Investigation of the effects of sildenafil on liver and remote organ in hepatic ischemia-reperfusion damage

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Introduction
The aim of this study was to demonstrate whether sildenafil is effective in minimizing and/or eliminating hepatic ischemia/reperfusion injury effects. For this purpose, the authors experimentally performed biochemical and histopathological examinations of the included rats using the hepatic ischemia/reperfusion model.

Materials and methods
The authors used 40 animals, with 10 rats in each group, in this study. Ischemia was applied 30–45 min with the hepatoduodenal ligament clamping, and then reperfusion is started. The rats were grouped as follows: the first group, only laparotomy; the second group, laparotomy and sildenafil; the third group, hepatic ischemia-reperfusion; and the fourth group, hepatic ischemia-reperfusion and sildenafil. During experimental studies, sildenafil capsules were opened, and appropriate dose required for animals had been created with the weighing scales. Then, the powder was diluted with saline. The authors gave sildenafil through oral gavage 15 minutes before the ischemia. 60 min after starting the experiment in 1–2 groups and 30 min after beginning reperfusion in 3–4 groups (60 min after beginning the experiment in all groups), blood was taken from the animals for biochemical analysis, and the animals were sacrificed. Simultaneously liver, lung, and kidney tissues were removed for biochemical and histopathological examination.

Results
Based on plasma evaluation, total antioxidant status was lower (P=0.0274) in ischemia/reperfusion group compared with ischemia/reperfusion+sildenafil group. However, there was no difference between the groups regarding total oxidant status values (P=0.0274). When comparing total antioxidant status and oxidative stress index in liver tissue, a statistically significant difference was observed between groups (P=0.012766 and P=0.004081), but on comparing histopathological scores, there was no difference between groups (P=0.1244).

Conclusion
Sildenafil partly reduced the effects of hepatic ischemia-reperfusion injury on the liver and distant organs, although this difference was not statistically significant.

Keywords:
hepatic ischemia-reperfusion, liver, remote organ, sildenafil

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Introduction

Liver ischemia/reperfusion injury may occur during liver trauma, large tumor resections, and liver transplantation. Reactive oxygen radicals cause more damage during reperfusion, as ischemia-induced oxygen deprivation causes severe damage [1]. In animal studies, sildenafil has been shown to protect alveolar growth and angiogenesis and reduce inflammation [2]. Inhibition of c-GMP metabolism leads to smooth muscle relaxation around the arterioles feeding the corpus cavernosum with nitric oxide-dependent mechanism. PDE-5 inhibition locally increases c-AMP and c-GMP concentrations, leading to the relaxation of pulmonary vascular smooth muscles [3]. Sildenafil causes the formation of e-NOS and iNOS with NO-dependent mechanism. c-AMP and c-GMP are the most important intracellular secondary messengers that play an active role in cellular events such as inflammation [4,5]. Studies have shown that sildenafil reduces oxidative stress and inflammation [6,7]. In another animal study, the protective effect of sildenafil against oxidative stress and inflammation has been demonstrated in diabetic rats [8]. Many studies have been done to minimize ischemic liver injury and reperfusion injury that

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develops afterward. The purpose of this study was to investigate the efficacy of anti-inflammatory and antioxidant effects in the foreground by giving sildenafil to the rats in preventing hepatic ischemia/reperfusion injury after Pringle maneuver by preventing and evaluating its effects on distant organs such as lung and kidney. Experimental ischemia/reperfusion was created in rats. The efficacy of sildenafil which was given by oral gavage before the injury, was determined in real terms by examining the biochemical and histopathological analysis of tissue and blood samples taken from the liver, kidneys, and lungs, and it was compared with a control group.

Materials and methods

Animals

Rats were preferred as the experimental animals because of being the main species used in biomedical research, their size, and ease of care. This study was prepared in accordance with the Declaration of Helsinki. It was approved by Dicle University Prof. Dr. Sabahattin Payzın Health Sciences Research and Application Center Local Ethics Committee for Animals Experiments (protocol no: 2013/54). For this purpose, under the conditions of Dicle University Health Sciences Research and Application Center (DÜSAM), 40 Wistar Albino rats of average weight (250–300 g) were randomly selected. All rats were kept in a cage for five rats throughout the study. Rats were fed with water for 12 h daytime at 25°C and standard rat food for 12 h night periods. Chlorinated tap water was used as water.

Experimental design and surgical procedures

Animals were intramuscularly injected with 50 mg/kg ketamine hydrochloride (Ketalar, Parke Davis, ECZACIBAŞI, Istanbul, Turkey) and 10 mg/kg Xylazine (Rompun, Bayer AG, Leverkusen, Germany) for anesthesia, and the experimental procedure was started. Following the shaving of the abdomen, the skin was cleaned with a 10% povidone-iodine solution (Betadine). In sterile conditions, the abdomen of the rats was opened with a midline incision. The liver, diaphragm, hepatoduodenal ligament, and adjacent organs were carefully dissected. Hepatoduodenal ligament (v. port, arteria hepatica communis, and choledochal canal) was revealed. After a pack of rubber was placed on top of the hepatoduodenal ligament, it was suspended by turning around the hepatoduodenal ligament with 3.0 silk sutures, and the ischemia period was initiated by performing a pringle maneuver. Following the 30-min ischemic period, the rubber was opened, and the 30-min reperfusion period was initiated. At the end of this period, blood was taken from the heart and the animals were sacrificed. A total of 40 Wistar Albino female rats were included in the study, and the groups were formed as follows:

(1) Group I (Sham): hepatoduodenal ligament dissection was performed, and no medication was given.
(2) Group II (control): in addition to group 1, sildenafil was given orally at a dose of 200 mg/kg by oral gavage (according to T_{max}) 15 min before starting an experimental study.
(3) Group III (Ischemia/reperfusion): following the 30 min Pringle maneuver, 30 min reperfusion was performed, and no medication was given.
(4) Group IV (Ischemia/reperfusion+sildenafil): in addition to the performed procedures in group 3, sildenafil was given orally at the dose of 200 mg/kg (according to T_{max}) 15 min before the ischemia period was started.

At the end of the experimental procedures in each group, blood samples were taken for biochemical tests and histopathological examinations, and tissue samples were taken from the liver, both lungs, and kidneys. The plasma obtained by rapidly centrifuging the blood was transferred to plastic Eppendorf capped tubes for biochemical analysis and stored in a −80°C freezer. The tissues were separated according to biochemical analysis. Blood and foreign tissue residues were removed by washing with saline and subsequently placed in suitable storage containers (plastic Eppendorf capped tubes) and transferred to −80°C freezer. Moreover, for pathological evaluation, the tissues in question were taken in appropriate amounts in plastic and capped containers in 10% formaldehyde solution and stored for analysis of parameters specific to that sample in the relevant plasma and tissue samples.

Homogenization of tissues

Until the day of analysis, tissues were stored at 80°C and were removed from the freezer on the working day and brought to the laboratory in dry ice environment. A volume of 2 ml Tris-HCl buffer was added to ~0.30–0.50 g tissue piece, which was taken into the tube by paying attention to the cold chain. The tissue was treated in 14 000 cycles (rpm) in mmol/l pH 7.0 PBS. During this period, a buffer was added to the final volume 10 times the tissue weight. Homogenate was centrifuged at +4 for 30 min. Samples were taken from the supernatant for total oxidant status (TOS) and TAS analysis.

Biochemical analysis

Total antioxidant status (TAS) and TOS analyses were performed in the blood and the tissues. Moreover, the oxidative stress index (OSI) was calculated for the tissues.

Assessment of total oxidant activity

It is a fully automatic colorimetric method developed by Erel (50). Although the oxidants in the examples oxidize the ferrous ion-o-dianisidine complex to the ferric ion, the glycerol in the environment accelerates this reaction and triples it approximately three times. Ferric ions form a colored complex with 'xylenol orange' in an acidic environment. The intensity of the color related to the amount of oxidants in the sample was measured spectrophotometrically. Tissue TOS values were calculated as nmolH_2O_2Equiv/mg protein.

Assessment of total antioxidant capacity

This method is a current method that can measure the body's total antioxidant capacity against fully automatic and powerful free radicals developed by Erel (50-51). In this method, hydrogen peroxide with the complex of Fe^{2+}-o-dianisidine reacts with Fenton and causes the formation of OH radical. This OH radical is reduced and interacts with the colorless o-dianisidine molecule at low pH to form yellow-brown dianisidyl radicals. Dianisidyl radicals increase this color formation by participating in further oxidation reactions. However, antioxidants in the analysis samples suppress and prevent these oxidation reactions and reduce color formation. This reaction was measured spectrophotometrically on an automated analyzer. While blood TAS values were calculated, μmol was calculated as Trolox Equiv/L and tissue TAS values were calculated as nmol Trolox Equiv./mg protein.

Assessment of the OSI is an indicator parameter of the degree of oxidative stress; its formulation is as follows (52): OSI=(TOS/TAS)×100.

Histologic examinations

It was revealed by scoring from mild to severe injury in the liver, kidney, and lung tissues. Tissues were prepared in 10% formalin solution and placed in paraffin blocks; 4-μm sections were prepared, and a standard protocol was applied by staining with hematoxylin-eosin. Hepatic ischemia-reperfusion injury was graded as follows: grade 0, minimal or no damage; grade 1, mild damage including cytoplasm vacuolization and focal nuclear pianos; grade 2, moderate-severe damage, including enlarged nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular boundaries; and grade 3 was classified as severe necrosis accompanied by the disintegration of hepatic ligaments, severe damage, including bleeding and neutrophil infiltration. Lung injury secondary to hepatic ischemia/reperfusion was graded as follows: grade 0, no change; grade 1, mild neutrophil leukocyte infiltration and mild to moderate interstitial congestion; grade 2, moderate neutrophil leukocyte infiltration, perivascular edema formation, and fragmentation of the pulmonary structure; and grade 3 was defined as intense neutrophil leukocyte infiltration and complete damage to the pulmonary structure. Kidney damage related to hepatic ischemia/reperfusion was graded as follows: Grade 0; no diagnostic changes, grade 1; swelling of tubular cells, loss of brush edges, and nuclear condensation with up to 1/3 of tubular profile showing nuclear loss, grade 2; greater than 1/3 and less than 2/3 of tubular profile showing nuclear loss, grade 3; more than 2/3 of tubular profile showing nuclear loss.

Statistical analysis

The data obtained from the groups were transferred to the computer. The data were entered into the Medcalc for Windows 11.5 (SPSS Inc., Chicago, Illinois, USA) package program. Kruskal–Wallis variance analysis was used for comparison between groups. Mann–Whitney *U* test was used for double comparison. $P<0.05$ level was accepted as significant. Spearman correlation test was used to evaluate the relationship between the parameters. Average (minimum–maximum) values were used for the histopathological and biochemical values [9].

Result

When the sham and sildenafil groups were compared in terms of TAS and TOS values, no significant difference was observed ($P=0.6365$ and 0.9164, respectively). When ischemia/reperfusion and ischemia/reperfusion+sildenafil groups were compared with sham the group, TAS and TOS values were significantly lower in the sham group ($P=0.0157$ and 0.0274 for the ischemia/reperfusion group and $P=0.0016$ and 0.0117 for the ischemia/reperfusion + sildenafil group, respectively). When comparing ischemia/reperfusion and ischemia/reperfusion+sildenafil groups with sildenafil group, TAS and TOS values were lower in the sildenafil group ($P=0.0054$ and <0.001 for TAS, and $P=0.0033$ and <0.001 for TOS, respectively). When the ischemia/reperfusion and ischemia/reperfusion +sildenafil groups were compared, TAS values were significantly lower in the ischemia/reperfusion group

Table 1 Distribution of TAS and TOS values by groups in tissues

	Groups	Median value	Minimum	Maximum
	Sham	2.140	1.7100	2.530
Liver				
TAS (nmol Trolox Equiv./mg)	Sildenafil	1.530	0.9500	1.910
	IR	2.375	1.3400	2.620
	IR+Sildenafil	2.540	1.6200	2.950
	Sham	59.805	42.1700	108.440
Liver				
TOS (nmol H_2O_2 Equiv./mg)	Sildenafil	58.5 00	32.8400	106.550
	IR	60.465	29.9700	148.180
	IR+Sildenafil	43.720	22.6400	61.800
	Sham	2.140	1.7100	2.530
Lung				
TAS (nmol Trolox Equiv./mg)	Sildenafil	1.530	0.9500	1.910
	IR	2.375	1.3400	2.620
	IR+Sildenafil	2.540	1.6200	2.950
	Sham	59.805	42.1700	108.440
Lung				
TOS (nmol H_2O_2 Equiv./mg)	Sildenafil	58.500	32.8400	106.550
	IR	60.465	29.9700	148.180
	IR+Sildenafil	43.720	22.6400	61.800
	Sham	2.140	1.7100	2.530
Kidney				
TAS (nmol Trolox Equiv./mg)	Sildenafil	1.530	0.9500	1.910
	IR	2.375	1.3400	2.620
	IR+Sildenafil	2.540	1.6200	2.950
	Sham	59.805	42.1700	108.440
Kidney				
TOS (nmol H_2O_2 Equiv./mg)	Sildenafil	58.500	32.8400	106.550
	IR	60.465	29.9700	148.180
	IR+Sildenafil	43.720	22.6400	61.800

TAS, total antioxidant status.

(P=0.0274), whereas there was no significant difference in TOS values (P=0.0274). Distribution of TAS and TOS values of plasma in liver, lung, and kidney tissue according to groups is given in Table 1.

When the sham and sildenafil group were compared in terms of plasma interleukin (IL)-6 level, it was significantly higher in the sham group (P=0.0283). When sham group and ischemia/reperfusion group and ischemia/reperfusion+sildenafil group are compared regarding plasma interleukin (IL)-6 level, there was no significant difference between the groups (P>0.05). When the ischemia/reperfusion and ischemia/reperfusion+sildenafil groups were compared regarding plasma interleukin (IL)-6 level, no significant difference was observed between them (P=0.0547). When sham and sildenafil groups were compared in terms of plasma tumor necrosis factor (TNF)-α levels, no significant difference was observed between them (P=0.3472). When sham group and ischemia/reperfusion and ischemia/reperfusion +sildenafil groups were compared, TNF-α levels were significantly higher in ischemia/reperfusion and ischemia/reperfusion+sildenafil groups (P=0.0446 and 0.0285, respectively). When the ischemia/reperfusion and ischemia/reperfusion+sildenafil groups were compared, no significant difference was observed between them (P=0.88728). Rats treated with sildenafil had significant I/R injuries in the liver.

Discussion

Ischemia-reperfusion injury a complex process involving numerous intracellular signaling pathways, mediators, cells, and pathophysiological disorders [1]. Many studies have shown that the negative factor that causes damage is not caused by hypoxia but by adverse reactions that occur during the return of oxygenated blood to ischemic tissue. The damage that occurred during reperfusion is a two-phase model, starting with reoxygenation, the early phase, and the delayed phase. Early stage is associated with cellular damage and is between 2 and 6 h after reperfusion (reoxygenation), and late phase appears 18–24 h after reperfusion and is accompanied by neutrophil infiltration. Although free oxygen radicals

mediate injury in the early stage (acute phase), damage in the late phase (subacute phase) is associated with an inflammatory response caused by neutrophil activity [9]. Free oxygen radicals can also initiate oxidative stress by causing lipid peroxidation [10].

Our study aimed to investigate the efficacy of sildenafil in the hepatic ischemia/reperfusion model, which was created by using the Pringle maneuver, owing to its antioxidant effects in preventing or minimizing the effects of free radical damage on the distant organs such as liver, kidney, and lung in the early stage of ischemia and reperfusion injury.

When plasma TAS values were compared, values were higher in study groups compared with sham and control groups. It was observed that the difference in value between sham and sildenafil group and study groups was significant. This is a result of the antioxidant systems being activated as a natural result of the resulting oxidative stress. Higher TAS values than silica/reperfusion group in the sildenafil group and ischemia/reperfusion+sildenafil group can be attributed to the antioxidant effect of sildenafil.

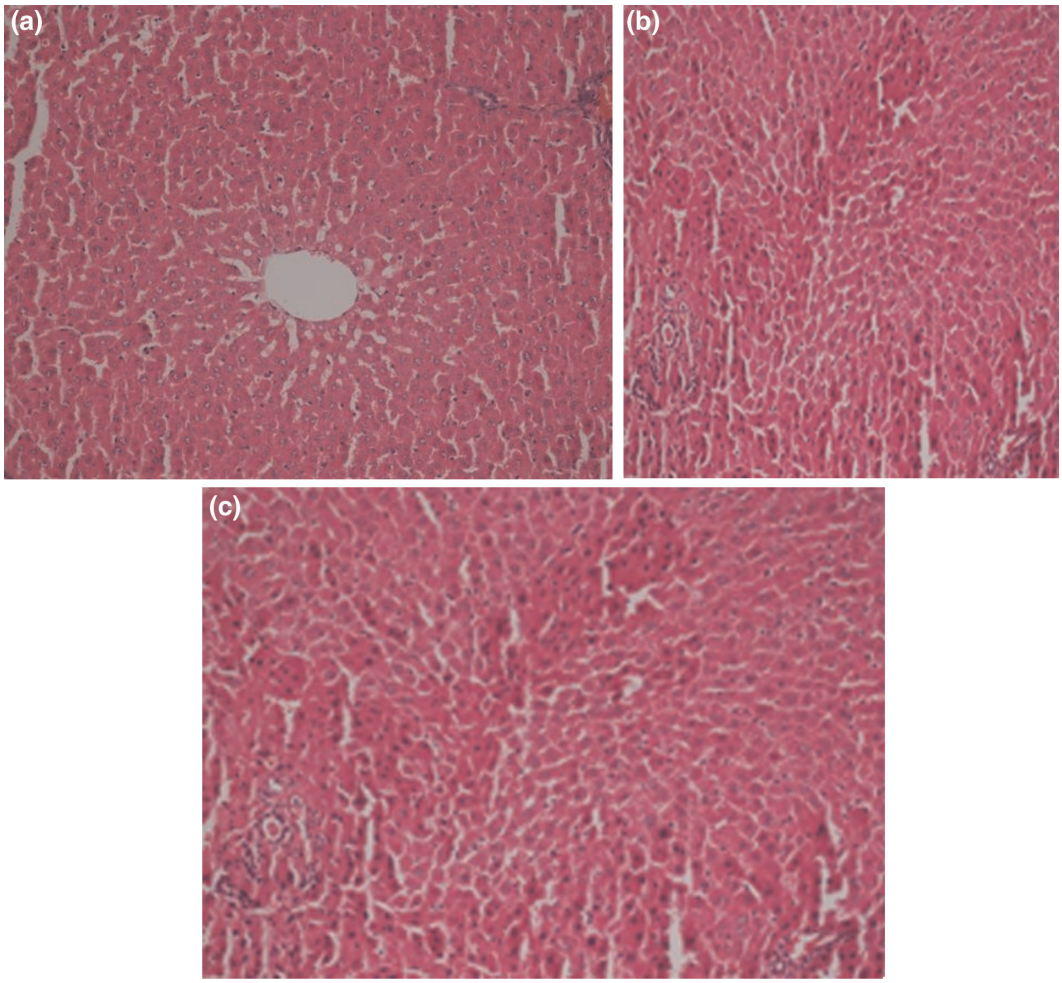
Hepatic ischemia-reperfusion injury causes distant organ damage, which includes organs such as the heart, lung, and kidney, as well as the liver [11]. In the light of the information obtained in our study, when the total oxidant activity in the liver tissue and the data from kidney and lung tissue were compared, it was observed that there was a positive correlation between them, but this relationship was statistically significant only with the kidney tissue, and the relation in the lung tissue was extremely weak. Abnormal activation of the radicals and related Kupffer cells plays a role [12]. This causes structural and functional changes in the liver [1]. In our study, to reveal liver damage, besides TOS and TAS values, histopathologically, cytoplasm vacuolization, nuclear pyknosis, cytoplasmic hypereosinophilia, loss of intercellular boundaries, necrosis, bleeding, and neutrophil infiltration were also investigated. Although TOS values similar to the sham group were found in the control group, TAS values were significantly higher. In the sham group, both TOS and TAS values were lower than the ischemia/reperfusion group. Although the TAS values were found to be significantly higher in the ischemia/reperfusion + sildenafil group compared with the ischemia/reperfusion group, this difference was not statistically significant although the TOS values were lower. Histopathologically normal findings except focal nuclear pyknosis and cytoplasmic vacuolization were found in sham and control groups. Neutrophilic infiltration with moderate-severe damage, loss of intercellular boundaries, and bleeding areas were observed in the study groups (Fig. 1). These results showed that sildenafil had a moderate positive effect on hepatic damage, but the level of significance of this effect was low.

The development of acute renal failure after major liver ischemia/reperfusion is extremely common (40–85%), resulting in high mortality and morbidity in the perioperative period [11]. It is stated that hepatic ischemia/reperfusion moderately impairs kidney function [13]. Zahran *et al.* [14] showed that sildenafil activates antioxidant and antiapoptotic genes and inhibits proinflammatory cytokine genes in a rat model in which they studied renal ischemia/reperfusion injury. Medeiros *et al.* [15] showed that sildenafil had a protective effect in normothermic I/R-exposed kidneys by scintigraphy and histomorphometry. TOS values were significantly higher in the ischemia/reperfusion group.

When the groups are evaluated histopathologically, although mild swelling in tubular cells is found infrequently with normal findings in the sham and control group, in the study groups, swelling of tubular cells, loss of brush edges, nuclear condensation showing nuclear loss and nuclear losses up to 2/3 of tubular structures are observed, in addition to the ischemia/reperfusion group. It was observed that nuclear losses included more than 2/3 of tubular structures, but this difference was not statistically significant (Fig. 1). The effects of sildenafil in kidney damage after hepatic ischemia/reperfusion were moderate, but limited.

In our study, it was proved that TNF and IL-6 levels and W/D weight ratio increased in lung tissue of sildenafil applied before the procedure. This showed that the inflammation of the lungs increased in the early reperfusion injury of sildenafil applied before the procedure. As a result, sildenafil applied before the procedure increases the ischemia-reperfusion damage in the lung early. In our study, it was also observed that the TOS and TAS values were lower in the sham and control groups compared with the study groups, whereas the ischemia/reperfusion+sildenafil group had moderate lower TOS and higher TAS values compared with the ischemia/reperfusion group. Histopathologically in lung tissue, although normal findings and mild neutrophil leukocyte infiltration were detected in the sham and control groups, mild to moderate interstitial congestion was detected. Although mild-moderate neutrophil leukocyte infiltration was detected in interstitial

Figure 1



(a) Near-normal liver tissue in a rat in the control group (H&E, 200×). (b) Near-normal liver tissue in a rat in the SL group (H&E, 200×). (c) Liver tissue with score 2 changes in one rat in the IR group (H&E, 200×). H&E, hematoxylin and eosin.

congestion, it was seen that perivascular edema formation and fragmented pulmonary structures were accompanied in part by the ischemia/reperfusion group.Alper *et al.* [5] have shown that rats with bleomycin-induced lung fibrosis are useful by preventing lipid peroxidation, cytokine production and/or release, and neutrophil accumulation in sildenafil. However, our data support these findings, unlike the literature; although there was a correlation with total oxidant activity occurring in kidney tissue, it did not find a significant correlation with total oxidant activity measured in liver tissue. Ota *et al.* [16] showed that the main factor determining lung damage in the hepatic ischemia-reperfusion model is ventilation, and in cases with low tidal volume ventilation, there is no lung damage, and in high tidal volume ventilation, the damage is higher. The fact that there were better histopathological findings in the sham and control groups compared with the study groups is a natural result of ischemia/reperfusion injury. However, as there is no significant difference in ischemia/reperfusion and ischemia/reperfusion+sildenafil group, it can be attributed to the fact that there is no change in the ventilation volumes of animals and no effective effect on changes in sildenafil.

Conclusion

In our experimental study, as a result of hepatic ischemia-reperfusion injury we created owing to Pringle maneuver, significant damage was also observed in distant organs, especially the liver, kidney, and lung. Although sildenafil appears to partially reduce this damage in the liver and distant organs, this difference is not statistically significant. Larger comparative experimental and clinical studies are needed to clinically recommend the use of sildenafil alone or for nutritional support during the preoperative preparation phase.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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