Effect of curcumin-containing chitosan nanoparticle on caspase-3, carcinoembryonic antigen in colorectal cancer induced by dimethylhydrazine

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Background and aim
Colon cancer is the third most common cancer for males and the fourth for females in Egypt. Previous studies have demonstrated that curcumin (CUR) is safely used against different cancers. This study was performed to evaluate the effect of curcumin-containing chitosan nanoparticles (CUR-CS-NP) against colon cancer induced by dimethylhydrazine (DMH) in rats.
Material and methods
The rats were randomly divided into four groups. The first group served as control group. The second group received DMH (20 mg/kg, subcutaneously) once a week. The third group was given DMH and CUR. Group 4 received DMH and CUR-CS-NP. The animals were sacrificed at the end of 10 weeks. Caspase-3 expression in colon tissues was determined by quantitative real-time PCR. Plasma levels of carcinoembryonic antigen (CEA) were determined by enzyme-linked immunoassay. Malondialdehyde (MDA), nitric oxide, and reduced glutathione were determined in plasma and colorectal tissues. Histopathological examinations of colon tissues were done.
Results
DMH treatment decreased caspase-3 expression, increased CEA, and oxidative stress levels. Pathologic lesions in the form of dysplasia and lymphocytic infiltration were seen in DMH-treated group. CUR-CS-NP and CUR treatments reduced the pathologic changes and increased caspase-3 expressions. Each treatment increased glutathione and reduced MDA, nitric oxide, and CEA levels.
Conclusions
Our study reveals that CUR and CUR-CS-NP have antioxidant and proapoptotic effects. So, it provides an insight towards the use of biological sources as promising anticancer agents.

Keywords:
apoptosis, colon cancer, curcumin-containing chitosan nanoparticle, dimethylhydrazine, oxidative stress

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Introduction

In Egypt, colon cancer (CC) is the third most common cancer for men and the fourth for women. Also, high CC percentage occurs in children and in individuals below 40 years of age [1]. It ranks fourth in cancer deaths worldwide [2]. Environmental, genetic, and epigenetic factors can initiate CC [3]. Slattery *et al.* [4] hypothesized these factors work together to induce CC through an oxidative balance mechanism. The antioxidant agents can protect the colon against DNA mutations by scavenging the free radicals [5].

Tumor cells need to disrupt apoptosis pathways to escape the cytotoxic action of oncogene activation and microenvironmental stress during the carcinogenic process. However, the cytotoxic action of classical chemotherapy and radiotherapy includes the induction of apoptotic cell death [6]. Furthermore, apoptosis can increase the sensitivity of cancer cells to chemotherapeutic agents as 5-fluorouracil [7]. Caspases belong to a cysteine protease family that play an important role in programmed cell death. Caspase-3 is a key executioner of apoptosis. It is activated by initiator caspase 8 or caspase 9.Cancer cells can escape apoptosis by downregulation or complete loss of the caspase-3 expression [8].

1,2 dimethylhydrazine (DMH) is able to induce precancerous lesions and cancers of the colon that are similar to human CC. The liver activates it into intermediates like azoxymethane and methylazoxymethane. These intermediates are converted into the carcinogenic metabolite methyldiazonium ion which causes oxidative stress and inflammation of colon epithelial cells [9].

Curcumin (CUR) is a natural polyphenolic compound extracted from the plant *Curcuma longa*. It possesses multiple biomedical functions, such as antibacterial, antioxidant, anticancer, and anti-inflammatory [10]. CUR can regulate tumor progression [10]. However, CUR has a low oral bioavailability due to low solubility and poor intracellular uptake [11]. The use of mucoadhesion delivery system allows better bioavailability of CUR and enhances its delivery to inflamed colorectal tissue [12]. Chitosan (CS) is a nontoxic mucoadhesive polymer and curcumin-containing chitosan nanoparticles (CUR-CS-NP) delivered efficiently to colon tumor cells enhancing the anticancer effect of CUR [13].

In the current study, we investigated the possible chemopreventive effects of CUR-CS-NP against animal model of CC induced by subcutaneous injection of DMH for 10 weeks.

Materials and methods

The present study included 37 male white Albino rats aged about 6–8 weeks. The body weights were about 110–130 g. Rats were obtained from the Animal House of the Faculty of Medicine at Assiut University. They were kept in metal cages in healthy conditions according to the classification of groups of the study. Natural light–dark cycle and free access to food and water ensured. They acclimatized to environmental conditions for 2 weeks before the experiment. Care and treatment of animal were provided according to the guidelines of Animal House of Assiut University.

DMH was purchased from Sigma (St Louis, Missouri, USA). CUR obtained from Egyptian herbal market (Cairo, Egypt). All other chemicals and reagents were of analytic grade. DMH was weighed and dissolved immediately just before use in 1 mM EDTA to ensure the stability of the chemical. The pH was adjusted to 6.5 with 1 mM NaOH. CUR was weighed and dissolved in diluted dimethyl sulfoxide (DMSO) (1 ml DMSO + 3 ml distilled water). Each 100 mg CUR dissolved in 5 ml of diluted DSMO. Every rat received about 0.5 ml of dissolved CUR orally for 10 weeks. CUR-CS-NP was formulated as described by Chuah *et al.* [12]. It was prepared by addition of CUR to ethanol, then this solution was added to CS in acetic acid (2%, v/v) solution and the pH adjusted to 5 with 2 M NaOH. Tripolyphosphate solution (0.133%, w/v) was added under constant magnetic stirring. The solution was stirred further for 1 h and then centrifuged at 10 000 rpm for 60 min to pellet the NP. Trehalose was added before centrifugation to prevent aggregation of the NP.

The animals divided randomly into four groups as follow:

(1) Control group (six rats): they received (vehicle) 0.5 ml of 25% DMSO orally once daily
(2) DMH group (10 rats): they were given a subcutaneous injection of DMH (20 mg/kg, subcutaneously) once a week for 10 weeks [14]
(3) DMH and CUR group (10 rats): rats were given DMH (20 mg/kg, subcutaneously) once a week and received CUR orally at a dose of 100 mg/kg body weight per day [15] for 10 weeks
(4) DMH and CUR-CS-NP group (10 rats): they were given a subcutaneous injection of DMH (20 mg/kg, subcutaneously) once a week and oral CUR-CS-NP at a daily dose 100 mg/kg body weight for 10 weeks.

The rats were sacrificed by cervical dislocation at the end of 10 weeks and 1 day after the last dose treatment. The rat's colons were quickly removed, washed with iced cold saline 0.9%, examined grossly for pathological lesion, and divided into three parts. The first part was snap frozen in liquid nitrogen then stored in −80°C until the assay of caspase-3 gene expression by real-time PCR. The second part was homogenized in (0.1 M) phosphate buffer, and used for the biochemical assay of malondialdehyde (MDA), nitric oxide (NO), glutathione (GSH), and the third part was fixed in 10% neutral buffered formalin for histopathological examination. Blood samples were collected in tubes containing an anticoagulant. Then plasma was separated after centrifugation at 3000 rpm for 10 min and stored at −20°C until the biochemical assay of MDA, NO, reduced GSH, and carcinoembryonic antigen (CEA).

Total RNA was extracted from homogenized colon tissue by use of Genezol CT RNA Extraction Reagent (catalog no. PG-100103; Puregene Genetic Brand, Delhi, India). RNA quality was assessed by a 260/280 nm ratio. Total RNA concentrations were determined using a nanodrop spectrophotometer (SPECTROstar Nano, Microplate and cuvette Spectrophometer; BMG LABTECH, Ortenberg, Germany).

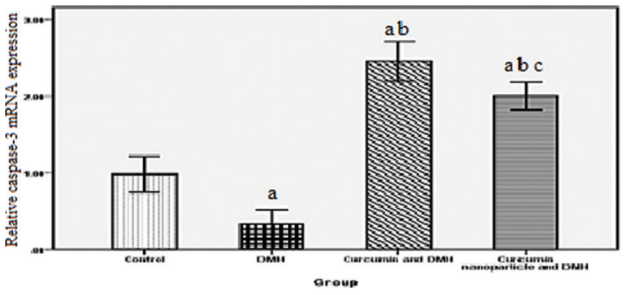
Reverse transcription was performed with High-Capacity cDNA Reverse Transcription Kit (catalog no. 4368814; Applied Biosystems, Foster City, California, USA). The resulting complementary DNA was then used for amplification. Real-time PCR was done in the thermal cycler (Applied Biosystems Step One Plus, Real-Time PCR Systems, California, USA) using SYBR GREEN

PCR master mix (catalog no. #K0251; Thermo Scientific Maxima SYBR Green PCR Master Mix (2×) kit; Thermo Scientific, Massachusetts, USA). PCR reaction volume was 10 μl. It contained 2 μl of cDNA, 5 μl of 2 × SYBR Green PCR master mix, 0.25 μl of forward, 0.25 μl of reverse primers, and up to 10 μl of nuclease-free water. Thermocycling for denaturation 40 cycles (95°C for 25 s) then 60°C for 1 min for annealing and extension. Real-time PCR primers for caspase-3 were forward, 5-GAG CTT GGA ACG CGA AGA AA-3 and reverse, 5-TTG CGA GCT GAC ATT CRCA GT-3. Primers for GAPDH were forward, 5-GCA TCT TCT TGT GCA GTG CRC-3 and reverse 5-ACRC AGC TTC CRCA TTC TCA GC-3. The mRNA levels were normalized to GAPDH. The gene expression levels were assessed using the comparative cycle threshold (CT) method (ΔΔCT method). ΔCT was calculated using the formula: ΔCT = CT (target gene)–CT (endogenous reference gene, GAPDH). ΔΔCT method was calculated using the formula ΔΔCT method=ΔCT target–ΔCT control. The relative fold change in expression was calculated by $2^{-\Delta\Delta CT}$ where ΔΔCT=ΔCT diseased–ΔCT control.

CEA plasma level was detected by using CEA assay kit (catalog no. PT-CEA-96, Germany), according to the manufacturer instructions. Oxidative stress markers as MDA and NO in plasma and colon tissue were measured according to the methods described by Wills [16] and Guevara *et al.* [17], respectively. GSH was assessed as described by Bulaj *et al.* [18]. The protein content was determined as described by Lowry *et al.* [19].

Colon tissues were collected for histological examinations. They were fixed in 10% neutral buffered formalin solution, embedded in paraffin wax, cut into 5 μm thick sections and stained with hematoxylin and eosin for examination by light microscopy.

Figure 1



Mean fold change of caspase-3 mRNA. Each value represents means ± SD of each group. *P* value less than 0.05 considered significant. (a) Compared with control group, (b) compared with DMH group, (c) compared with CUR group. DMH, dimethylhydrazine.

Statistical analysis

The statistical analysis performed with the statistical package for the social science (SPSS), version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY, USA: IBM Corp.), software. All results presented as mean ± SD. One-way analysis of variance was used to compare between all groups followed by Student's unpaired *t* test. *P* value of 0.05 was considered as statistically significant.

Results

Fig. 1 shows mean fold change of caspase-3 mRNA in control, DMH, DMH and CUR group, and CUR-CS-NP and DMH group. Comparing the gene expression level of caspase-3 in DMH group to control group, the caspase-3 expression level was significantly decreased in the DMH-treated group with mean fold change 0.33 ± 0.09. The expression level of caspase-3 was significantly increased in CUR and CUR-NP-treated groups in comparison with DMH group with mean fold change 2.5 ± 0.13, 2 ± 0.09, respectively. CUR-treated group showed statistically significant elevation in the caspase-3 expression level in comparison with CUR-NP-treated group ($P < 0.001$).

The levels of plasma CEA in control and experimental groups are given in Table 1. CEA plasma levels were significantly higher in DMH group in comparison with control, but significantly lower in DMH and CUR and DMH and CUR-CS-NP group when compared with DMH-treated group. CEA levels were significantly decreased in DMH and CUR-CS-NP group in comparison with DMH and CUR group.

The levels of MDA, NO, and GSH in plasma and colon of control and experimental groups are given in Table 2. DMH treatment resulted in significant increase of plasma and colon MDA and NO in comparison with control group. GSH level in plasma and tissue was reduced by DMH treatment. The CUR and CUR-CS-NP treatments reduced the plasma and colon tissue oxidative stress parameters when compared with DMH group.

MDA levels were significantly reduced in DMH and CUR group in comparison with DMH and CUR-CS-NP group. NO levels showed no significant difference between DMH and CUR group and DMH and CUR-CS-NP group. GSH was significantly lower in DMH group in comparison with control, but significantly higher in DMH and CUR group and DMH and CUR-CS-NP group when compared with DMH group. However, GSH levels showed no significant difference between DMH and CUR group and DMH and CUR-CS-NP group.

Table 1 Effect of dimethylhydrazine, curcumin, and curcumin-containing chitosan nanoparticles on carcinoembryonic antigen plasma level

Groups	Control group (*n*=6)	DMH group (*n*=10)	DMH and CUR group (*n*=10)	DMH and CUR-CS-NP group (*n*=11)
Plasma CEA (ng/ml)	0.55±0.05	2.99±0.44[a]	1.29±0.22[ab]	1.09±0.13[abc]

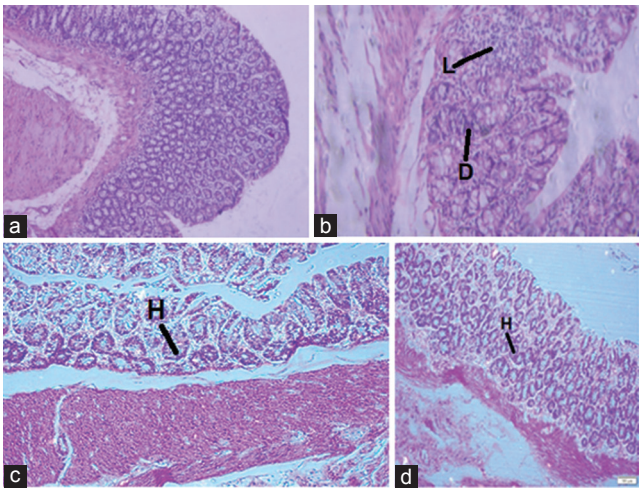
The results were expressed as means±SD. CEA, carcinoembryonic antigen; CUR, curcumin; CUR-CS-NP, curcumin-containing chitosan nanoparticle; DMH, dimethylhydrazine. [a]Compared with control group. [b]Compared with DMH group. [c]Compared with CUR group. *P* value less than 0.05.

Table 2 Plasma and colorectal levels of malondialdehyde, nitric oxide, and glutathione

Groups	Control group (*n*=6)	DMH group (*n*=10)	DMH and CUR group (*n*=10)	CUR-CS-NP and DMH group (*n*=11)
Plasma MDA (μmol/l)	5.05±0.15	5.93±0.22[a]	5.18±0.14[b]	5.30±0.12[abc]
Tissue MDA (nmol/mg protein)	13.7±0.9	18.2±0.76[a]	14.56±0.89[b]	15.58±1.04[abc]
Plasma NO (μmol/l)	34.9±0.4	38.4±2.1[a]	35.6±0.7[b]	36.3±1.9[ab]
Tissue NO (nmol/mg protein)	585.6±11.3	1130.6±29.8[a]	608.3±9.8[ab]	611.4±11.5[ab]
Plasma GSH (μmol/l)	59±1.3	44.5±3.2[a]	54.8±2.3[ab]	54.1±2.9[ab]
Tissue GSH (nmol/mg protein)	715.7±3.3	632.3±4.9[a]	702±3.3[ab]	699.9±4.8[ab]

Level in control, DMH, CUR and DMH, CURCS-NP and DMH-treated groups. The results were expressed as mean±SD. CUR, curcumin; CUR-CS-NP, curcumin-containing chitosan nanoparticle; DMH, dimethylhydrazine; GSH, glutathione; MDA, malondialdehyde; NO, nitric oxide. [a]Compared with control group. [b]Compared with DMH group. [c]Compared with CUR group.

Figure 2



(a) A photomicrograph of control animals revealed transverse sections in the colon lined with mucosa is formed of crypts with lining simple columnar epithelium with goblet cells (H and E, ×100). (b) A photomicrograph of dimethylhydrazine treated group animals revealed transverse section in the colon showing D, dysplastic changes in some crypts, L, lymphoid aggregate is observed with mono nuclear cells mostly lymphocytes (H and E, ×400). (c) A photomicrograph of curcumin treated animals revealed transverse section in the colon appeared more or less similar to those of control, however some crypts exhibit hyperchromasia (H) (H and E, ×100). (d) A photomicrograph of curcumin-nanoparticle-treated animals revealed transverse section in the colon appeared more or less similar to those of control with intact muscularis mucosa (MM), however many crypts exhibit hyperchromasia (H) (H and E, ×100). H and E, hematoxylin and eosin.

Histopathological examinations of colon tissues of different treatment groups are shown in Fig. 2. Control rats showed a normal architecture of colorectal mucosa with goblet cells and intact muscularis mucosa. DMH treatment resulted in dysplasia and lymphocytic infiltration. CUR-treated rat group showed more or less similar crypts to control with intact muscular mucosa. CUR-CS-NP treated animals revealed that the colon mucosa appeared more or less similar to those of control with intact muscularis mucosa, however some crypts exhibits hyperchromasia.

Discussion

CC is a leading cause for cancer-related deaths and its prevention is of great importance throughout the world. The use of natural products provides efficient and safe chemopreventive agents to inhibit the progression of precursor lesions of CC [20].

In the current study colon tissue homogenate of DMH group treated rats showed lower expression of caspase-3 mRNA as one of the apoptotic markers when compared with the control rats. This can be explained as a mechanism of malignant cells to resist apoptosis, so can increase the size of the tumor [21]. The current study showed that CUR administration to DMH-treated rats resulted in up regulation of caspase-3 mRNA when compared with DMH-treated rats. Induction of apoptosis in colon epithelial cells of rat received DMH inhibited aberrant colorectal epithelial cell progression. CUR can induce apoptosis of different cancer cells as it is able to identify abnormal mediators produced by cancerous cells and initiates the process of apoptosis leading to self-destruction [22]. CUR effectively modulated the number of apoptotic cells and increased caspase-3 activity [23]. In the present study, the increase of the caspase-3 expression by CUR-CS-NP treatment indicted that the CS-CUR-NP can prevent the growth of cancer cells by induction of apoptosis.

CEA is a 180–200 kD glycosylated protein. It is the most useful tumor marker to distinguish between benign and invasive carcinomas of the colon as benign adenomas do not cause an increase in CEA levels [24]. In the current study, there is a significant elevation in plasma CEA in DMH group rats in comparison with control group animals, mostly due to increase in its production by malignant cells. These data are in approval with a previous study [25]. This result is also consistent with the histological result which revealed dysplasia, hyperplasia, hyperchromatic, ulceration, and erosion in the lining epithelium of the colon in addition to dense lymphocytic infiltration in the submucosa. CUR and CUR-CS-NP supplements to DMH-treated rats significantly decreased plasma CEA level when compared with DMH-treated rats. Histopathological examinations revealed decreased hyperplasia, dysplasia, ulceration of mucosa, with some residual of hyperchromasia in colon of CUR and CUR-NP-treated group. This result reflects the anticancer activity of CUR, which lead to reduction in malignant cells with decrease CEA release.

In addition, our study showed that plasma CEA was significantly decreased in CUR-NP-treated rats in comparison with CUR-treated animals. This may be due to the improved anticancer effect of CUR-NPs. This result reflects the anticancer activity of CUR and CUR-CS-NP which lead to the reduction in malignant cells with decrease CEA release.

Lipid peroxidation induces DNA mutation and cell proliferation, which can lead to the development of colorectal cancer [26]. The present study showed that MDA levels in colorectal tissue were significantly elevated in DMH-treated rats in comparison with control group rats. As, DMH can induce oxidative stress [27], the reactive oxygen species readily react with lipid bilayers and release products such as MDA, hydroperoxides, and hydroxyl radicals. Reactive oxygen species disintegrate and loosen of the cell membranes, which in turn allow them to invade the intracellular molecules thus lead to mutations favoring the development of cancer [28].

An increase in plasma and colon MDA and decreased GSH antioxidant potential were observed after DMH injection in rats. The present study showed that plasma MDA was significantly elevated in DMH-treated rats group in comparison with control group animals. Tumor cells produce significant amount of hydrogen peroxide that is released into the circulation. Hydrogen peroxide increase in the blood may be responsible for increase lipid peroxidation [29].

In this study rats treated with CUR and CUR-CS-NP showed a reduced level of MDA, NO and an increase of the levels of GSH. This indicates that CUR and CUR-NP have antioxidant effect as they can scavenge free radicals and prevent lipid peroxidation [30]. CUR is a polyphenol, which can easily donate electrons to reactive free radicals because of the resonance stability of phenoxy radical and thus retard radical chain reactions, in the same manner CUR-CS-NP has radical scveing activity [31].

In conculsion, the present study indicated that CUR and CUR-CS-NP exhibit inhibitory effects against precancerous pathological lesions of colorectal induced by DMH treatment. Our study proves that inhibition of oxidative stress and induction of apoptosis may be two important mechanisms for prevention of CC.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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