloklardan alınan kesitlere histokimyasal (Mayer's Hemotoksilen-Eozin, PAS) ve immunohistokimyasal (kaspaz-3) boyama yöntemleri uygulandı. Boyanan kesitler ve Leica DFC-280 ışık mikroskobunda incelendi.
Bulgular: Kontrol ve AMG grubu normal histolojik görünümdeydi. Bununla birlikte STZ grubunda parankima içerisinde inflamatuvar hücre içeren multifokal nodüller izlendi. Ayrıca sağlam hepatositler arasında yerleşmiş asidofil sitoplazmalı ve heterokromatik nükleuslu nekrotik hücrelere ve hemorajik alanlara rastlandı. Lobülün periferinde yer alan hepatositlerde glikojen kaybı gözlendi. Ayrıca kaspaz-3 boyama metodu uygulanan kesitlerde kaspaz-3 (+) boyanan hücre sayılarının STZ grubunda, kontrol grubuna göre anlamlı derecede arttığı tespit edildi. STZ uygulanan sıçanlara AMG verilmesiyle karaciğerde izlenen histolojik hasarın düzeldiği izlendi. Ayrıca kaspaz-3 (+) hücre sayılarının azaldığı tespit edildi.
Sonuç: Kronik aminoguanidin uygulaması, STZ ile sıçanlarda oluşturulan diyabetin neden olduğu karaciğer hasarını azalttı. Sonuç olarak aminoguanidinin diyabetik karaciğer hasarının gelişimini önleyeceğini düşünmekteyiz.
Anahtar Kelimeler: Aminoguanidin; Diabetes Mellitus; Streptozotosin; Sıçan.

INTRODUCTION

Resulting from the absence, deficiency or inefficiency of insulin hormone secreted by the pancreas, involving metabolical changes in the protein, fat and carbohydrate, and characterized by hyperglycaemia, diabetes mellitus (DM) is a metabolic disorder with high morbidity and mortality (1). A major health problem in the world, diabetes is one of the main diseases that affect individual's quality of life and life expectancy negatively in both developed and developing countries due to the acute and chronic complications it brings forward (2, 3). Because of limited research options on humans for ethical reasons, a variety of experimental models have led to development in diabetes mellitus related researches. It is agreed that examples of animal models used in diabetes researches are similar to characteristics of diabetes in humans in many aspects (4). Streptozotocin (STZ) is an agent used on laboratory animals to form chemical diabetes (type I) by damaging the beta cells of the pancreas (5, 6). By affecting glycogen and lipid metabolism, diabetes causes structural and functional abnormalities in the liver (7). Also hyperglycaemia, that occurs as a result of oxidative stress, leads to liver damage (8). Further reports indicate

that genes controlling apoptosis are affected due to elevated blood glucose in diabetes (9). It has also been shown that hyperglycaemia reduces anti-apoptotic protein bcl-2 gene expression while temporarily increasing the mRNA levels of apoptotic genes (10). It is known that aminoguanidine (AMG) inhibits the formation of reactive oxygen compounds while it also has a scavenging effect on hydroxyl radicals, which are hydrogen peroxide derivatives (11). Besides, because it is structurally similar to L-arginine amino acid, AMG decreases nitric oxide synthesis by selectively inhibiting nitric oxide synthesis (through iNOS) (12).

The aim of this study is to have a histochemical and immunohistochemical examination of antiapoptotic and therapeutic effects of aminoguanidine on histological changes in the liver in experimental model of diabetes.

MATERIAL AND METHODS

Thirty-two male Sprague Dawley rats obtained from the Experimental Animal Research Center of Inonu University were used in the present study. Rats were randomly selected and divided into 4 groups: Control group (n=8), Aminoguanidine (AMG) group (n=8), Streptozotocin (STZ) group (n=8), STZ + AMG Group (n=8).

Blood glucose levels of the rats before the experiment were determined after measurement results of samples received from tail vein that were measured with the blood glucometer (Roche Accu-Chek glucometer). Rats, which would be exposed to diabetes, were administered intraperitoneal injection with insulin syringes containing 45 mg of streptozotocin (STZ) (Sigma, USA) that was thoroughly dissolved in 10 cc of distilled water (45 mg/kg single dose) in accordance with animal body weight. 72 hours after the STZ injection, blood samples were collected from STZ and STZ+AMG and their blood glucose levels were measured with the help of the glucometer. Those with 270 mg/dL blood glucose levels and above were included in the study. AMG was prepared by dissolving it in tap water by 1 g/l and was given to the subjects every day at 9:00. Throughout the 10-week experimental period, all rats were fed with standard rat pellet and received normal drinking water except for those who were given AMG.

At end of the experiment, blood glucose levels of the animals were measured. Under ketamine/xylazine anesthesia rats were sacrificed. Liver tissue was fixed in 10% formalin solution and embedded in paraffin. Tissue sections were cut at 5 µm, mounted on slides, stained with hematoxylin-eosin (H-E) for general liver structure, periodic acid schiff (PAS) to demonstrate the glycogen deposition in hepatocytes. The sections were examined by Leica DFC 280 light microscope by a histolog unaware of the status of animals.

Immunohistochemical Staining

5 µm sections taken from hepatic tissues were moved on the polylysine coated slides for immunohistochemical staining. After the deparaffinization, the preparations were brought to water, put into 0.05% tween 20-citrate buffer (pH 7.6), and then heated in the microwave for 30 mins. After the cooling at room temperature, the sections were washed with phosphate buffered saline (PBS). Then the sections were applied 0.3% of hydrogen peroxide. The sections were incubated for 1 hour at room temperature with primary rabbit polyclonal caspase 3 antibodies in the following step (Neomarkers, USA). After another set of PBS washing, they were re-incubated with biotinylated anti-polyvalent for 30 mins followed by another 30 minutes' incubation with streptoavidin-peroxidase. Staining was completed by soaking the sections in chromogen+substrate for 15 minutes.

Histological evaluation

The sections underwent histological analysis for inflammation, eosinophilic cytoplasm and pyknotic nucleated cells, hemorrhagic areas and loss of glycogen deposition in the hepatocytes cytoplasm. For histological evaluation, a total of 10 areas were examined at each x20 magnification. The severity of damage and glycogen loss were graded as: 0= no change, 1= mild, 2= moderate, 3= severe. A scoring chart was prepared for each rat and, for each group, mean values were determined. For immunohistochemical evaluation, caspase-3 (+) stained cells in 10 areas acquired from each rat liver were taken into consideration at x40 magnification.

In all the preparation analyses and evaluation, the Leica Q Win Image Analysis System (Leica Micros Imaging Solution Ltd., Cambridge, UK) was used.

Statistical Evaluation

Statistical analyses were carried out in SPSS 13.0 software by using Kruskal-Wallis and Mann-Whitney-U tests. With Kruskal-Wallis test, mean values of the groups were calculated while Mann-Whitney-U was used to test the significance of differences between groups. The change test within the groups were made with two-sample Wilcoxon paired tests. All results were expressed as mean±standard error (SE). At the end, $p<0.05$ values were considered statistically meaningful.

RESULTS

Blood-Glucose Levels

Hyperglycaemia was observed in the STZ group rats from the beginning all throughout the experiment. On the other hand, the difference between the final-initial blood glucose levels in the STZ+AMG group was reduced statistically significantly compared to those of the STZ group ($p = 0.001$). No significant difference was found between the STZ+AMG and control groups in terms of initial-final blood glucose levels ($p> 0.05$). The blood glucose levels of the rats at the start and at the end of the experiment are given in Table 1.

Table 1. The initial-final blood glucose levels of the groups

Groups	Initial blood-glucose (mg/dl)	Final blood-glucose (mg/dl)	Blood-glucose difference (mg/dl)
1-Control	128.6±2.5	154.4±3.4	25.7±3.5
2-AMG	132.25±6	167.0±13.2	32.7±8.7
3-STZ	383.3±14.1	587.0±5.0	203.6±11.9
4-STZ+AMG	283.58±6.1	334.6±7.8	51.1±8.5
P=0.001	1-3	1-3	1-3
	3-4	3-4	3-4

Histological Findings

Control and AMG groups:

In hematoxylin-eosin staining implemented sections, it has been observed that the liver had regular hexagonal structure and that it was composed of lobules with central veins in the centre and portal areas in the corners. Moreover, some cords of hepatocytes extending radially from the veins towards the periphery of the lobules along with sinusoidal capillaries among these cords were also identified (Figure 1A, B). In the PAS method applied sections, pink coloured glycogen granules were widely visible in the cytoplasm of hepatocytes (Figure 1C, D). Immunohistochemical analysis did not reveal any cells with positive reaction in the caspase-3 immunostaining applied sections (Figure 1E, F).

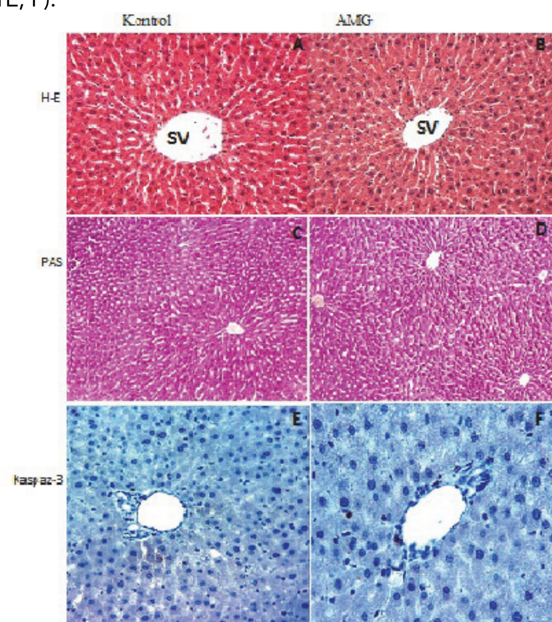

Figure 1. Control and AMG groups (A, B): the central vein observed in the liver (SV) and the general appearance of the hepatocyte cords H-E×20; (C, D) the general appearance of the PAS (+) hepatocytes. PAS ×10; (E, F) no caspase 3 (+) cells are visible. Caspase-3 ×20

Diabetes group:

In this group, we determined a number of focal area that contained inflammatory cells (1.37 ± 0.48) (Figure 2A) and some hemorrhagic areas (1.93 ± 0.51) (Figure 2B) in the liver parenchyma. In addition to that, there were necrotic cells with eosinophilic cytoplasm and heterochromatic nuclei that were settled among intact hepatocytes (1.31±0.46) (Figure 2C).

A significant decrease in glycogen content was observable in the hepatocytes located at the periphery of lobules of PAS staining method applied sections compared to those of the control group (1.93±0.51) ($p < 0.05$) (Figure 2D). The cells showing positive reaction to caspase-3 immunostaining method were stained in shades of brown. In this group, the caspase (+) cell number was 4.07±1.30 (Figure 2E).

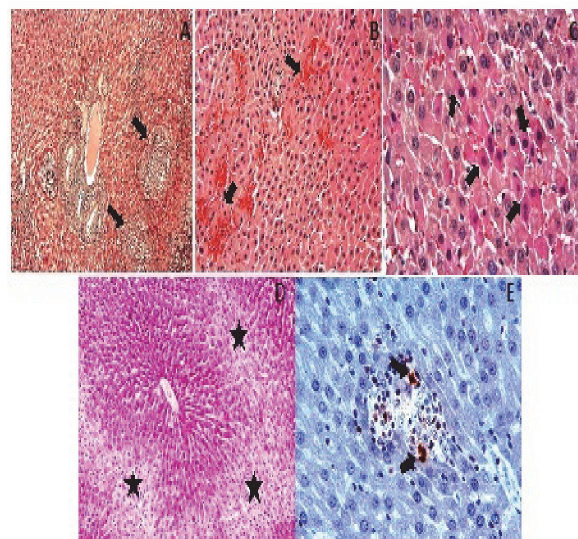

Figure 2. STZ group: (A) focal areas with inflammatory cells in liver parenchyma (arrows) H-E×20; (B) hemorrhagic areas in liver parenchyma (arrows) H-E×20; (C) necrotic cells with eosinophilic cytoplasm and heterochromatic nuclei among intact hepatocytes in different shapes (arrows) H-E×40; (D) glycogen loss in the hepatocytes located at the periphery of lobule (stars). PAS×10; (E) caspase-3 (+) cells near the portal area (arrows). Caspase-3 ×40

Diabetes-AMG group:

Excluding some small hemorrhagic areas, liver parenchyma had normal histological structure. This group proved to contain a statistically notable reduction in terms of inflammation compared to the diabetes group (0.91±0.39) ($p < 0.05$) (Figure 3A). In addition, cells with heterochromatic nuclei and eosinophilic cytoplasm were rarely viewed in this group (0.81±0.39) ($p<0.05$) (Figure 3 B). The glycogen loss in PAS staining method applied sections was 1.28±0.45. This loss was found to be significantly reduced compared to that of the STZ group ($p < 0.05$) (Figure 3C).

The number of cells reacting positively to caspase-3 immunostaining method was 2.31±0.46. Contrasted with the STZ group, this group showed statistically important decrease in caspase-3 (+) cell number ($p < 0.05$) (Figure 3D). The histopathological scores and the average caspase (+) cell numbers of all the groups are shown in Table 2.

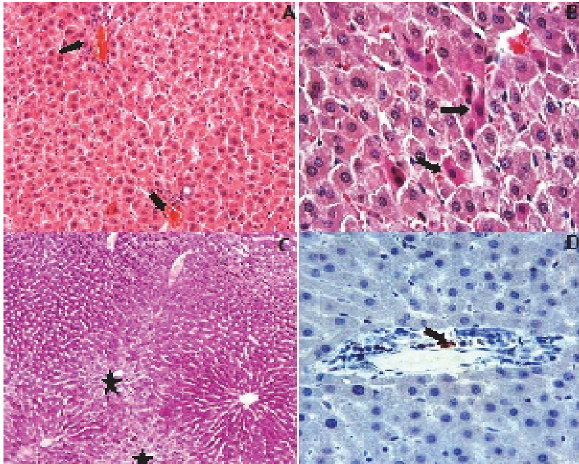

Figure 3. STZ+AMG group: (A) normal histological structure apart from some small hemorrhagic areas (arrows) H-EX20; (B) cells with heterochromatic nuclei and eosinophillic cytoplasm were rarely viewed (arrows); (C) The glycogen loss in PAS staining method applied was found to be significantly reduced compared to that of the STZ group. PAS ×10; (D) caspase-3 (+) stained cells. Caspase-3 ×40

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Table 2. The histopathological scores and the average caspase (+) cell numbers of the groups

Groups	Hemorrhage	Inflammation	Necrotic Cells	Glycogen Loss	Caspase-3 (+) cells
1-Control	0.11±0.30	0.25±0.43	0.00±0.00	0.10±0.30	0.00±0.00
2-AMG	0.16±0.37	0.35±0.47	0.12±0.33	0.16±0.37	0.00±0.00
3-STZ	1.93±0.51	1.37±0.48	1.31±0.46	1.93±0.51	4.07±1.30
4-STZ+AMG	1.28±0.45	0.91±0.39	0.81±0.39	1.28±0.45	2.31±0.46
P<0.0001	1-3,4 3-1,2,4	1-3,4 3-1,2,4	1-3,4 3-1,2,4	1-3,4 3-1,2,4	1-3,4 3-1,2,4

DISCUSSION

In this study, we have investigated the anti-apoptotic and curative properties of aminoguanidine, which is known to have antioxidant effects on the damage in the liver of rats with experimental diabetes, by using histochemical and immunohistochemical methods.

Widely used in creating experimental diabetes, STZ is effective with its decreasing corollaries in insulin receptors of target tissue cells which bring about the inhibition of pancreatic insulin secretion as well as tyrosine kinase activity (13, 14). During the STZ metabolisation, alkaline agents such as methyl cations and methyl radicals are produced in addition to reactive oxygen radicals (RR). Because of their low antioxidative capacity, these newly produced materials directly affect pancreatic beta cells (15).To induce diabetes in rats, STZ is administered in a single injection in doses of 40 to 60 mg (16, 17, 18). We also used 45 mg/kg of STZ in a single dose in this study. Throughout our study, we observed the development of hyperglycaemia from the 3rd day of the experiment onwards in rats with STZ injection. In the group treated with AMG, the blood glucose values significantly decreased compared to those of the STZ group. These results are in line with other works (20,21) like that of Liptakov et al. (19) which report meaningful decline in plasma glucose levels following the 8-week AMG treatment applied to diabetic rats.

The developing hepatocellular damage in the liver after STZ administration in rats has been shown by several authors (22, 23, 24). Vardi N. et al. have reported deterioration in the radial placement of hepatocytes starting from the central veins towards the periphery in the liver and hydropic change in hepatocytes located at the periphery of the lobules (22). Hamadi N et al. have given an account of STZ, administered as single dose (60 mg/kg), generating inflammation, necrosis in the liver of diabetic rats, and vacuolisation in hepatocytes (23). Similarly, Raw et al. have outlined inflammation in the liver, dilatation and formation of necrotic cells with pyknotic nuclei in the central veins after applying 50 mg/kg STZ to rats (24). In our study, too, we observed hemorrhage, inflammation and necrotic cells with eosinophilic cytoplasm and heterochromatic nuclei in the liver parenchyma.

The study we conducted assessed glycogen content in the hepatocyte cytoplasm by PAS staining method. With this method, at the periphery of the lobule in the STZ injected groups, a noteworthy glycogen loss was observed. Glycogen, which is the main energy source of hepatocytes, is an important parameter indicating liver damage. It is believed that the glucose-dependent state of cells, a consequence of abnormality in the energy

metabolism, play an important role in the reduction of glycogen stores in the cell (25). Liver, as the gluconeogenesis, glycogenolysis, glycogenesis, and blood-glucose level regulator of the body, is the most affected organ by insulin changes (26). As an outcome of lack of insulin in diabetes, the hexokinase activities such as glycogen synthase activity are decreased, and in turn, glucose cannot be converted to glycogen and glycogenesis is reduced (27). Furthermore, in type I diabetes, as a result of the increase in glucagon levels, the existing glycogen stores in the liver are consumed through glycogenesis. Eventually, glycogen granules in hepatocytes are notably lowered (28). Doi et al. (29), Kume et al. (30), and Malekinejad et al. have all concluded that STZ causes remarkable decrease in terms of glycogen contents in hepatocyte cytoplasms (31).

In this study, we have identified apoptotic cells in the liver tissue with the help of caspase-3 activities. With this method, it has been found out that caspase-3 (+) cell count in the STZ group control was higher compared with other groups. The increase in the number of caspase-3 stained cells in the liver makes us assume that STZ causes apoptosis through caspase activation. It has formerly been reported that the increase in free radical formation in the hyperglycemia participates in the development of diabetic complications and that the newly formulated oxidative stress activates apoptotic pathway (32, 33). It is also known caspases, by initiating proteolytic cleavage cascade during the apoptosis, play a critical role in the development of apoptotic events. Caspase-3, one of the members of 14-member caspase family, is a key protease in the early stages of apoptosis (34). Haligur et al. in their experimental STZ-induced diabetic study, disclosed an exceptional rise of apoptotic cells in liver sections of rats (35). Oxidative stress is known to have an important role in the pathophysiology of chronic complications in diabetes (36). The idea that there is a close connection between hyperglycemia and oxidative stress has been supported by some in vivo studies (37). Increased glycolysis in hyperglycaemia, intracellular sorbitol (polyol) pathway activation, glucose auto oxidation, and nonenzymatic glycosylation are the four different ways that are known to increase free radical formation. Under increased oxidative stress, ROS induce oxidative damage on proteins and DNA, and, by way of various mechanisms such as membrane lipid per oxidation, they cause further cellular damage that comprises apoptosis (38, 39).

Throughout this study, it has been observed that the liver damage caused by STZ could improve through AMG treatment. AMG, a hydrazine compound, is a specific, inducible nitric oxide synthase inhibitor (40). As a powerful antioxidant, AMG has proved to have potential to cease diabetic complications as shown in different animal models (41). Okomato et al. in their attempt to study the effects of AMG on a concanavalin A-applied liver damage, have reported notable reduction in congestion, necrosis, and infiltration in the liver after the administration of aminoguanidine (42). In our study, likewise, we have determined statistically significant decrease in the findings in the AMG group in contrast to the STZ group.

In other words, we have noticed a suggestive decrease in glycogen loss in diabetic rats following the administration of AMG. Brunet et al. too, have published about decreased glycogen loss after AMG administration in the cytoplasm of hepatocytes that were exposed to PAS staining method in schistosoma-mansion-related liver damage in rats (43). AMG, being a phenyl hydrazine compound and selectively inhibiting nitric oxide synthesis, decreases the production of NO (12). As one of the free oxidant radicals, nitric oxide (NO) is produced from the arginine by nitric oxide synthase activity (44). Studies have shown NO to inhibit glycogen synthesis (45, 46). Aminoguanidine is a nitric oxide synthase inhibitor, which in turn makes us think that it may have caused such reduction in glycogen loss.

AMG application, with caspase-3, brought about a notable decrease in the number of positively reacting cells. Similarly, Yang et al. have reported a parallel loss in the number of caspase 3 (+) cells in the retina of ischemia-reperfusion-implemented rats with the use of AMG (47). Dingman et al. following the implementation of cerebral ischemia in rats, have underlined a comparable loss in the number caspase-3 (+) cells by the administration of aminoguanidine (48). AMG's protective effect against apoptosis is explained by the fact that it inhibits ROR formation and that it has a scavenging impact on hydroxyl radical, which are hydrogen peroxide derivatives (49,50).

In conclusion, it has been observed that the liver damage in STZ-induced diabetic rats is reduced by AMG administration. AMG 's protective effect on the apoptotic cell death and liver damage caused by diabetes in rats may be elucidated both by its being an antioxidant agent and the inhibition of NO it causes. However, further studies on the efficacy of AMG on diabetes are needed.

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