Research Article

Isoflavones-Enriched Soy Protein Prevents CCL$_4$-Induced Hepatotoxicity in Rats

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The burden of liver disease in Egypt is exceptionally high due to the highest prevalence of hepatitis C virus (HCV) resulting in rising rates of hepatocellular carcinoma (HCC). The aim of the current study was to determine the isoflavones in soy and to evaluate the protective role of soy against CCl$_4$-induced liver damage in rats. Four experimental groups were treated for 8 weeks and included the control group, soy-supplemented diet (20% w/w) group, the group treated orally with CCl$_4$ (100 mg/kg bw) twice a week, and the group fed soy-supplemented diet and treated with CCl$_4$. Blood and liver tissue samples were collected for biochemical analyses and histological examination. The results indicated that protein content was 45.8% and the total isoflavones recorded 167.3 mg/100 g soy. Treatment with CCl$_4$ resulted in a significant biochemical changes in serum liver tissue accompanied with severe oxidative stress and histological changes. Supplementation with soy succeeded to restore the elevation of liver enzymes activities and improved serum biochemical parameters. Moreover, soy supplementation improved the antioxidant enzymes, decreased lipid peroxidation, and improved the histological picture of the liver tissue. It could be concluded that soy-protein-enriched isoflavones may be a promising agent against liver diseases.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world with an estimated 473,000 new cases annually [1]. Most patients survive less than 1 year after diagnosis. In Egypt, the annual prevalence of HCC has increased significantly during the past decade [2]. HCC was reported to account for about 4.7% of chronic liver disease (CLD) patients where its epidemiology is characterized by marked demographic and geographic variations [3]. The cancer is more common in men with a male: female ratio exceeding three in high incidence areas.

Liver is subjected to a number of malignant and benign tumors. There are three common tumors of the liver, hemangiomas, adenomas, and focal nodular hyperplasias. Etiology of these benign tumors are either congenital or due to oral contraceptive intake. There is no evidence that any of these benign lesions progress to malignant tumors [4]. Malignant tumors include hepatocellular carcinoma, cholangiocarcinoma, hepatoblastoma, and angiosarcoma. The most common and important primary malignant tumor is hepatocellular carcinoma. This tumor is one of the major malignant diseases in the world today. It occurs most frequently in Asia and sub-Saharan Africa. Cholangiocarcinoma, hepatoblastoma, and angiosarcoma are less common but still important malignant tumors. Metastatic spread of tumors from elsewhere in the body to the liver is frequent [5].

The etiology of HCC seems to be multifactorial, and several events seem to be necessary for malignant transformation to occur. Large geographic differences in the incidence of

HCC suggest that environmental factors often contribute to its development. One common factor is the association with chronic liver disease, most frequently cirrhosis. Cirrhosis is seen in about 70–80% of HCC and it has been considered as a preneoplastic condition. The incidence of HCC varies depending on the type and etiology of associated cirrhosis [6].

Soybeans and soy products, which are relatively enriched in isoflavones, are of particular interest due to the fact that they make up a significant dietary protein source in some areas of the world [7]. Moreover, soy is rich in phenols which have been reported to exhibit antioxidant activity [8]. Soy isoflavones have been reported to decrease LDL oxidation both *in vitro* and *in vivo* [9]. Dietary flavonoids from a variety of fruits, vegetables, and beverages have also been shown to be potent inducers of this enzyme [10, 11]. Soy phytochemicals can modulate both phase I and II enzymes in the xenobiotic metabolizing system [12–15]. *In vivo* and *in vitro* experiments have demonstrated that soy and soy phytochemicals specifically induce QR activity [16–18]. The aim of the current study was to evaluate the protective effect of soy against CCl_4-induced liver damage in rats.

2. Materials and Methods

2.1. Chemicals and Kits. Carbon tetrachloride (CCl_4) was obtained from Morgan Chemical Co. (Cairo, Egypt). Transaminase (ALT and AST) kits were purchased from Spectrum Diagnostics (Cairo, Egypt). Alpha fetoprotein (AFP) was purchased from Immunospec (CA, USA). Total protein, albumin, Cholesterol, triglycerides, superoxide dismutase, glutathione peroxidase, and lipid peroxidase kits were purchased from Biodiagnostic Co. (Giza, Egypt).

2.2. Plant Materials. Soy bean (*Glycine max*) was purchased from the Crop Institute, Agricultural Research Center, Giza, Egypt in the season 2010.

2.3. Extraction and Determination of Soy Protein and Isoflavones. Soy samples were ground to a fine powder to pass through a 60-mesh sieve and the soy flour was used for the determination of protein and isoflavones. Crude protein was determined according to the method described by AOAC [19]. The soy flour was extracted by methanol 80% using a magnetic stirrer and the extract was used for the determination of daidzin, genistein, genistin, daidzein by HPLC according to the method described by Duke et al. [20]. The isoflavones were detected using a UV detector (Water 486) and online monitoring was done at 260 nm. The data were integrated and recorded using a Millennium chromatography manger software 2010 (Water Milford MA O1757).

2.4. Experimental Animals. Three-month old Sprague-Dawley male rats (100–120 g, purchased from animal house colony, Giza, Egypt) were maintained on standard lab diet (protein: 160.4; fat: 36.3; fiber: 41 g/kg and metabolizable energy 12.08 MJ) purchased from Meladco Feed Co. (Aubor City, Cairo, Egypt). Animals were housed in a room free from any source of chemical contamination, artificially illuminated and thermally controlled, at the Animal House Lab., National Research Centre, Dokki, Cairo, Egypt. After an acclimatization period of one week, the animals were divided into four groups (10 rats/group) and housed in filter-top polycarbonate cages. All animals received human care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Center, Dokki, Cairo, Egypt.

2.5. Experimental Design. Animals within different treatment groups received their respective treatment for 8 weeks as follows: group (1), untreated control; group (2), fed soy supplemented diet (20% w/w); group (3), treated orally with CCl_4 (100 mg/kg bw) twice a week for 8 weeks and group (4), treated orally with CCl_4 and fed soy-supplemented diet. At the end of the treatment period (i.e., day 57), all animals were being fasted for 12 hrs and blood samples were collected from the retroorbital venous plexus under diethyl ether anesthesia. Sera were separated using cooling centrifugation and stored at $-20°C$ until analysis. The following serum biochemical analyses were carried out: ALT and AST [21], total protein [22], albumin [23], cholesterol [24], triglycerides [25], and alpha fetoprotein [26]. After blood samples were collected, all animals were killed and sample of liver tissues of each animal was dissected, weighed, and homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate [27]. This homogenate was centrifuged at 11500 ×g and 4°C for 10 min and the supernatant was stored at $-70°C$ until analysis. Lipid peroxide (LP) was estimated by measuring the formed malondialdehyde (MDA) using thiobarbituric acid reactive substances method according to the spectrophotometric method of Buege and Aust [28] and Ruiz-Larrea et al. [29]. The level of lipid peroxide was expressed as nmol MDA per gram tissue. Hepatic glutathione peroxidase (GPX) activity was determined by a spectrophotometric method, using reduced glutathione and cumene hydroperoxide as substrate and 20 μL diluted liver homogenate, according to the modified method of Paglia and Valentine [30]. Hepatic superoxide dismutase (SOD) activity was assayed spectrophometrically by the red formazan dye reduction procedure [31] using 50 μL diluted liver homogenate. The specific activity of hepatic glutathione peroxidase and superoxide dismutase was expressed as unit/mg liver protein.

Other samples of the liver from all animals within different treatment groups were excised and fixed in 10% neutral formalin followed by dehydration in ascending grades of alcohol, clearing in xylene and embedding in paraffin wax. Liver sections (5 μm thickness) were stained with hematoxylin and eosin (H&E) for the histological examination [32].

2.6. Statistical Analysis. All data were statistically analyzed by analysis of variance (ANOVA) using the General Linear Model Procedure of the Statistical Analysis System [33]. The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio [34]. All statements of significance were based on probability of $P \le 0.05$.

TABLE 1: The effect of soy supplementation on different treatments on the biochemical serum parameters in rats treated with CCl_4.

Parameters	Groups			
	Control	Soy	CCl_4	CCl_4 + Soy
ALT (U/mL)	176.88 ± 3.54^a	173.88 ± 4.88^a	264.33 ± 7.43^b	174.25 ± 6.98^a
AST (U/mL)	65.25 ± 3.8^a	63.88 ± 5.04^a	263.63 ± 20.2^c	63.75 ± 3.95^a
Albumin (g/dL)	3.64 ± 0.09^a	3.95 ± 0.23^a	1.43 ± 0.12^b	2.98 ± 0.26^c
Total protein (g/dL)	9.54 ± 0.66^a	9.32 ± 0.7^a	5.94 ± 0.2^b	8.78 ± 0.58^a
Cholesterol (mg/dL)	62.5 ± 4.54^a	54.75 ± 3.03^b	103 ± 4.53^c	67.5 ± 6.07^a
Triglycerides (mg/dL)	63.15 ± 4.04^a	56.71 ± 6.23^b	117.83 ± 1.67^c	107 ± 6.51^d
AFP (IU/mL)	10.04 ± 2.42^a	6.39 ± 0.73^b	15.71 ± 1.07^c	7.16 ± 0.80^d

Within each row, means superscript with different letters are significantly different ($P \geq 0.05$).

TABLE 2: The effect of soy supplementation on oxidative stress markers in rat liver treated with CCl_4.

Parameters	Groups			
	Control	Soy	CCl_4	CCl_4 + Soy
GPX (U/g tissue)	2512.00 ± 53.59^a	2939.78 ± 521.99^b	949.10 ± 29.39^c	2390.68 ± 561.57^d
SOD (U/g tissue)	1289.63 ± 92.3^a	4102.94 ± 191.76^b	834.44 ± 48.56^c	3425.42 ± 205.44^d
MDA (nmol/g tissue)	48.48 ± 0.58^a	41.52 ± 5.89^b	139.01 ± 9.43^c	58.27 ± 2.22^d

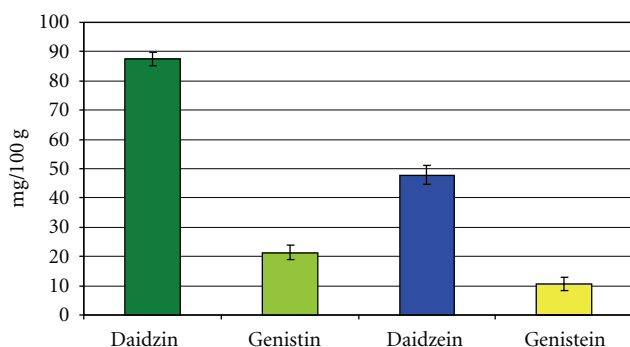Within each row, means superscript with different letters are significantly different ($P \geq 0.05$).

FIGURE 1: Isoflavones concentration in soy flour extract determined by HPLC (means ± SE).

3. Results

The results of crude protein indicated that crude protein in soy was high and recorded 45.8% based on dry matter contents and the total isoflavones content recorded 167.3 mg/100 g soy (Figure 1).

The results of the current study revealed that treatment with CCl_4 resulted in a significant increase in ALT and AST activities, AFP and cholesterol level accompanied with a significant decrease in albumin, total protein, and triglycerides (Table 1). Animals fed soy-supplemented diet showed a significant decrease in AFP, cholesterol, and triglycerides however; ALT and AST activities and albumin and total protein were comparable to the control. ALT, AST, total protein, and cholesterol in animals treated with CCl_4 and fed soy-supplemented diet were comparable to the control however; a significant improvement in albumin and triglycerides was observed although this treatment did not normalize them.

The current results revealed that treatment with CCl_4 resulted in a severe stress in liver as indicated by the significant increase in MDA and the significant decrease of GPX and SOD in liver tissue (Table 2). Animals fed soy-supplemented diet showed a significant decrease in MDA accompanied with a significant increase in GPX and SOD. However, animals treated with CCl_4 and fed soy-supplemented diet showed a significant improvement in the oxidative stress marker as well as the antioxidant enzymes activity although these values were still different significantly than the control group.

The biochemical analyses were further confirmed by the histological examination of the liver section. The results indicated that liver tissue in the control group showed normal central vein and hepatocytes (Figure 2(a)). Animal fed soy-supplemented diet also showed normal central vein and hepatocytes (Figure 2(b)). The liver section in the animals treated with CCl_4 showed an increase in fibrous tissues and inflammatory cells around the congested blood vessel; moreover, a marked hepatocellular fatty degeneration was also seen (Figure 2(c)). The hepatocytes of same group were observed with large fatty droplets and nuclear pleomorphism (Figure 2(d)). The liver of animals treated with CCl_4 plus soy showed marked improvement in most of hepatocytes and prominent decrease in connective tissue (Figure 2(e)) accompanied with a marked improvement in hepatocytes and portal vein area (Figure 2(f)).

4. Discussion

The current study revealed that soy is enriched in protein and isoflavones which were found at high concentrations.
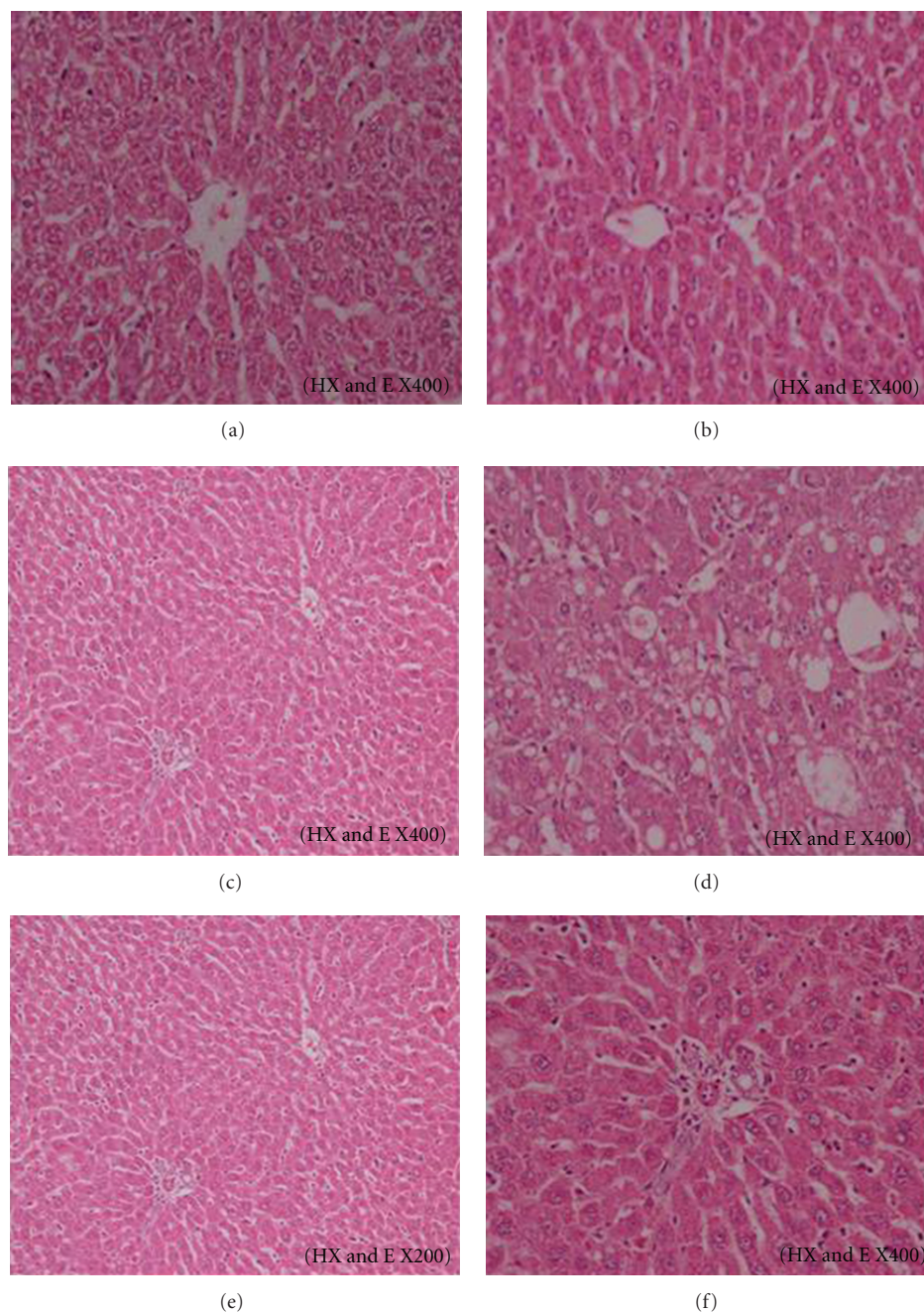
FIGURE 2: A photomicrograph in liver section of (a) control rat showing the normal central vein and the hepatocytes, (b) rat fed soy-supplemented diet showing the central vein and the hepatocytes, (c) rat treated with CCl_4 showing increase in fibrous tissues and inflammatory cells around the congested blood vessel. Marked hepatocellular fatty degeneration also seen, (d) rat treated with CCl_4 showing hepatocytes with large fatty droplets and nuclear pleomorphism, (e) rat treated with CCl_4 and fed soy supplemented diet showing marked improvement in most of hepatocytes and prominent decrease in connective tissues, and (f) rat treated with CCl_4 and fed soy supplemented diet showing marked improvement in hepatocytes and in the portal vein area.

Similar to these results, Simmen et al. [35] reported that soy contained high protein content. Moreover, King and Bignell [36], Hou et al. [37], and Duke et al. [20] reported that soy contained a high content of isoflavones as determined by HPLC. The isoflavones genistin and daidzin constitute 99% of the total isoflavones [38].

In the current study we evaluated the protective role of soy against CCl_4-induced liver damage in rats. The selected doses of CCl_4 and soy were literature based [39, 40]. It is well known that CCl_4 is one of the most extensively studied hepatotoxins. The mechanism by which CCl_4 causes hepatotoxicity is well documented in a series of reports.

The hepatotoxicity of CCl_4 undergoes two phases, the first results from its metabolic conversion to free radical product CCl_3^- by Cyt P-450 [41]. Once CCl_3^- has been formed it reacts very rapidly with O_2 to produce CCl_3OO^-, a much more reactive radical than CCl_3^- [42]. These free radicals attack microsomal lipids leading to its peroxidation and also covalently bind to microsomal lipids and proteins. This results in the generation of reactive oxygen species (ROS), which includes the superoxide anion O_2, H_2O_2 and the hydroxyl radical.

In the current study, CCl_4 caused significant changes in serum biochemical parameters typical to those reported in the literature [43, 44]. The liver is considered to be the principal target organ for CCl_4 and the activity of transaminases are sensitive indicators of acute hepatic necrosis [45, 46]. The elevated levels of ALT, AST, triglycerides, and cholesterol reported herein indicated severe hepatic parenchymal cells injury [47–49], whereas the decrease in total protein and albumin indicated liver necrosis and/or kidney dysfunction [48]. MDA, an end product of lipid peroxidation, is widely used as a marker of lipid peroxidation. It is well documented that CCl_4 enhanced lipid peroxidation [44, 48, 50] that is an indication of free radical mediated toxicity.

Free radicals are known to attack the highly unsaturated fatty acids of the cell membrane and induce lipid peroxidation that is considered a key process in many pathological events induced by oxidative stress [51]. In the present study, MDA was found to be significantly higher in the animals treated with CCl_4 suggesting a significant effect on lipid peroxidation and supporting the earlier findings [52, 53].

Alteration in the hepatic antioxidant status may be a manifestation of oxidative stress caused by CCl_4 and their metabolites. Both GPX and SOD are considered enzymatic free radical scavengers in cells. In the present study, GPX and SOD were found to decline significantly in rats treated with CCl_4. It is well known that SOD plays an important role in the elimination of ROS derived from the peroxidative process in liver tissues [50]. Moreover, SOD removes superoxide by converting it to H_2O_2, which can be rapidly converted to water by CAT [54]. Taken together, the increased level of MDA and the decreased activity of antioxidant enzymes GPX and SOD may be attributed to free radical formation which initiates chain reactions of direct and indirect bond formation with cellular molecules (nucleic acids, proteins, lipids, and carbohydrates) impairing crucial cellular processes that may ultimately culminate.

In the current study, the elevated serum level of AFP in the animals treated with CCl_4 indicated that it is a potent hepatocarcinogen, enhances reactive oxygen species (ROS) formation, and causes oxidative DNA damage, which may play a role in its carcinogenicity [44, 48, 55, 56]. Therefore, the current study affirmed that CCl_4 can induce hepatotoxicity and degeneration in liver cells in rats as indicated by the elevation of AFP level in serum. Similarly, Engelhardt et al. [57] reported that AFP was much higher after the exposure to CCl_4 for 3-4 days.

The biochemical study was further confirmed by the histopathological examination of the liver tissue. The liver section in CCl_4-treated group showed an increase in fibrous tissues and inflammatory cells around the congested blood vessel and a marked hepatocellular fatty degeneration. The hepatocytes of same group were observed with large fatty droplets and nuclear pleomorphism. These histological changes were similar to those reported earlier [44, 58]. Animals fed soy-supplemented diet showed a significant improvement in all the biochemical parameters tested and the histological picture of the liver. Possible explanation for the differential effects of soy on the activities of AST and ALT in plasma is that isoflavones and soy protein may inhibit the liver damage induced by CCl_4.

On the other hand, supplementation of the diet with soy protein significantly lowered plasma cholesterol and triglycerides levels caused by CCl_4. It is indicated recently that soy protein intake decreased the hepatic lipid depots of triacylglycerols and cholesterol and decreased the concentrations of lipid peroxides [59]. The mechanism by which soy protein decreases serum lipids is not yet fully established. A possible mechanism of this decrease is the enhancement of bile acid excretion, by which soy protein acts as dietary fiber to promote bile acid excretion and increase the rate of cholesterol resynthesis in the liver [60].

Previous report has shown that soy protein may improve hyperlipidemia due to its effects on the transcription factors, called sterol regulatory element binding proteins, which are important in the regulation of enzymes involved in lipid metabolism *in vivo* [61]. Moreover, Tovar et al. [62] found that soy protein lowered sterol regulatory element binding protein-1 expression in adipocytes, which also helped prevent the development of hepatic lipotoxicity.

AFP was higher in animals treated with CCl_4. This is likely related to the progression of these agents in liver cirrhosis [63]. Soy ingestion significantly lowered AFP levels in the serum of rats. One of the mechanisms for lowering AFP level may have been the anti-inflammatory properties of soy. Previous studies have indicated that the isoflavones in soy may regulate the inflammatory response and immune function [64, 65]. Moreover, genistein and secoisolariciresinol have anticancer properties on MCF-7 and BT20 *in vitro* [66] and have been suggested as the most potent inhibitors of cancer cell growth [67].

In the current study, soy supplementation resulted in a significant decrease in lipid peroxidation measured as MDA concentration in liver. This decrease was accompanied with a significant increase in GPX and SOD activity. Moreover, diet supplemented with soy succeeded to induce a significant improvement in the oxidative stress markers and antioxidant status of the liver tissue in rats treated with CCl_4. These results suggested that soy protein may prevent oxidative damage in the liver by lowering plasma-free fatty acids and decreasing CYP2E1 expression. Similar observations were reported recently by Yang et al. [59]. The histopathological study of liver confirmed these findings. The liver section of the animals treated with CCl_4 and fed soy-supplemented diet showed marked improvement in hepatocytes and in the portal vein area. According to Lila and Raskin [68], the antioxidative effects of several plant compounds have a potentiating effect of other compounds found in plants. A mixture of compounds rather than a single compound may

be the key to full antioxidant potency. In this concern, soy contains other bioactive phytochemicals in addition to isoflavones [69]. Consequently, the antioxidative effects of soy reported in the current study may be due to several compounds present in soy.

5. Conclusion

The current study revealed that CCl_4 induced severe hepatotoxicity typical to those reported in the literature. Supplementation with soy succeeded to protect the liver from the toxic effects and the oxidative stress of CCl_4. These effects may be due to the antioxidant activity, the anticancer effect, and the enhancement of immune response of soy components since it increased the antioxidant capacity of the body and reduced the oxidative stress and tumor markers. The current results concluded that soy contained a combination of active compounds that may be more efficacious and safer as chemopreventive agents than individual compounds.

Conflict of Interests

The authors declare that there is no conflict of interests.

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