

# Evaluation of Luteolin in the Prevention of *N*-nitrosodiethylamine-induced Hepatocellular Carcinoma Using Animal Model System

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**Abstract** Hepatocellular carcinoma (HCC) is one of the commonest tumors worldwide. The treatment of HCC is vital for disease diagnosis and prognosis, as the liver is the most important organ controlling metabolic functions. Now-a-days, western folklore medicines are largely dependent on the phyto compounds which are highly effective in therapy and with low side effects. Luteolin is a flavonoid (3,4,5,7-Tetrahydro flavones) possess anti-inflammatory, anticancer and anti allergic property. The present study evaluates the efficacy of luteolin against *N*-nitrosodiethylamine (DEN) induced HCC in albino rats. In the highlight of the above, luteolin was evaluated for its efficacy against DEN induced HCC in male Wistar albino rats. The Biochemical parameters such as tissue damaging enzymes viz., AST, ALP, LDH and  $\gamma$ -GT, enzymatic antioxidants viz., SOD, CAT, GSH and GPx and histopathological changes have been estimated. The tissue damaging enzymes were found to be high in DEN alone treated group whereas the enzymatic antioxidants decreased destructively. Severe lesions and cirrhosis were observed in the toxin (DEN alone) treated group. The luteolin treated DEN group altered the tissue damaging enzymes and the enzymatic antioxidants. The damaged lesion in the histoarchitecture of DEN treated rat liver was almost completely restored. Finally this study strongly demonstrates that luteolin has potent curative property against HCC in albino rats.

**Keywords** Hepatocellular carcinoma · Luteolin · DEN · Enzymatic/Non-enzymatic Antioxidants · Ultrastructural study

## Introduction

Hepatocellular carcinoma (HCC) is a major malignancy worldwide and is increasingly associated with cancer related death [1]. The treatment of cancer is still a big challenge in medicine. HCC is a heterogeneous disease in the expression of etiology and underlying associations as well as biological and clinical behavior.

Several chemicals are known to possess chemo preventive property against a broad spectrum of cancers. Chemoprevention serves as an attractive alternative to control malignancy [2]. Several herbal drugs have been evaluated for their potential as liver protectant against NDEA-induced hepatotoxicity in rats [3]. Luteolin is a bioactive flavonoid, chemically (3,4,5,7-Tetrahydroxy flavones) an important member of the flavonoid family. It is present in various fruits, and vegetables. It exhibits a wide spectrum of pharmacological property including anti-inflammatory and anti allergic property [4, 5]. Much attention has been recently paid to its antioxidant property and to its proliferative effect.

DEN (*N*-Nitrosodiethylamine) is a strong hepatocarcinogenic dialkyl nitrosoamine present in tobacco smoke, water, cheddar cheese, curd and fried meals and in a number of alcoholic beverages [6]. NDEA is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication and producing reproducible HCC after repeated administration in experimental animals [7]. Plenty of reports give evidence that that NDEA causes a wide range of tumors in all animal species and such compounds are hazardous to human health [8]. The formation

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of reactive oxygen species (ROS) is apparent during the metabolic biotransformation of NDEA resulting in oxidative stress. Oxidative stress leads to carcinogenesis by several mechanisms including DNA, lipid and protein damage, change in intracellular signaling pathways and even changes in gene expression. Lipid peroxidation (LPO) may also result in several changes, including structural and functional membrane modifications, protein oxidation and generation of oxidation products such as acrolein, crotonaldehyde, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which are considered strong carcinogens. Efforts to develop less toxic drugs that affect antioxidant system, malignant cells and mechanism-based approach are necessary in prevention and therapy of cancer.

The majority of HCC occurs in patients with liver cirrhosis and consequent hepatic dysfunction, which complicates safe administration of systemic therapy and poses a challenge to conducting clinical trials in this patient population. Therefore this paper emphasizes the *in vivo* antitumor efficacy of the phyto compound luteolin (flavonol) against DEN induced HCC in male albino Wistar rats.

## Materials and Methods

### Animals

Male Wistar albino rats (130–150 g) were procured from Kerala Agriculture University, Thrissur, Kerala. The animals were housed in polypropylene cages at an ambient temperature of 25–30°C and 45–55% relative humidity with a 12 h each of dark and light cycle. Rats were fed pellet diet and water *ad libitum*. The study was approved by the Institutional Ethical Committee. (743/03/abc/CPC-SEA dated 3.3.03).

### Source of Chemicals

Luteolin and DEN were purchased from Sigma Aldrich, USA and all other chemicals used were of analytical grade.

### Experimental Design

The experimental animals were divided into four groups, each group comprising of six animals for a study period of 16 weeks. Group I, control rats (untreated) were fed with standard diet and water *ad libitum*. Group II rats were administered with luteolin alone (0.2 mg/kg b.w.daily) was administered by intraperitoneally. Group III rats were induced intraperitoneally with DEN (100 mg/kg b.w.) once in a week for a period of 6 weeks. To the group IV rats, luteolin (0.2 mg/kg b.w.daily) was administered by intraperitoneally to DEN induced group of rats after the

proliferation tumor from 6th week onwards up to 16 weeks. At the end of the experimental period, the rats were euthanized by cervical dislocation. The blood was collected, processed and stored for further analysis. The liver was excised immediately, rinsed in ice cold saline and was homogenized in 0.1 M Tris buffer (pH.7.4) for further biochemical analysis.

### Preparation of Hepatic Tissue Homogenate

Hepatic tissues from control and experimental group of rats were excised, rinsed with ice-cold saline and homogenized in Tris–HCl buffer (100 mM, pH 7.4) using Teflon homogenizer and centrifuged at 12,000×*g* for 30 min at 4°C. The supernatant was pooled and used for the further estimations. The protein content in the tissue homogenate was measured by the method of [9].

### Liver Injury Markers

The activities of aspartate transaminase (AST) and Alanine transaminase (ALT) were estimated by the method of [10], while alkaline phosphatase (ALP) and glutamyl transferase (GT) were estimated by methods of [11, 12].

### Antioxidant Enzyme Assay

The activity of superoxide dismutase (SOD) in the hepatic tissue was assayed by the method of [13]. Catalase (CAT) activity was measured according to the method of [14]. Reduced glutathione (GSH) level was measured by the method of [15]. Glutathione peroxidase (GPx) activity was measured by the method of [16] and the activity of glutathione-*S*-transferase (GST) was measured according to the method of [17].

### Histopathological Assessment

Liver sections were prepared from different groups of rats, fixed in 10% formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene, and embedded in paraffin. The pathological changes were observed microscopically after staining with hematoxylin and eosin (H–E).

### Transmission Electron Microscopic Study

For Ultrastructural study, a portion of liver (about 1 mm) from control and experimental groups of rats were fixed in 3% glutaraldehyde in sodium phosphate buffer (200 mM, pH 7.4) for 3 h at 4°C. Tissue samples were washed with the same buffer, placed in 1% osmium tetroxide and sodium phosphate buffer (200 mM, pH 7.4) for 1 h at 4°C. The samples were again washed with the same buffer

for 3 h at 4°C, dehydrated with graded series of ethanol and embedded in Araldite. Thin sections were cut with LKBUM4 ultra microtome using a diamond knife (Diatome, Aldermaston, Berkshire, England), mounted on a copper grid and stained with 2% uranyl acetate and reynolds lead citrate [18]. The grids were examined under a Philips EM201C transmission electron microscope (TEM) (Philips, Eindhoven, Netherlands).

#### Statistical Analysis

The data were analysed using SPSS/16.0 software. Hypothesis testing methods were included with analysis of variance (ANOVA) followed by least significance difference (LSD). *P* values of > 0.05 were considered statistically significant. The data were expressed as mean ± S.D with six animals in each group.

### Results

Table 1 illustrates the activity of liver enzymes LDH, ALT, AST and ALP in experimental group of rats. DEN alone treated group-III showed significant increase in the liver enzymes viz., LDH, ALT, AST and ALP compared to group-I control and group-II luteolin alone treated group. The enzyme activities in the DEN induced luteolin treated group was similar to that of the group-I (control) rats.

Table 2 shows the content of antioxidants in liver of treated group. The levels of antioxidant enzymes viz., CAT, Glutathione peroxidase (GPx), SOD were found to be more in the DEN induced group-III treated rats, whereas glutathione reductase (GR) and GST levels significantly decreased in these groups. In the luteolin treated group, the antioxidants enzyme levels were slightly lower than the control group. The DEN induced luteolin treated group restores the changes to near normalcy by its antioxidant efficacy.

The antioxidant potential was further confirmed by the non-enzymatic antioxidants such as Vitamin-C, Vitamin E, GSH and MDA levels. The DEN induced treated group showed significantly decreased levels of Vitamin-C,

Vitamin-E, GSH and MDA (Table 3). The luteolin treated group possessed slightly elevated levels of non-enzymatic antioxidants than the control group-I untreated rats. The non enzymatic antioxidants in the DEN induced luteolin treated group IV rats were found to be similar to that of control (normal) rats.

The histology of the liver tissue was examined under a light microscope and represented in Fig. 1 (a–d). Group I (control rats) indicated in the Fig. 1a revealed the normal architecture of the liver cells and group-II (Fig. 1b) rats treated with luteolin alone showed the normal histological appearance of liver cells further ascertaining its nontoxic property. Group-III (Fig. 1c) DEN alone treated groups depicted the area of severe hepatocellular necrosis with the adjacent liver cells. It is interesting to observe that, in the DEN induced luteolin treated group (Fig. 1d) the damaged liver architecture was altered, necrosis healed and the cellular degeneration was found to be lower.

The ultra structural changes occurred in hepatocytes of control and experimental groups of rats were shown in Fig. 2 (a–d). The Fig. 2a depicts the electron micrograph of hepatocyte of control group of rats. From the figure, it could be seen that the normal cellular organelles, mitochondria (M), rough endoplasmic reticulum, golgi complex (GC), nucleus (N) with intact nuclear membrane (NM) and nuclear chromatin were visible. Similar architecture was observed in the electron micrograph of control group of rats treated with luteolin Fig. 2b. The electron micrograph of hepatocyte of DEN induced HCC group III of rats in Fig. 2c revealed the decrease of organelles regeneration, swelling in the cisternae of the rough endoplasmic reticulum and mitochondrial cristae, fusion or disappearance of mitochondrial crests, degranulation of rough endoplasmic reticulum, pyknotic nuclei with damaged NM, extensions in perinuclear area, increased smooth ER and lipid accumulation, cells with dark and light cytoplasm.

Figure 2d shows an apparent appearance of NM and chromatin, either absent or significant reduction in the swelling in the cisternae of the rough endoplasmic reticulum and mitochondrial cristae, dilation in the perinuclear space, presence of few pyknotic nuclei and reduction of smooth ER. This finding shows the *in vivo* antitumor

**Table 1** Effect of luteolin on liver enzyme profiles of untreated and treated rat groups

Group	LDH (U/l)	ALT (U/l)	AST (U/l)	ALP (u/l)
Control	182 ± 5.6	32.21 ± 3.2	74.21 ± 4.5	128.22 ± 0.9
Luteolin alone	178 ± 2.6	31.66 ± 3.2	76.52 ± 3.5	130.47 ± 2.6
DEN alone	230 ± 13.5 <sup>a</sup>	192.20 ± 4.8 <sup>a</sup>	273. ± 10.5 <sup>a</sup>	242.6 ± 0.6 <sup>a</sup>
DEN + luteolin	162 ± 3.9 <sup>b</sup>	69.00 ± 5.1 <sup>b</sup>	123 ± 4.89 <sup>b</sup>	163.14 ± 14 <sup>b</sup>

Values of results are expressed as mean ± .SD for six rats. In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

**Table 2** Effect of luteolin on liver antioxidant enzymes of untreated and treated rat groups

Groups	Catalase	GPx	GRD	SOD	GST
Control	18.52 ± 0.052	437.84 ± 2.0	25.07 ± 0.45	468.04 ± 23	0.34 ± 0.01
Luteolin alone	17.61 ± 0.42	440.12 ± 27	24.97 ± 0.21	465.65 ± 15	0.33 ± 0.04
DEN alone	13.58 ± 0.21 <sup>a</sup>	262.61 ± 4.1 <sup>a</sup>	15.42 ± 0.41 <sup>a</sup>	269.84 ± 16 <sup>a</sup>	0.15 ± 0.08 <sup>a</sup>
DEN + luteolin	16.85 ± 0.17 <sup>b</sup>	390.45 ± 3.6 <sup>b</sup>	22.15 ± 0.45 <sup>b</sup>	442.23 ± 11 <sup>b</sup>	0.28 ± 0.01 <sup>b</sup>

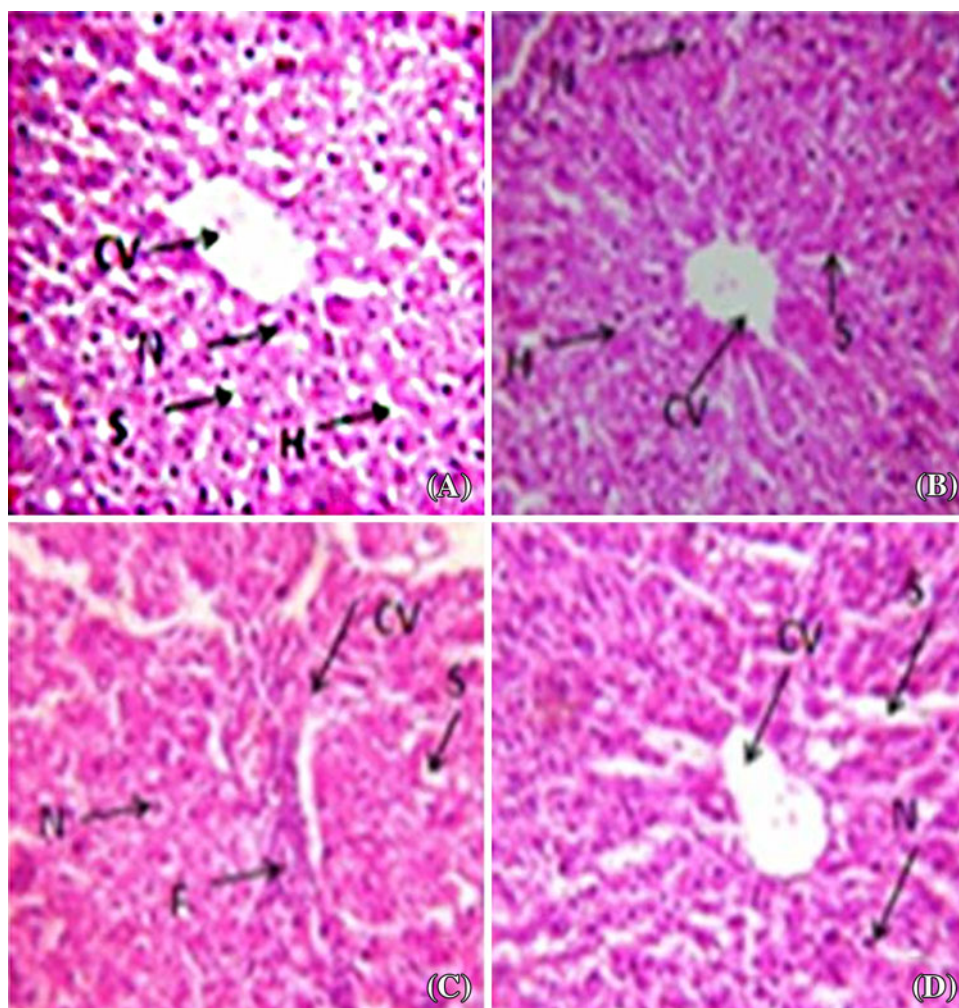
Catalase (U/mg of protein), GPx (U/mg of protein), GR (nmol NADPH/min/mg protein), SOD (U/mg of protein), GST (μmol 1-chloro-2, 4-dinitrobenzene conjugate formed/min/mg of protein)

Values of results are expressed as mean ± .SD for six rats. In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

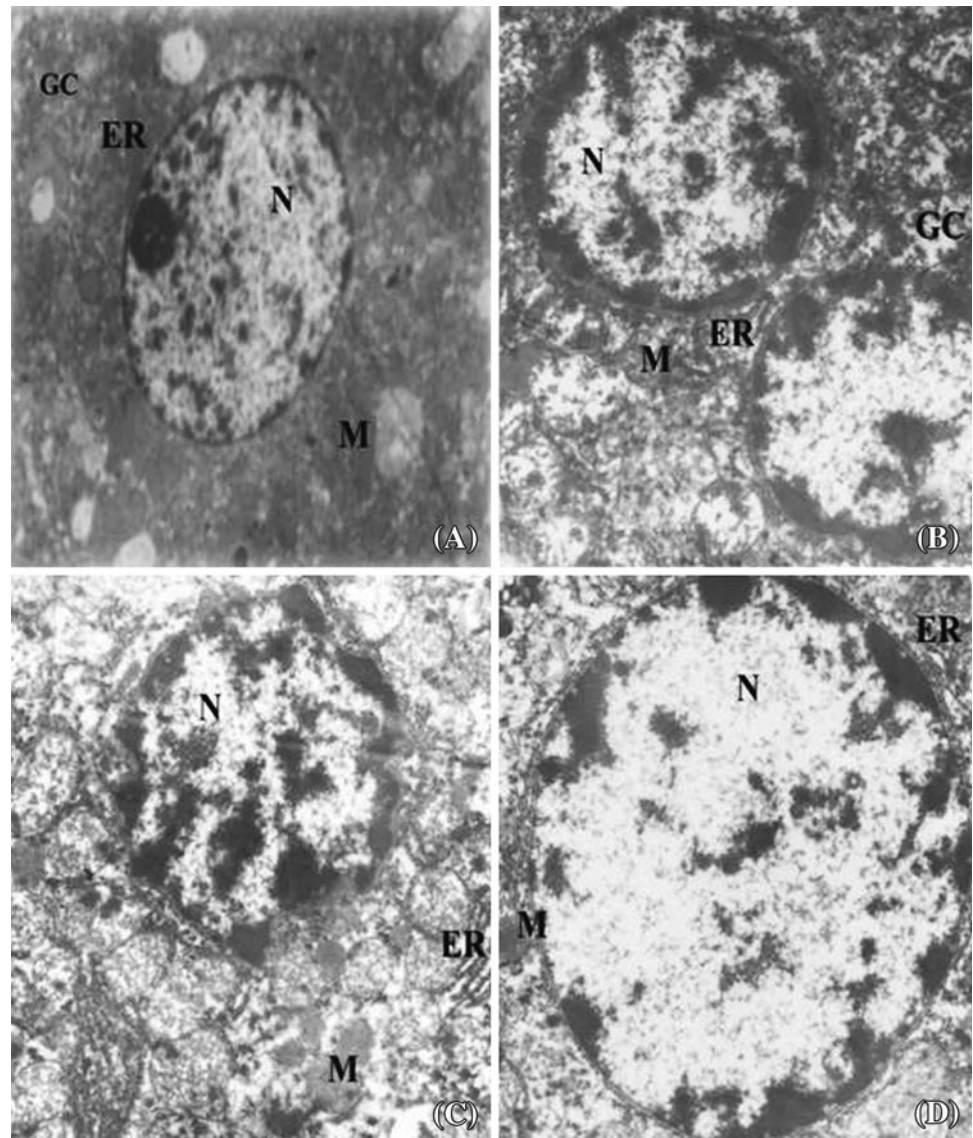
**Table 3** Effect of luteolin on non-antioxidant enzymes in liver of untreated and treated rat groups

Groups	Vitamin C (μg/mg of protein)	Vitamin E (μg/mg of protein)	GSH (mg/100 g of wet tissue)	MDA (nm/mg of protein)
Control	1.23 ± 0.05	1.56 ± 0.04	40.23 ± 1.6	2.17 ± 0.41
Luteolin alone	1.28 ± 0.02	1.26 ± 0.01	46.21 ± 1.8	2.04 ± 0.12
DEN alone	0.56 ± 0.08 <sup>a</sup>	0.62 ± 0.07 <sup>a</sup>	20.56 ± 2.1 <sup>a</sup>	8.16 ± 0.41 <sup>a</sup>
DEN + luteolin	1.16 ± 0.06 <sup>b</sup>	1.38 ± 0.09 <sup>b</sup>	38.26 ± 2.4 <sup>b</sup>	4.12 ± 0.36 <sup>b</sup>

Values of results are expressed as mean ± .SD for six rats. In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

**Fig. 1** Light microscopic analysis of control and treated group of rats

**Fig. 2** TEM image of HCC in control and experimental rats. **a** Control. **b** Luteolin alone. **c** HCC. **d** HCC + luteolin. Hepatic tissue sections were showed at 15000× magnification. The organelles such as ER, GC, N, NM and M were displayed



activity of luteolin in the experimental group of animals. Specifically it confirms the HCC protective nature of luteolin in DEN induced HCC group of rats.

## Discussion

Luteolin is a naturally occurring flavonoid, is a known biochemical target other than the fact that it induces topoisomerase-II mediated apoptosis. Similar activity has been reported by inhibition of invasive activity in Mia-PaCa-2 cancer cells [19–21]. Luteolin exhibits wide spectrum of anti-tumor activities, but little is known about its anti-cancer mechanisms.

Liver damage caused by DEN generally reflects instability of liver cell metabolism which leads to distinctive changes in the serum enzyme activities [22]. AST, ALT,

LDH and ALP are representative of liver function. Their increased levels are indicators of liver damage. The elevation of ALT activity is repeatedly credited to hepatocellular damage and is usually accompanied by a rise in AST. Increase in ALP reflects the pathological alteration in biliary flow. In the present study, treatment with luteolin induced the increased activities of these enzymes and were normalized. This suggested that the luteolin played a role in parenchymal cell regeneration in liver, thus protecting membrane integrity, thereby decreasing enzyme leakage.

SOD acts as the first line of defense against superoxide free radicals, which dismutates two superoxide radicals to  $H_2O_2$  and  $O_2$ . Besides CAT and GPx act as supporting antioxidant enzymes by converting  $H_2O_2$  to  $H_2O$ , thereby providing protection against ROS [23]. The reduction in activity of these enzymes may be caused by the increase in radical production during DEN metabolism. In the present

investigation, a raise in MDA formation was presumably associated with increased ROS, consistent with the observation that these free radicals reduce the activity of hepatic SOD [24]. In the present study the reduction in these antioxidant enzymes was due to the action of luteolin on the DEN induced HCC. Biochemical results of hepatic SOD showed a decrease in activity of SOD in DEN-induced rats compared to control and luteolin treatment on the DEN induced HCC group rats. GPx is one more endogenous antioxidant seleno protein present in the cytosol and mitochondrial matrix that participate in the defense mechanism. GPx was activated before the initiation of chronic oxidative stress and catalyzes the reduction of lipid and non-lipid hydro peroxides using two molecules of GSH and thereby curtails the quantity of biomolecules having destructive properties [25]. Similarly, GST is a soluble protein situated in cytosol and plays a vital role in detoxification and excretion of xenobiotics [26].

GST catalyzes the conjugation of the thiol functional groups of GSH to electrophilic xenobiotics and results in escalating solubility. The xenobiotic–GSH conjugate is then either eliminated or converted to mercapturic acid [27]. Since GST increases solubility of hydrophobic substances, it plays an important role in storage and excretion of xenobiotics. Induction of xenobiotic detoxifying enzymes is an additional mechanism by which antioxidant rich extracts may act as anticarcinogens as they compete with steps in xenobiotic activation and metabolize toxic compounds to non-toxic ones [28]. As the activity of GST increased in luteolin treated rats, it appears that the drug induces greater coupling of electrophilic intermediates with GSH.

In summary, luteolin stabilizes and restores the antioxidant defense system viz., GSH, CAT, SOD, GPx and GST. These antioxidant enzymes protect cells from ROS damage in DEN-induced HCC. Luteolin, a bioflavonoid protects the activities of liver injury and tumor markers by decreasing MDA. The research findings clearly indicate that luteolin eliminates the oxidative state induced by the initiator DEN, and it interacts directly with ROS (e.g., OH<sup>•</sup>), as well as indirectly by activating the antioxidant defense system. Luteolin thus reduces the DEN induced increased ROS generation during hepatocarcinogenesis and promotes the enzymatic and non-enzymatic antioxidant defense system and has potentiality in chemoprevention.

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