The potential protective effect of propolis on experimentally induced hepatitis in adult male albino rats. Histological and immunohistochemical study

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Abstract

Background: In Egypt, liver diseases are one of the most prominent killers especially hepatitis virus infection, fibrosis and cirrhosis. Hepatitis has a serious health effects and alter the functions of the liver. D-galactosamine (D-GalN) and Lipopolysaccharide (LPS) induced hepatitis in rats are closely resembling human viral hepatitis. Propolis is honey bee product with a wide range of beneficial therapeutic effects.

Objective: To evaluate the possible protective effect of propolis on experimentally induced hepatitis in adult male albino rats.

Materials and methods: Forty adult male rats included and divided equally into 4 groups (10 rats each). group I (control group), group II (Propolis group): The rats received daily oral dose of the propolis (200mg/Kg) by gastric tube for 2 weeks, group III (Hepatitis model group): The rats received single intraperitoneally injection of D-GalN and LPS (300 mg/kg and 30 μg/kg) 18 hours before the end of experiment, group IV (Propolis and hepatitis model group): The rats received daily oral dose of propolis for 2 weeks and D-GalN and LPS 18 hours before the end of experiment. Liver specimens were taken and processed for histological and immunohistochemical study.

Results: Group III showed signs of degeneration and necrosis as some swollen hepatocytes had finely granular cytoplasm, other hepatocytes had small hyperchromatic or karyolytic nuclei. Dilated congested, proliferation of endothelial cells of central vein were seen and its wall showed inflammatory cells. There were apparent increase of collagen fibers, significant increase of anti-proliferating cell nuclear antigen (PCNA) positive nuclei among hepatocytes and strong immunoreaction for anti-Transforming growth factor (TGF-β) in the wall of portal vein. Group IV showed improvement of histological and immunohistochemical changes described before.

Conclusion: Propolis has potential protective effect against D-GalN/LPS induced hepatotoxicity in rats as it has antioxidant, anti-inflammatory and antiapoptotic activities.

Keywords: Acute hepatitis, D-galactosamine, lipopolysaccharide, immunohistochemistery, propolis

Introduction

The liver is a vital organ that plays a key role in the detoxification of exogenous and endogenous substances. It also performs a wide range of metabolic activities required for homeostasis, nutrition and immune defense. A variety of pathological factors including viral hepatitis (especially hepatitis B and C), alcohol and drug abuse, metabolic diseases, autoimmune diseases and congenital abnormalities can cause hepatic injury. Liver fibrosis is the final stage of all chronic hepatic disease. Hepatic stellate cells (HSCs) play a principal role in liver fibrosis. It is a well-known fact that fibrosis has lots of important complications such as portal hypertension, hepatocellular carcinoma, hepatic encephalopathy, spontaneous bacterial peritonitis and hepatorenal syndrome [1,2].

Hepatitis is a major public health problem worldwide, responsible for considerable morbidity and mortality from liver disease. Common causes of hepatitis include viral infection, side effects of certain prescription drugs and overdoses of the...
over-counter drugs [3].

Among the numerous models of experimental hepatitis, Lipopolysaccharide (LPS)/D-galactosamine (D-GalN) are a well-established hepatotoxicant, inducing liver damage which closely resembling to human viral hepatitis in its morphological and functional features [4].

LPS is a toxic component of the cell walls of gram-negative bacteria and is widely present in the digestive tracts of humans and animals [5]. D-GalN is an amino sugar selectively metabolized by hepatocytes [6]. The toxicity of D-Gal N is mainly related to depletion of uridine pools that are associated with limited ribonucleic acid (RNA), glycogen and protein synthesis, thus altering hepatocellular function [4].

Currently, hepatoprotective drugs are inadequate, so far no therapy has successfully prevented the progression of hepatic disease; even though newly developed drugs have been used to treat chronic liver disorders, these drugs have often have side effects. Therefore, a special interest has been directed to the use of dietary antioxidants of natural products in the treatment of such disorders and may have a significant role in maintaining health [7,8].

Propolis is considered as one of the most promising natural products has therapeutic and preventive actions. It contains more than 300 compounds from different groups. It contains mostly a mixture of polyphenols, flavonoids (major ingredients). Propolis can be used for treatment of cardiovascular, blood system, respiratory disorders, cancer, digestive tract disorders and dermatological disorders [9-11].

Propolis exhibits a wide spectrum of pharmacological properties such as antioxidant, anti-inflammatory, antibacterial, antiviral, anti-ulcerous, anticarcinogenic properties and immune system support [10,12].

The aim of this study was to evaluate the possible protective effect of propolis on experimental induced hepatitis in adult male albino rats.

Materials and methods
In this study, 40 adult male rats of average weight 150-250 g were used. The animals obtained from the animal house, Moshtohor faculty of Veterinary Medicine, Benha University, Moshtohor city, Egypt. Animals were housed in the animal laboratory at the medical research center of Benha faculty of medicine. Strict care and cleaning measures were utilized to keep the animal in a normal healthy state, the animals were kept in animal cages under the prevailing atmospheric conditions and also were fed to standard basal diet and liberal supply of tap water. All ethical protocols for animal treatment were followed and were supervised by the animal facilities. The experimental protocol was approved by the Ethical Committee of Benha faculty of medicine.

Used drugs
LPS (lipopolysaccharide from Escherichia coli, O55:BS), and N-acetyl D-GaIN, were purchased from Sigma-Aldrich Company-Saint Louis, Missouri, USA. Propolis was purchased from a beekeeper. It was dissolved in 1% gum acacia, forming an aqueous solution.

Diagnostic kits
1. For the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin using kits were purchased from Bio diagnostic Co, Cairo, Egypt.
2. For the determination of Tumour necrosis factor α (TNF-α): using the a commercially available enzyme-linked immunosorbent assay (ELISA) Ray Bio Rat TNF-α kit (Catalogue number ELR-TNF-α 001, Ray Biotech Inc., 3607 Parkway Lane, Suite 100, Norcross GA 30092, USA).

Experimental procedure
Rats were divided into 4 groups, included 10 rats for each as follows

Group I (Control group)
The 5 rats received intraperitoneal injection of phosphate-buffered saline as vehicle for D-GalN and LPS. They were sacrificed at the same time as the corresponding experimental groups. The other 5 rats received 0.5 ml gum acacia dissolved in distilled water as vehicle for propolis. They were sacrificed at the same time as the corresponding experimental groups.

Group II (Propolis group)
Rats received an aqueous suspension of propolis 200 mg/kg orally by gastric tube daily for 2 weeks [7].

Group III (Hepatitis model group)
Rats received single intraperitoneal injection of D-GalN and LPS (300 mg/kg body weight and 30 μg/kg body weight) dissolved in phosphate-buffered saline 18 hrs before the end of experiment [8].

Group IV (Propolis and hepatitis model group)
Rats received an aqueous suspension of propolis 200 mg/kg orally by gastric tube daily for 2 weeks and received single intraperitoneal injection of D-GalN and LPS (300 mg/kg body weight and 30 μg/kg body weight) dissolved in phosphate-buffered saline 18 hrs before the end of experiment. The blood was drained from the tail vein by using capillary tubes immediately before the end of the experiment into Eppendorf tubes containing heparin (20 ml, 200 IU/ml). The plasma was separated by centrifugation (5000 rpm for 5 min) and used for biochemical analysis.

Biochemical measurements
Liver function tests
Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) [13], total bilirubin [14], albumin [15] were measured.

Tumor necrosis factor α (TNF-α)
Quantitative determination of serum (TNF-α) a hepatic proin-
Immunohistochemistry study
1. Immunohistochemical staining for detection of anti-proliferating cell nuclear antigen (PCNA); the primary monoclonal antibody used was anti-PCNA IgG antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The cellular site of the reaction was nuclear and brown color.
2. Immunohistochemical staining for detection of transforming growth factor (TGF-β1); the primary monoclonal antibody used was TGF-β1 IgG antibody (Biogen Inc., Cambridge, Massachusetts, USA). The cellular site of the reaction was cytoplasmic and brown color.

Immunohistochemical study was conducted using the avidin-biotin peroxidase method. Briefly, sections of about 5 μm were deparaffinized, rehydrated, rinsed in tap water, and embedded in 3% H2O2 for 10 min to block endogenous peroxidase. The sections were treated initially with 2% trypsin at 37°C for 10 min in order to increase the sensitivity of the immunoperoxidase staining method. Sections were immersed in an antigen retrieval solution (10mmol/l sodium citrate buffer, pH 6) and subjected to heat-induced antigen retrieval for 20 min in a microwave. Nonspecific protein binding was blocked by a blocking solution (phosphate buffer solution (PBS) and 10% normal goat serum). The slides were incubated for 30 min with the diluted primary antibody using PBS. Drops of streptavidin peroxidase were added to the slide, left for 20 min and then washed with PBS for 5 min. Diaminobenzidine (Dakopatts, Glostrup, Denmark) was added to slides as a chromogen, after which the slides were washed with distilled water. Finally, the sections were counterstained with hematoxylin, dehydrated, rendered transparent with xylene, mounted and observed under a light microscope. For the negative control the specific primary antibody was replaced by phosphate-buffered saline.

Morphometric study
The mean area percentage (%) of PCNA and TGF-β1 reaction were quantified in 10 images for each group using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA). Mean area percentage of PCNA reaction and TGF-β1 in group IV (Propolis and hepatitis model group) compared with group III (Hepatitis model group) using the t-test, with P<0.05 as the level of statistical significance.

Statistical analysis
Statistical analyses were carried out using IBM SPSS statistics software for Windows, Version 20 (IBM Corp., Armonk, NY, USA).

All data were expressed as mean±SD. The significance of differences between mean values was analyzed by using the t-test, with P<0.05 as the level of statistical significance.

Result
Biochemical results
As shown in Table 1, in group III compared to control group, the liver injury markers of ALT, AST, total Bilirubin and TNF-α were very highly significant increase (p<0.001), but albumin was very highly significant decrease (p<0.001). In group IV compared to group III, ALT was significant decrease (p<0.05), AST was very highly significant decrease (p<0.001), total Bilirubin was highly significant decrease (p<0.01) and TNF-α was very highly significant decrease (p<0.001), but albumin was highly significant increase (p<0.01).

Histological results
Haematoxylin and eosin
Group I (Control group)
The liver sections of the group I showed cords of normal hepatocytes radiating from central vein. Hepatocytes are polyhedral in shape with central vesicular nuclei with prominent nucleoli and acidophilic cytoplasm. Some hepatocytes may be binucleated. Silt like spaces lined by endothelial cells and kupffer cells representing blood sinusoid were seen between the cords of hepatocytes (Figure 1A). The portal tract contained a branch of portal vein, a branch of hepatic artery and a branch of bile duct (Figure 1B).

Group II (Propolis group)
No histological changes were observed in the group II as compared to that of the control group.

Group III (Hepatitis model group)
Showed signs of degeneration and necrosis as some swollen
hepatocytes, central vein and blood sinusoids were seen (Figure 1F).

Masson’s trichrome stain

**Group I (Control group)**
The liver sections of the group I showed little amount of collagen fibers around central vein and portal tract (Figures 2A and 2B).

**Group II (Propolis group)**
No detectable changes were observed in the distribution of collagen fibers in the liver of the group II as compared to that of the control group.

**Group III (Hepatitis model group)**
Showed apparent increase of collagen fibers around the central vein and portal tract and between cords of hepatocytes and blood sinusoids (Figures 2C and 2D).

**Group IV (Propolis and hepatitis model group)**
Showed preserved hepatic architecture as more or less normal hepatocytes had finely granular cytoplasm, other hepatocytes had small hyperchromatic nuclei. Monocellular infiltration and collapse of sinusoids between hepatocytes were seen (Figure 1C). Empty hepatocytes that contained wisp of cytoplasmic remnants were seen. The nuclei of some hepatocytes showed karyolytic changes (*) and the wall of blood vessel is thickened by inflammatory cells (I). Notice collapse of sinusoids between hepatocytes. [H&E X400].

**Figure 1. Photomicrograph of liver sections stained with hematoxylin and eosin.**

(A) Liver section from group I showing cords of normal hepatocytes (H) radiating from central vein (V). Hepatocytes are polyhedral in shape with central vesicular nuclei and acidophilic cytoplasm. Slit like spaces lined by endothelial cells representing blood sinusoid are seen between the cords of hepatocytes (↑) [H&E X400]. (B) Liver section from group I showing portal tract with portal vein (V), hepatic artery (A) and bile duct (*) [H&E X400]. (C) Liver section from group III showing monocellular infiltration (I). Some swollen hepatocytes show finely granular cytoplasm (↑). The hepatocytes show small hyperchromatic nuclei (arrow head ^). Notice collapse of sinusoids between hepatocytes. [H&E X400]. (D) Liver section from group III showing empty hepatocytes that contain wisp of cytoplasmic remnants (*). The nuclei of some hepatocytes show karyolytic changes (↑) and the wall of blood vessel is thickened by inflammatory cells (I). [H&E X400]. (E) Liver section from group III showing dilated congested central vein (V). Notice swelling and proliferation of endothelial cells of central vein (*) and its wall show inflammatory cells (I). [H&E X630]. (F) Liver section from group IV showing more or less normal hepatocytes (H), central vein (V) and blood sinusoids (↑). [H&E X400].

**Figure 2. Photomicrograph of liver sections stained with masson trichrome.**

(A) Liver section from group I showing few collagen fibers around central vein (↑). [Masson’s Trichrome X400]. (B) Liver section from group I showing few collagen fibers around portal tract (↑). [Masson’s Trichrome X400]. (C) Liver section from group III showing apparent increase of collagen fibers around dilated congested central vein and blood sinusoids. [Masson’s Trichrome X400]. (D) Liver section from group III showing apparent increase of collagen fibers around portal area and blood sinusoids. [Masson’s Trichrome X400]. (E) Liver section from group IV showing apparent scanty collagen fibers around central vein (↑). [Masson’s Trichrome X400]. (F) Liver section from group IV showing apparent scanty collagen fibers in the portal area (↑). [Masson’s Trichrome X400].
Group IV (Propolis and hepatitis model group) showed apparent scanty collagen fibers around the central vein and portal tract (Figures 2E and 2F).

Immunohistochemistry results

*PCNA immunohistochemical stain*

**Group I (Control group)**
The liver sections of the control group showed few brown PCNA positive nuclei among few hepatocytes (Figure 3A).

**Group II (Propolis group)**
No detectable changes were observed to PCNA expression in the group II as compared to that of the control group.

**Group III (Hepatitis model group)**
Showed increase the number of brown PCNA positive nuclei among hepatocytes (Figure 3B).

**Group IV (Propolis and hepatitis model group)**
Showed decrease the number of brown PCNA positive nuclei among hepatocytes (Figure 3C).

*TGF-β1 immunohistochemical stain*

**Group I (Control group)**
The liver sections of the control group showed weak positive immunoreaction for TGFβ1 in the wall of central vein and blood sinusoids (Figure 4A).

**Group II (Propolis group)**
No detectable changes were observed to TGFβ1 expression in the group II as compared to that of the control group.

**Group III (Hepatitis model group)**
Showed strong positive immunoreaction for TGF-β1 in the wall of central vein, portal vein and blood sinusoids (Figures 4B and 4C).

**Group IV (Propolis and hepatitis model group)**
Showed moderate positive immunoreaction for TGF-β1 in the
wall of central vein and blood sinusoids (Figure 4D).

**Morphometric results**
The mean area % of PCNA and TGF-β1 reaction for studied experimental groups was represented in (Tables 2 and 3) and Histograms 1 and 2. There were significantly decreased (P<0.05) in PCNA and TGF-β1 reaction in group IV compared with group III.

**Table 2. Showing the mean area % and ±SD of PCNA reaction in all experimental groups.**

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<th>Group I</th>
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<th>Group III</th>
<th>Group V</th>
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<tr>
<td>Mean area %</td>
<td>0.06</td>
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<tr>
<td>±SD</td>
<td>0.027</td>
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SD: Standard deviation; S: Significant at P<0.05 Group IV compared with group III.

**Table 3. Showing the mean area % and ±SD of TGF-β1 reaction in all experimental groups.**

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<tr>
<td>Mean area %</td>
<td>2.16</td>
<td>2.02</td>
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<tr>
<td>±SD</td>
<td>0.161</td>
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SD: Standard deviation; S: Significant at P<0.05 Group IV compared with group III.

**Discussion**
Hepatitis remains as a clinical challenge and a problem of great importance in the worldwide today and affect a great number of population especially in Egypt [18].

In the present study our group III revealed a very highly significant increase of AST, ALT, total bilirubin and TNF-α but there was a very highly significant decrease of albumin as compared to control group.

Serum ALT and AST a cytoplasmic enzymes were released into circulation after cellular damage in acute hepatotoxicity and were considered the most sensitive markers used in the diagnosis of hepatic damage [19].

Our results were in agreement with the findings of some investigators who reported that a liver damage induced by D-GalN/LPS reflected the disturbances of liver cell metabolism which leads to characteristic changes in the liver serum enzymes and albumin [3,4,18,20].

Serum bilirubin was considered as an index for the assessment of hepatic function and any abnormal increase indicates hepatobiliary disease [21].

D-galactosamine (D-GaIN) together with lipopolysaccharide (LPS) can lead to pronounced secretion of pro-inflammatory cytokine such as TNF-α by Kupffer, stellate and sinusoidal endothelial cells which had been shown to be early and important mediators of liver injury and hepatic necrosis [3,22]. Some authors clarified that the D-GaIN/LPS induced hepatocyte apoptotic changes and enhance the expression of cytokines TNF-α [6,18].

The histological examination of liver sections of group III of the present study revealed signs of degeneration and necrosis such as some swollen hepatocytes had finely granular cytoplasm, other hepatocytes had small hyperchromatic nuclei or karyolytic changes. Monocellular infiltration and collapse of sinusoids between hepatocytes were seen. Empty hepatocytes that contained wisp of cytoplasmic remnants were seen. The wall of blood vessel was thickened by inflammatory cells. Central vein was dilated and congested and its wall infiltrated by inflammatory cells. Swelling and proliferation of endothelial cells of central vein (central phlebitis) were seen.
These results were in agreement with previous studies indicating that during D-GalN/LPS induced hepatitis, there were severe hepatocellular degeneration, necrosis and mononuclear cellular infiltration [20,23,24]. Some authors clarified that the D-GalN/LPS induced marked cellular infiltration, is due to ROS production that indirectly regulate chemokine receptor expression and promote cytokine IL-6, IL-8 and TNF-α which are key modulators of inflammatory response [4]. Some investigators reported that central phlebitis characterized by swelling and proliferation of endothelial cells of central vein [25]. The release of aminotransferases by the damaged hepatocytes was strongly supported the previous histological observations. An apparent increase in collagen fibers around the central vein and the portal areas was seen in the present study. This was supported with strong positive immunoreaction for TGF-β1 in the wall of central vein, portal vein and blood sinusoids.

Our results were in accordance with other researchers who stated the expression of TGF-β1 could be correlated with the degree of liver damage to cirrhosis in chronic viral hepatitis [26]. Liver fibrosis, an early stage of cirrhosis, was a common consequence of chronic injury from many causes as infection and toxic drugs and characterized by the accumulation of extracellular matrix (ECM) proteins which; due to imbalance in their production, deposition, and breakdown. Prolonged liver injury result in hepatocytes damage, which triggers activation of hepatic stellate cells (HSCs). TGF-β1 was produced by HSCs and Kupffer cells and was believed to be involved in the synthesis and deposition of extracellular matrix components like fibronectin, collagens type I, III, and IV. TGF-β1 had a dual impact on the progression of liver disease by promoting fibrogenesis and inducing hepatocytes apoptosis [27-29].

Liver fibrosis was caused by a combination of multiple mechanisms, such as acute and chronic hepatocyte damage, recruitment and activation of inflammatory cells as macrophages, eosinophil and neutrophils, release of inflammatory and fibrogenic cytokines, production of reactive oxygen species; activation of hepatic stellate cells (HSCs), periportal or perivenular fibroblasts, circulating fibrocytes, and bone marrow cells that transdifferentiate into collagen producing myofibroblasts [29-31].

Portal hypertension was a major complication of advanced liver cirrhosis. The main reason of portal hypertension was pathologically elevated intrahepatic resistance to portal blood flow due to fibrosis or cirrhosis caused by different chronic mainly inflammatory stimuli. The site of the increased resistance may be prehepatic (portal vein obstruction) or posthepatic (hepatic vein obstruction) [32]. An increase in the number of brown PCNA positive nuclei among hepatocytes were seen in group III of the present study. Proliferating cell nuclear antigen (PCNA) was a marker of cell proliferation.

PCNA found in cell nucleus and was directly involved in DNA replication. Its expression was increased at late G1 stage and early S stage. Previous studies had demonstrated that PCNA labeling index increased sequentially from normal tissue through premalignant stage to carcinoma of various tumors [33,34].

Some investigators reported PCNA had been also found to induce apoptosis in cells subjected to oxidative stress [35]. Our findings were in accordance with those of previous studies who suggested that the ability of cell proliferation become stronger and this was closely related to malignant cell proliferation and carcinogenesis in livers of D-GalN/LPS treated rats [36-38].

In many liver disorders, inflammation, fibrosis and apoptosis are important pathogenic components, finally leading to acute liver failure [39].

Some researchers demonstrated that the mechanism of action of D-GalN/LPS induced hepatitis mediated through release of the oxidized products of oxidative stress such as reactive oxygen species (ROS) and different cytokines released by Kupffer cells (KC) of the liver such as tumor necrosis factor (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6). These ROS of oxidative stress were altering and damaging the cell compounds as protein and lipid as well as membranes and leading to increase lipid peroxidation and hepatic damage characterized by hepatocellular necrosis, inflammation and apoptosis [4,5,22,40].

In the present study group IV revealed a marked improvement of biochemical parameter, histological changes of liver. Moreover, a decrease in the number of brown PCNA positive nuclei among hepatocytes and moderate positive immunoreaction for TGF-β1 in the wall of portal vein and blood sinusoids.

These results are in agreement and confirming the findings of some researchers who reported the protective effect of propolis against hepatotoxicity as it contains higher content of total phenolic compounds [7,9,41]. Some researchers reported that the protective effect of propolis was due to its antioxidant properties, as it acts as a free-radical scavenger and lipid peroxidation inhibitor. Further it was increasing the intracellular concentration of glutathione and superoxide anions and antioxidant enzymes [42]. Moreover propolis was improving the activity of hepatic microsomal drug metabolizing enzymes [10].

Propolis had potent anti-inflammatory and immunomodulatory agents which acted through inhibiting neutrophil infiltration, suppression of proinflammatory cytokines or mediators as tumor necrosis factor-α and interleukins and stimulation of macrophages and thus influencing specific and nonspecific immune defense mechanisms [11,43]. Some researcher reported that propolis had been shown to ameliorate fibrosis due to its antioxidant properties [12]. Other investigators had reported that propolis might exert a modulatory effect on cytokine TGF-β1 induced fibrosis [44]. Some
investigators reported that the propolis had antiproliferative and anticarcinogenic activity [34,45].

**Conclusion**

Acute D-GALN/LPS induced hepatitis model caused an increase of oxidative stress and degenerative changes of liver. Propolis had a potential protective effect and preventing degenerative changes of liver caused by D-GALN/LPS as it had antioxidant, anti-inflammatory enzyme and antiapoptotic activities. These results should provide a new insight in treating patients with acute hepatitis and thus the intake of propolis as supplement should be advised and might serve as clinically useful hepato-protective natural product in future.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contribution**

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