The Effect of Chrysin on Liver Damage Induced By Subchronic Toxicity of Formaldehyde in Rats

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Objective: This study examines the adverse effects of intraperitoneally administered formaldehyde (FA) on liver and potential protective effects of chrysin (CH) against FA exposure.

Methods: 42 Wistar albino male rats were divided into 6 groups as follows: group I: control; group II: CH (50 mg/kg); group III: 0.1 mg/kg Formaldehyde (FA-0,1); group IV: 1 mg/kg Formaldehyde (FA-1); group V: CH (50 mg/kg) treatment and 0,1 mg/kg formaldehyde application (FA-0,1+CH); group VI: CH (50 mg/kg) treatment and 1 mg/kg formaldehyde application (FA-1+CH). At the finish of the investigation, the livers were removed.

Results: The levels of thiobarbituric acid reactive substances (TBARS), decreased glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) in liver sections were analyzed. In the groups that received only FA, a significant increase in the levels of TBARS, GSH and CAT was observed as markers of oxidative stress, while the SOD levels significantly decreased. In the groups treated both with FA and CH, the biochemical values were partially corrected towards those of the control group. In addition, the liver tissues were examined histologically. Histopathological damage was observed in the livers of rats treated with FA alone, but the lesions were less severe or were absent in the rats treated with both FA and CH.

Conclusion: FA exposure causes severe damage to liver and CH can be said to have a protective effect against such damages.

Keywords: Formaldehyde, chrysin, liver, antioxidant, histopathological damage, biochemistry.

Introduction

Formaldehyde (FA) is a colorless chemical that is readily soluble in water and has a very strong and distinctive odor. It is the simplest member of the aldehyde family [1, 2]. Exposure to FA occurs through skin contact, inhalation or through oral exposure. After entering into the body, FA is oxidized to formic acid in liver and erythrocytes. It is the enzyme formaldehyde dehydrogenase (FDH) that oxidizes FA [3]. In the reaction in which FDH takes a catalytic role, glutathione acts as a cofactor. Formic acid excreted in the urine or feces, or broken down to carbon dioxide and eliminated via lungs [1-5]. FA has toxic effects on some systems including the respiratory, central nervous and digestive systems [6, 7]. FA is widely used in many substances that we use in our daily lives such as industrial products, cleaning materials and cosmetic products, and in most of the work areas. The toxic effects of FA exposure on skin, eyes, testes, respiratory system, central nerve system and digestive system have been confirmed by various studies [5].

Experimental studies have reported that FA causes centrilobular vacuolization and focal cellular necrosis in the liver. Administration of FA to rats has been to cause

mononuclear cell infiltration in liver tissues in the portal area and around central veins [8].

Given their chemical structure and biological functions, flavonoids are among the most important compounds in the phenol groups [9, 10]. The potent antioxidant activity they have is considered as one of the most important properties of flavonoids. Studies have shown that they are usually distributed in plants and cannot be synthesized by humans [10-14]. Chrysin (CH) is one of the flavonoids on which a large number of studies have been conducted in recent years. There are various studies on the effects of CH, that sought to detect the mechanisms of how these effects occur in the target systems. CH is believed to contribute to the prevention of toxic effect and cancer development by means of decreasing the level of free radicals and inactivating carcinogens Based on the findings of these studies, mostly conducted on animals, CH is believed to have anticarcinogenic [15-18], antioxidant [19-21], anti-inflammatory [22] and antiviral [23] properties.

In the light of this information, we studied the liver damage that might be induced by exposure to low concentrations of FA. As a protective substance, we used CH which has potent antioxidant properties against liver damage.

Material and Methods

This study was performed with permission in Inonu University Experimental Animals Ethic Committees (Protocol no: 2011/A-58). In our study, three-month-old male Wistar albino rats, weighing between 250 and 300 g, were used and were divided into 6 groups with 7 rats in each group. The rats were housed in separate cages in standard conditions, with a 12/12 h light–dark cycle and were given standard rat chow and water ad libitum in Inonu University Experimental Animals Laboratory. Two different concentrations of FA were administered.

Group 1: Control group was treated orally with 50 mg/kg corn oil.

Group 2: Group CH was treated orally with 50 mg/kg CH (CH 97%, Sigma-Aldrich C80105, Germany) dissolved in corn oil [19, 20].

Group 3: Group FA-0.1 received intraperitoneal injection of 0.1 mg/kg FA (formalin, Sigma-Aldrich Formaldehyde 37% solution, Deisenhofen, Germany).

Group 4: Group FA-1 received intraperitoneal injection of 1 mg/kg FA.

Group 5: Group FA-0.1+CH was treated with both CH (50 mg/kg) and FA (0.1 mg/kg).

Group 6: Group FA-1+CH was treated with both CH (50 mg/kg) and FA (1 mg/kg).

Treatment with FA and CH was given three times a week for a period of 60 days. In the groups that received both FA and CH, CH was administered one day earlier. At the end of the experimental period, the rats were decapitated and liver tissues were dissected out for biochemical (TBARS, GSH, CAT and SOD) and histological analysis.

Biochemical Analysis

The liver tissue samples stored in a deep freezer at -80 °C were thawed and weighed on the day of analysis. The tissues were homogenized in ice-cold 10% phosphate buffer and the homogenate was centrifuged at 14968 xg (RCF) for 1 to 2 minutes (IKA, Germany). The tissue homogenates were then centrifuged at 3885 xg (RCF), at +4 °C for 30 minutes and the supernatant was collected.

Measurement of TBARS Levels: TBARS levels were determined using the method developed by Esterbauer and Cheeseman [24]. Malondialdehyde reacting with thiobarbituric acid in the acidic environment at 90-95°C was rapidly cooled following the formation of pink-colored chromogen. After 10 minutes, absorbance of the samples was read at a wavelength of 532 nm in a spectrophotometer. The results were expressed as nmol/g wet tissue weight.

Measurement of GSH Levels: GSH analysis was conducted using Ellman's reagent and the level of reduced glutathione was measured through reading the absorbance of yellow-green substance formed after reaction of glutathione with 5.5 dithio-bis-2-nitrobenzoic acid at a wavelength of 410 nm in a spectrophotometer [25].

Measurement of CAT Activity: Catalase activity was measured using the method developed by Aebi, through recording the decrease in absorbance that occurred after adding tissue samples to 50 mM phosphate buffer (pH 7.0) containing H2O2 (0.500) at 240 nm for 10 seconds [26].

Measurement of SOD Activity: SOD activity was measured based on the method developed by Sun et al., through determining the inhibition of nitroblue tetrazolium (NBT) reduction with an O_2^--generator [27].

The Method of Histological Analysis: Extracted liver tissues were fixed in 10% formaldehyde solution. After being washed in tap water, the samples were dehydrated and cleared, and then embedded in paraffin. 4-5 μm thick tissue sections were cut from the paraffin blocks. After the deparaffinization and rehydration processes, the tissue sections were stained with hematoxylin-eosin (H-E) and periodic acid-Schiff (PAS). Stained preparations were examined using a Leica DFC-280 light microscope. To detect liver damage, hepatocytes were examined based on the manifestations of swelling of cells, increased eosinophilia of the cytoplasm and loss of the glycogen content. The tissues were scored as follows: 0: no damage, 1: mild damage, 2: moderate damage and 3: severe damage. Kupffer cells were counted in 10 different areas of the tissues stained with periodic acid-Schiff (PAS) under x40 magnification.

Statistical Analysis

Normality of the data was analyzed with Shapiro-Wilk test. Mann Whitney U test was applied because the data did not show normality. IBM SPSS Statistics 22.0 software was used for the analysis. The data represented as arithmetic mean (X) +/- standard deviation (SD) and the significance level was set at 0.05.

Biochemical results

Biochemical results are given in Table 1. TBARS and GSH levels of FA-1 and FA 0,1 groups were significantly increased compared to control group. When the level of CAT of the FA-1 group was increased, SOD levels were significantly decreased compared to the control group.

The levels of the TBARS of the groups treated with FA and CH (Groups 5 and 6) were significantly decreased compared with the groups treated with FA only (Groups 3 and 4). The GSH levels of the FA-1+CH group were significantly decreased when compared to the FA-1 group.

Histological Results

Control and CH Groups
No abnormalities were discovered.

FA Groups
In sections stained with H-E, some hepatocytes had intensely eosinophilic cytoplasm and dark, pyknotic nuclei. Some hepatocytes were found to have pale and swollen cytoplasm due to hydropic changes (Fig. 1A, 1B).

Changes were more common and apparent in the hepatocytes from Group FA-1. The number of hepatocytes with increased eosinophilia was significantly increased in Group FA-1, as compared to Group FA-0.1 (p=0.001). There was no statistically significant difference between the groups in terms of hydropic changes (p<0.05) (Table 2).

In addition, some of the sections from the FA groups exhibited apoptotic cells. Apoptotic cells were detected through their pyknotic nuclei and eosinophilic cytoplasm surrounded by a clear halo.

Another remarkable finding in the FA groups was the large number of binuclear hepatocytes observed in the sections (Fig. 1C, 1D).

In the sections stained with periodic acid-Schiff (PAS), it was observed that the number of PAS-positive hepatocytes decreased in the FA groups, as compared to the Control Group and CH groups (p=0.005). Although the decrease is greater in the Group FA-1 than in Group FA-0.1, the difference between the groups was not statistically significant (p>0.05) (Table II).

In the FA groups, it was observed that the number of PAS-positive Kupffer cells increased significantly compared to the control group (Fig. 1E). The number of Kupffer cells in Group FA-1 increased significantly compared to Group FA-0.1

FA+CH Groups
Administration of CH did not have a statistically significant effect on the histological changes observed in Group FA-0.1 (p>0.05).

The number of hepatocytes with increased eosinophilic cytoplasm and decreased glycogen content observed in Group FA-1 decreased in Group FA-1+CH, but this decrease was not

Table 1. TBARS (thiobarbituric acid reactive substances), GSH (glutathione), SOD (superoxide dismutase) and CAT (catalase) analyses of liver tissues (The letters "a, b, c, d" in the same column show statistical differences).

Groups	TBARS (nmol/g tissue)	GSH (nmol/ml)	SOD (U/mg protein)	CAT (k/mg protein)
Control	3.62 ± 0.31[a]	96.2 ± 2.77[a]	4.04 ± 0.39[a]	0.144 ± 0.024[a,c]
CH	4.04 ± 0.32[a]	95.8 ± 4.15[a]	4.19 ± 0.40[a]	0.103 ± 0.015[c]
FA-0.1	5.43 ± 0.22[b]	123.2 ± 2.89[b,c]	3.15 ± 0.34[a]	0.166 ± 0.015[a]
FA-1	9.06 ± 0.45[c]	170.2 ± 2.77[d]	1.90 ± 0.26[b]	0.275 ± 0.026[b]
FA-0.1+CH	5.08 ± 0.27[b]	103.3 ± 2.40[a,b]	3.35 ± 0.33[a]	0.179 ± 0.016[a]
FA-1+CH	5.49 ± 0.19[b]	117.3 ± 3.15[b]	3.75 ± 0.26[a]	0.200 ± 0.016[a]
P value	0.001	0.002	0.001	0.003

a – Significantly increased as compared to the Control Group, p= 0.0014
b – Significantly decreased as compared to Group FA-1, p= 0.0014
c – No significant difference as compared to Group FA-0.1+CH, p>0.05
d – Significantly increased as compared to the Control Group, p= 0.0519
e – Significantly increased as compared to the Control Group, p= 0,0005
f – Significantly increased as compared to the Control Group, p< 0,0001
g – No significant difference as compared to Group FA-1, p>0.05

Table 2. Results of the histological analysis of groups.

Groups	Hydropic Changes	Eosinophilic Hepatocytes	Loss of Glycogen Content	Number of Kupffer cells
Control	0.43 ± 0.30	0.14 ± 0.14	0.43 ± 0.20	5.37 ± 0.36
CH	0.57 ± 0.20	0.29 ± 0.18	0.57 ± 0.20	5.64 ± 0.35
FA-0.1	1.29 ± 0.18[a,c]	0.86 ± 0.14[c,d]	1.57 ± 0.20[c,e]	7.44 ± 0.35[c,f]
FA-1	2.29 ± 0.29[a]	1.00 ± 0.3 d	1.86 ± 0.14[e]	11.80 ± 0.38[f]
FA-0,1+CH	1.14 ± 0.26[a]	0.71 ± 0.18	1.43 ± 0.20	6.90 ± 0.33
FA-1+CH	1.29 ± 0.18[a,b]	0.57 ± 0.20[g]	1.71 ± 0.29[g]	9.39 ± 0.35[b]
P value	0.0014	0.0519	0.0005	< 0.0001

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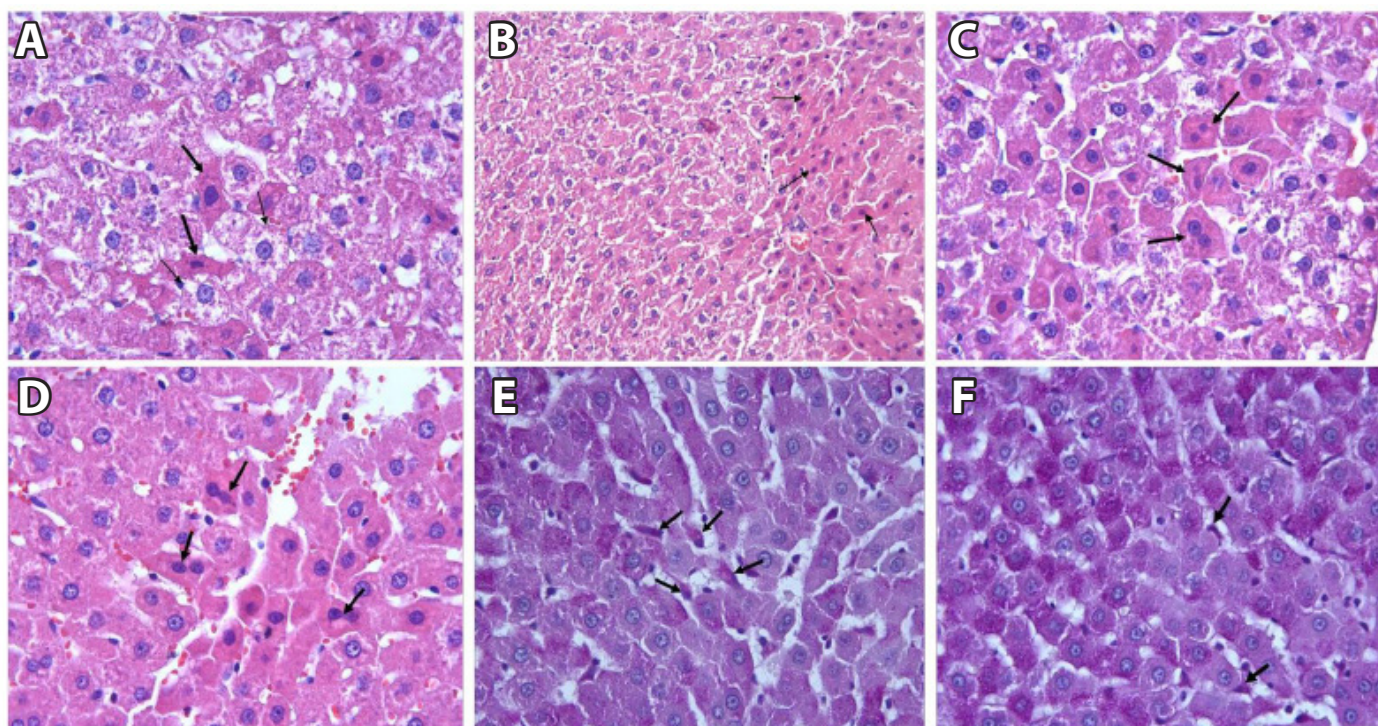Liver damage induced by subchronic toxicity of formaldehyde
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Figure 1.
A - Group FA-0.1- Hepatocytes with eosinophilic (thick arrows) and swollen (thin arrows) cytoplasm, H-E x40; B - Group FA-1- Hepatocytes with eosinophilic cytoplasm, H-E x20; C - Group FA-0.1- Binuclear hepatocytes (arrows); H-E X40; D - Group FA-1- Binuclear hepatocytes (arrows), H-E X40; E - Group FA-1- Kupffer cells (arrows), PAS X40; F - Group FA-1+CH- Decrease in the number of Kupffer cells compared to Group FA-1 can be seen (arrows), PASX40.

found to be statistically significant, though (p>0.05). However, CH treatment decreased the number of Kupffer cells and significantly reduced the number of hydropic (p=0.001 and p<0.0001, respectively) (Fig. 1F) (Table II).

Discussion

This experimental study examined the liver damage induced by low concentrations of intraperitoneally injected FA in terms of biochemical and histological parameters. Moreover, the protective properties of CH, which is an important flavonoid, against these adverse effects were assessed. The histological and biochemical findings obtained at the end of the study were discussed and compared with the findings of other relevant studies.

FA is widely used in many substances that we use in our daily lives such as industrial products, cleaning materials and cosmetic products, and in most of the work areas [5, 28-30].

The toxic effects of FA exposure on skin, eyes, testes, respiratory system, central nerve system and digestive system have been confirmed by various studies [5, 31-36].

In an organism, there is a systematic balance between antioxidants, which have protective effects, and free radicals formed under a physiological or pathological activity. The shift of this balance in favor of the free radicals results in oxidative stress. Formed as a byproduct of lipid peroxidation, TBARS are considered as an important indicator in detecting oxidative stress [37, 38].

In this study, TBARS levels in the liver tissues from Group FA-1 and FA-0.1 were found to have increased significantly compared to the control group. Enzymatic and non-enzymatic antioxidant systems have a protective role against oxidative stress. Enzymatic antioxidant defense systems include CAT, SOD and GSH [34, 35]. In this study, it was observed that CAT and GSH enzyme activity levels in the liver tissue samples from Group FA-1 increased significantly, while there was a significant decrease in the SOD levels. On the other hand, a significant increase was observed in the GSH activity levels in Group FA-0.1, effects on the CAT and SOD activity levels were not found to be statistically significant.

In their study on rats, Zararsiz et al. reported that high concentrations of intraperitoneally injected FA (10 mg/kg) increased the CAT, SOD and GSH-Px activity levels in the liver tissues as well as increasing the levels of MDA which is a product of lipid peroxidation [39]. Farooqui et al. reported that high concentrations of intraperitoneally administered FA (72 mg/kg) increased glutathione concentration in secretion of bile, but decreased the levels of glutathione in the liver tissues [40]. Similarly, Skrzydlewska indicates that methanol is oxidized to formaldehyde and formate and increases the SOD and CAT activities in the rat liver tissues [41]. In their experimental study on isolated rat hepatocytes, Teng et al. found that even low concentrations of FA (500 μl) caused oxidative stress [42]. Dobrzynska et al. reported an increase in the lipid peroxidation products in the livers of rats administered methanol (150 mg/kg) [43]. Such increase in the TBARS level is an indication of FA-induced lipid peroxidation

and oxidative stress in the liver tissues. In their experimental study, Gulec et al. found that there was a decrease in the SOD and CAT activity levels in the livers of rats administered FA (10 mg/kg) [44].

The biochemical data obtained in this study indicate that low concentrations of FA cause oxidative stress on liver, which is a finding compatible with the findings of the studies specified above [39-44]. Increased TBARS levels observed in this study indicate that low concentrations of FA can cause lipid peroxidation and oxidative stress in the liver tissues. A study reported that the levels of antioxidant enzyme SOD increased to balance the formation of excessive amounts of free radicals in acute pathologies.45 Since this study examines the subchronic toxicity, we believe that the decreased SOD levels observed are the result of the ongoing toxicity. Besides, increased transcription of GSH and CAT was observed due to the long-term exposure to FA. However, given the increased TBARS levels and other histological findings, we think that increased transcription was not sufficient to prevent toxicity.

Previous experimental studies found that FA also caused some changes in the microscopic structure of liver tissues. Beall and Ulsamer reported that exposure to formaldehyde can cause focal cellular necrosis and centrilobular vacuolization in the liver [8]. In the study by Zararsiz et al. light microscopic examination revealed vacuolization in the cytoplasm of some hepatocytes as well as some other hepatocytes with hyperchromatic nuclei [39]. Besides, they found that hepatocytes around the portal space were PAS-negative, which means there is no presence of glycogen. In this study, light microscopic examination of the H-E stained liver tissue sections from Group FA-1 and Group FA-0.1 revealed intense eosinophilia in the cytoplasm of some hepatocytes. It was found that changes were more common and apparent in the hepatocytes from Group FA-1. Besides, some of the sections from the FA groups exhibited apoptotic cells. In the FA groups, an increase was observed in the apparent number of Kupffer cells as compared to the control group. The apparent number of Kupffer cells in Group FA-1 increased significantly compared to Group FA-0.1. The findings of this study are compatible with those of previous studies in terms of the microscopic changes observed in the liver tissues after administrating FA.

Flavonoids are compounds with beneficial biochemical and antioxidants effects found mainly and abundantly in plants, and CH is one of the best defined flavonoids [46-48]. Due to such potent antioxidant property of CH, we believed it could prevent liver damage induced by FA exposure. We examined the protective effects of CH against the liver damage that might be induced by FA exposure. Pushpavalli et al. reported changes in the CAT, SOD and GSH in favor of the control group following the use of CH on d-galactosamine administered rats as a protective agent [20]. In that study, the effects of chrysin and sylimarin (silymarin is the most active ingredient of silibinin used in Amanita phalloides mushroom poisoning) were compared and the effect of chrysin was shown to be higher than that of sylimarin. Similarly, in another study, chrysin has also been shown to improve glutamic oxaloacetic transaminase and glutamic pyruvate transaminase levels and decreased SOD,

CAT and GSH levels due to tissue damage in the liver in rats exposed to CCl4 [49]. Forty to 50 grams of the Amanita phalloides fungus causes severe liver damage, sufficient to kill an adult man, due to the presence of a strong poison, α-amatine. In the treatment of these fungal intoxications, silibinin, which is a partially beneficial substance, is used. In an in-vitro study conducted in recent years, the efficacy of chrysin versus silibinin on hepatotoxic effect was compared and a slight reduction in the hepatoxic effect was seen in both drugs. This healing effect has been shown to be slightly greater with chrysin than with silibinin [50]. In a study conducted by Cuglan et al. in rats, it has been shown that damage to liver and kidneys occurs in fetuses due to formaldehyde exposure during pregnancy and decreases in given chrysin [51]. Sathiavelu et al. also reported increased CAT, SOD, GSH levels and decreased TBARS levels in favor of the control group as a result of CH treatment as antioxidant against ethanol-induced oxidative stress in rat livers [21]. In addition, they indicated that the histological changes observed in their study were correlated with the biochemical findings. Ciftci and Ozdemir also used CH to prevent the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced strong oxidative stress in rat livers and detected significant changes in the CAT, SOD, GSH and lipid peroxidation levels towards those of the control group [52]. They indicated that the histological changes observed in their study were correlated with the biochemical findings. In this study, biochemical analyses of the liver tissues revealed beneficial effects of chrysin in the FA-1+CH group. The TBARS, CAT and GSH levels significantly decreased, while the SOD activity levels significantly increased up to values close to the level of the control group. The CAT, SOD, GSH and TBARS levels in Group FA-0.1+CH also changed although the changes were not found to be statistically significant. It was observed that CH treatment prevented oxidative tissue damage in Group FA 1+CH at the biochemical level. Moreover, in groups treated with both FA and CH, the number of Kupffer cells decreased and cell swelling significantly reduced. However, there was no significant difference in terms of the number of eosinophilic hepatocytes and loss of glycogen content. The biochemical and histological findings obtained in this study regarding the antioxidant effect of CH are compatible with those of the previous studies.

Conclusion

The biochemical and histological findings obtained in this study reveal that FA exposure weakens the antioxidant defense system of liver, causing oxidative stress in the tissues. Furthermore, degeneration and apoptosis were observed in the histological structure of liver tissues following the FA administration. However, we found that chrysin treatment resulted in repression and regression of FA-induced oxidative tissue damage, microscopic changes and apoptosis in the liver tissues. In the light of these findings: It is important to investigate the therapeutic effect of chrysin depending on the dose. In addition, the use of formaldehyde should be reduced and occupational groups with higher exposure to formaldehyde should be encouraged to increase the number of investigations into chrysin's use as a preservative.

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