Genetic alterations and gene expression profile in male Balb/c mice treated with carbon tetrachloride with or without carboxymethyl chitosan

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Abstract: Carboxymethyl chitosan (CMC), which is a water-soluble derivative of chitosan, it has attracted much attention as a new biomedical material. **The aim** of the current study was to evaluate the chemopreventive effects of CMC against Carbon tetrachloride (CCl_4)-induced genotoxicity and alterations in gene expression in male Balb/c mice. **Materials and Methods**: Sixty male Balb/c mice were divided into six groups included the control group; the group treated orally with CCl_4 (0.5 ml/kg b.w) for three doses at 48 h intervals and the groups treated orally with CMC (140 and 280 mg/kg b.w.) alone for three weeks or in combination with CCl_4. **The results** indicated that treatment with CCl_4 resulted in increased caspase-3 activities, induction of micronucleus (MnPCEs), frequencies of sister chromatid exchanges (SCE's), total chromosomal aberrations in bone marrow, DNA fragmentation percentage in liver, comet formation in liver and bone marrow, over expression in *bax* and down expression in *Bcl-2*. CMC at the two tested doses succeeded to induce a significant improvement in all tested parameters in a dose dependent fashion. Moreover, CMC itself was safe at the tested doses. It could be concluded that CMC is a promise candidate against genotoxicity.

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Key words: genotoxicity, Carboxymethyl chitosan

1. Introduction

Chitosan (COS) is a modified, natural carbohydrate polymer derived by deacetylation of chitin [poly-β-(1 → 4)-N-acetyl-D glucosamine], a major component of the shells of crustacean such as crab; shrimp; crawfish and the 2nd most abundant natural biopolymer after cellulose (No and Meyers 1995). During the past several decades, COS has received increased attention for its commercial applications in the biomedical, food, and chemical industries (Li *et al.,* 1992). In 2005, shrimp-derived COS was approved as GRAS (generally recognized as safe) by USFDA based on the scientific procedures for use in foods in general, including meat and poultry, for multiple technical effects (No *et al.,* 2007). COS has been approved as a food additive in Korea and Japan since 1995 and 1983, respectively (Weiner 1992; KFDA 1995). COS has attracted notable interest due to its biological activities such as antimicrobial (No *et al.,* 2002; Zheng and Zhu, 2003), antifungal (Roller and Covill, 1999), antitumor (Qin et al.., 2002), and hypocholesterolemic functions (Sugano *et al.,* 1992). The antimicrobial activity of COS against a range of food borne filamentous fungi, yeast, and bacteria has attracted attention as a potential food preservative of natural origin (Sagoo *et al.,* 2002).

COS also protect normal cells from apoptosis challenged by exogenous stimuli (Chen *et al.,* 2006), and apoptosis induced by serum starvation in human astrocytes (Koo *et al.,* 2002). Moreover, COS are known to exert good anti-oxidative activities in either cellular studies or cell-free assay (Je *et al.,* 2004; Mendis *et al.,* 2007) and many COS derivatives were synthesized and their antioxidant activity was assessed accordingly (Esumi *et al.,* 2003; Sun *et al.,* 2004; Xing *et al.,* 2005). Although the effectiveness of COS for its ability to

enhance quality and shelf life of foods has been reported by numerous workers, the information about the chemoprotective effects is still limited. Therefore, the aims of the current study were to evaluate the protective role of carboxymethyl chitosan (CMC) against the development of comet images during apoptosis induced by CCl_4 and to establish a correlation between induction of apoptosis and comet formation through the measurements of several assays such as activation of caspase 3, and expressions of apoptosis related genes such as bcl-2 and bax, micronucleus formation, DNA fragmentation and cytogenetic analysis.

2. Material and Methods

2.1. Materials:

2.1.1. Chemicals

Pharmaceutical grade chitosan (90 % deacetylated) was obtained from the Naval Research Laboratory (Washington DC, USA). Carbon tetrachloride (CCl_4) was purchased from Merck/Schuchardt (Darmstadt, Germany). A protease inhibitor cocktail was purchased from Roche (Mannheim, Germany), Trizol, fluorogenic substrates (7-amino-4-methylcoumarin N-acetyl-L-aspartyl-L-glutamyl- L-valyl-L-aspartic acid amide [Ac-DEVD-AMC], Bcl-2, Bax and β -Actin were obtained from Life Technologies (Grand Island, NY, USA). Superscript II reverse transcriptase Fermentas kits, Bromodeoxyuridine, propidium iodine, Dimethyl sulfoxide (DMSO), fetal calf serum, normal melting point agarose, low melting point agarose, ethidium bromide and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used were analytical grade.

Synthesis of Carboxymethyl Chitosan (CMC):

Chitosan (2 g) was alkalized in NaOH (8 g) for 12 h in a 50-50 mixture of deionized water and isopropanol (20 ml). After heating the mixture to 60 ^{O}C, monochloroacetic acid (8 g) was dissolved in isopropanol (2 ml) and slowly added to the solution over 30 min. After 6 h the reaction was quenched by adding ethanol (50 ml) to the solution. The resulting CMC was repeatedly rinsed in ethanol and vacuum-dried until the pH of the filtered solution was neutral. The products were dissolved in water and centrifuged to separate the unreacted chitosan; the water soluble portion of the sample was removed, precipitated in ethanol and vacuum-dried. The sample was then placed in an oven at 50 ^{O}C to dry.

2.1.2. Experimental animals:

Eight-week-old male Balb/c mice (25 ± 3 g) were obtained from the Animal House Colony, Giza, Egypt and were maintained *ad libitum* 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 six groups (10 mice/group) and housed in filter-top polycarbonate cages. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre, Dokki, Cairo, Egypt.

2.2. Methods:

2.2.1. Experimental design

Animals within different treatment groups were maintained on their respective diets for 3 weeks as follows: group1, untreated control; groups 2 and 3 treated orally with CMC at 140 and 280 mg/kg b.w. respectively for three weeks; group 4, treated orally with CCl_4 (0.5 ml/kg) for three doses at 48 h intervals; groups 5 and 6, treated orally with the two tested doses of CMC plus CCl_4. At the end of the treatment period (day 22), the animals were kept fasting over night and sacrificed then the bone marrow and liver samples of each animal were removed for the genetic alteration and apoptotic gene expressions studies.

2.2.2. Caspase-3

Lysates were prepared by homogenizing liver tissue in 0.25 mM sucrose, 1 mM EDTA, 10 mM Tris, and a protease inhibitor cocktail. The lysates were then centrifuged at 14,000 g for 10 min at 48 ^{O}C and supernatants (50 µg of protein) were incubated for 1 h at 37^{O}C in HEPES buffer containing 100 µM concentrations of the specific fluorogenic substrates (7-amino-4-methylcoumarin N-acetyl- L-aspartyl- L-glutamyl- L-valyl-L-aspartic acid amide, Ac-DEVD-AMC). Cleavage of the caspase substrates was monitored using a spectrofluorimeter (Hitachi F-2000 fluorimeter; Hitachi LTD, Tokyo, Japan) at excitation/emission wavelengths of 380/460 nm. Activity was expressed as fluorescence units per milligram of protein per minute of incubation (UAF/min/mg protein).

2.2.3. RNA extraction and RT-PCR

Liver samples (200 mg) were quickly thawed and homogenized in 2 ml of Trizol and total RNA was isolated according to the manufacturer's directions. RNA was resuspended in RNase-free water, quantitated using UV spectrophotometer, and

stored at -80°C. The quality of the isolated RNA was assessed by measuring the absorbance at 260 nm, analyzing the A260/A280 ratio (1.7-2).

For cDNA synthesis, 3 µg of total RNA were heated to 70°C for 10 min then placed immediately on ice for 10 min. To each sample, 4 µl of 5x first strand buffer, 2 µl of 0.1 mol/l DTT, 4 AL of 2 mmol/l each deoxynucleotide triphosphate mix, 1 µL of oligo (dT) primer and 1 µl of Superscript II reverse transcriptase were added. Reverse transcription was then carried out at 42°C for 50 min, followed by heating to 70°C for 15 min and cDNA samples were stored at -20°C until assayed. cDNAs were amplified using specific primers for mouse Bcl-2 and *bax* **Table (1)**.

Table (1): Sequences of primers used for amplification

Gene	Sense and antisense	PCR product (bp)	Ref.
Bcl-2	Sense 5-TTGTGGCCTTCTTTGAGTTCG-3 antisense 5-TACTGCTTTAGTGAACCTTTT-3	332	Agarwal et al. (1999)
Bax	Sense 5-ACCAGCTCTGAGCAGATCATG-3 antisense 5-GGGATTGATCAGACACGTAAG-3	626	Zhang et al. (2006)
β -Actin	Sense 5'-CGTGACATCAAAGAGAAGCTGTGC-3', antisense 5'-CTCAGGAGGAGCAATGATCTTGAT-3'.	376	Baek et al.(2007)

Gene expression was assayed according to the manufacturer's instruction. The PCR program cycles were set as follows: initial denaturing at 95°C for 20 s, followed by 40 cycles (95°C for 3 s, 60°C for 30 s). *β* -actin mRNA was used as an internal standard, Bcl-2 and *bax* mRNA expressions were determined by quantitative reverse transcription-PCR (RT-PCR) and normalized against *β*-actin mRNA levels. The PCR product was run on a 2% agarose gel in Tris-borate-EDTA buffer and visualized over a UV Trans-illuminator. The ethidium bromide-stained gel bands were scanned and the signal intensities were quantified by the computerized Gel-Pro (version 3.1 for window 3). The ratio between the levels of the target gene amplification product and the *β*-actin (internal control) was calculated to normalize for initial variation in sample concentration as a control for reaction efficiency (Raben *et al*., 1996).

2.2.4. Micronucleus test:

Micronucleus assay was carried out on bone marrow according to the method described by Schmid (1975). The femurs were dissected out and the bone marrow was flushed out, vortexes and centrifuged. The pellet was resuspended in a few drops of fetal calf serum. Smears were made on pre-cleaned dry slides, air dried and fixed in absolute methanol and the slides were stained with Giemsa stain. At least 2000 erythrocytes were observed and the numbers of polychromatic erythrocytes were counted. The micronuclei were recorded and micronuclei per 1000 cells were calculated.

2.2.5. DNA Fragmentation Assay

2.2.5.1. *DNA extraction*

DNA fragmentation was used as a measure of apoptotic. The presence of DNA ladder was determined according to Sambrook *et al.*, (1989) and the modifications described by Xu *et al,*. (1996). The absorbance of the DNA solution was red spectrophotometrically at absorbances of 260 and 280 nm. Equal amounts of DNA were taken after spectrophotometric analysis as described by Kamalay *et al.*, (1990).

2.2.5.2. Agarose gel electrophoresis

A gel was prepared with 2% agarose containing 0.1% ethidium bromide. The gel was electrophoresed using the submarine gel electrophoresis machine. The DNA was visualized and photographed with illumination under UV light.

2.2.6. Comet assay (Single Cell Gel Electrophoresis)

DNA damage was measured using the comet assay under alkaline conditions and dim indirect light according to the method described by Singh *et al.*, (1988) with a few modifications. Briefly, 120 µl of 0.5% normal melting point agarose in Ca^{+2} and Mg^{+2} -free phosphate buffer at 56°C were quickly layered onto a fully frosted slide and immediately covered with a cover-slip. The slides

were kept at 4°C to allow the agarose to solidify. After gently removing the cover-slip a 50 µl aliquot of cell suspensions of either bone marrow or liver were mixed with an equal volume of 1% low melting point agarose at 37°C and quickly pipetted onto the first agarose layer in the same manner. Finally, 70 µl of 0.5% LMP agarose were added to cover the cell layer. The slide sandwiched without cover-slips and were immersed in freshly prepared, cold lysing buffer (2.5 mol/l NaCl, 100 mmol/l Na_2 EDTA, 10 mmol/l Tris, 1% *N* -Lauroyl sarcosine sodium salt, pH 10, with 1% Triton X-100 added just before use) and kept at 4°C for 45 min to 1 h. The slides were placed on a horizontal gel electrophoresis platform and were covered with cold alkaline buffer (300 mmol/l NaOH, and 1 mmol/l Na_2 EDTA) for 8 to 20 min in the dark at 4°C to allow DNA unwinding and expression of the alkali-labile sites. The timing for lysis and unwinding was determined empirically for each cell line. Electrophoresis was conducted at 4°C in the dark for 20 min at 25 V and 300 mA. The slides were then rinsed gently twice with neutralizing buffer (0.4 mol/l Tris, pH 7.5). Each slide was stained with 120 µl of propidium iodine at a concentration of 5 µg/ml and covered with a cover-slip. Comet tail lengths were quantified as the distance from the centrum of the cell nucleus to the tip of the tail in pixel units, with the mean tail length being determined as the mean length of twelve tails.

2.2.7. Sister chromatid exchanges (SCE's) and chromosomal abnormalities in bone marrow:

2.2.7.1. *Sister chromatid exchanges*:

The method described by Allen (1982), for conducting *in vivo* SCE's induction analysis in mice was applied with some modifications. Approximately 55 mg 5'-Bromodeoxyuridine tablets were inserted in mice subcutaneously (s.c.) 21-23 hrs before sacrifice. Mice were injected intraperitonealy with colchicine at a final concentration of 3 mg/kg b.w 2 h before sacrifice. Bone-marrow cells from both femurs were collected and the fluorescence-photolysis Giemsa technique was used (Perry and Wolff, 1974). Forty well spread metaphases were analyzed per mouse to determine the frequency of SCE's/cell.

2.2.7.2. *Chromosome abnormalities:*

For cells preparations, animals within different groups were injected i.p. with colchicines, 2 hrs before sacrifice. Chromosome preparations from bone marrow cells were carried out according to the method described by Yosida and Amano (1965). One hundred well spread metaphases were analyzed per mouse. Metaphases with gaps, chromosome or chromatid breakage, fragments and deletions were recorded.

2.2.8. Statistical analysis

Data were analyzed statistically by "Analysis of Variance" (ANOVA) and groups were compared by Duncan's Multiple Range Test (DMRT). p values $\leq$ 0.05 were considered as significant. The significance of the results from the control data was calculated using (t- test) for SCE's and chromosome abnormalities.

3. Results

3.1. The levels of caspase-3 activities:

The levels of caspase-3 activities showed high apoptotic rate in mice treated with CCl_4 compared to the control group. Animals treated with CMC at the two tested doses show no significant increase comparable to the control. The apoptotic rate was significantly reduced when CMC co-administrated with CCl_4. The high dose of CMC was more effective than the low dose to reduce apoptotic rate **(Fig. 1)** since these treatments decline the caspase-3 activities and resulted in inhibition reduced from 89.4 in CCl_4 to 65.2 in CMC1 and 54.1% in CMC2. Moreover, the activation of caspase 3 also showed a good correlation with comet tail formation.

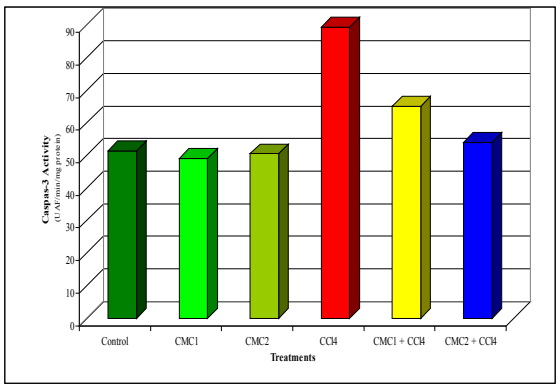

Fig. 1 Alterations of caspase 3 activity in mice treated with CCl_4 alone or in combination with CMC

3.2. Evaluation of genes expressions

The ratio between *bax*/ *β*-actin indicate an over expression in *bax* compared to the ratio between control/ *β*-actin **(Fig. 2 & 3)** which increased to reach 2 in the animals treated CCl_4 while in control it was 0.87. On the other hand, the ratio of Bcl-2/ *β*-actin was decreased compared to control/ *β*-actin ratio in mice treated with CCl_4 which decreased from 1.71 to 0.91 (Fig. 2 & 4). Treatment with CMC at the low dose reduced the ratio of expression of mRNA *bax* compared with CCl_4-treated group from 2 to 1.38.

While treatment with CMC at the high dose resulted in a further reduction in the ratio expression of mRNA *bax* to reach 1.01. Moreover, treatment with CMC at the two tested doses increased the expression of mRNA Bcl-2 ratio in a dose dependent fashion from 0.91 in CCl_4 group to reach 1.28 in the group treated with CCl_4 plus CMC at low concentration and increase the ratio to reach 1.46 in the group treated with CCl_4 plus CMC2 as shown from the results of image analysis

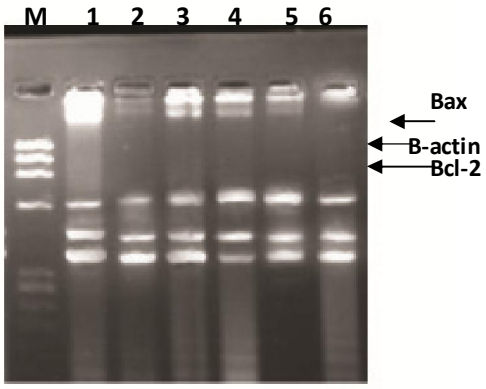

Fig.2. Effect of CCl_4 and CMC on expression pattern of *bax* mRNA level in liver. The 626 and 376, 332 bp fragments represent *bax* transcript, β -actin as internal standard and Bcl-2 respectively; lane M: molecular marker (Φx174 DNA HaeIII digest). Lane 1: control, lane 2: CMC1, lane 3: CMC2, lane 4: CCl_4, lane 5: CMC1 + CCl_4 and lane 6: CMC2 + CCl_4.

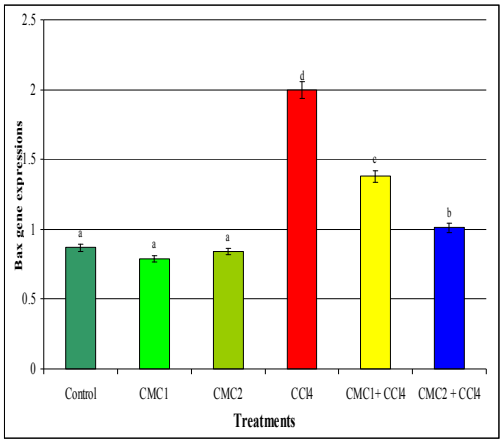

Fig 3. The ratio between *bax*/ β-actin in mice treated with CCl_4 alone or in combination with CMC1 and CMC2. Values represent mean ± S.E. for each group of mice.

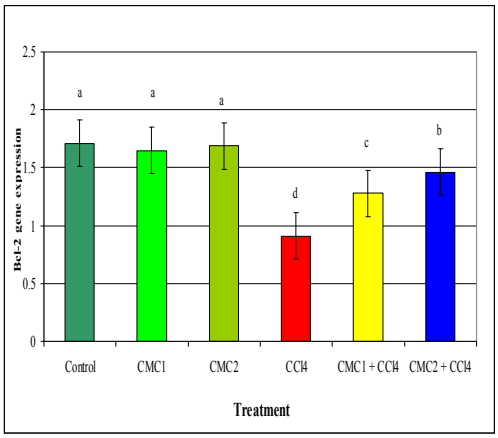

Fig 4. The ratio between Bcl-2/ β-actin in mice treated with CCl_4 alone or in combination with CMC1 and CMC2. Values represent mean ± S.E. for each group of mice.

3.3. Micronucleus

The results of MnPCEs are presented in **Table (2)** and indicated that mice treated with CCl_4 alone showed a high frequency of MnPCEs compared to the control group. However, animals treated with CCl_4 and CMC at the two tested doses showed a significant reduction in the mean of MnPCEs which reached 31.2 in CCl_4-treated group to 18.6 in CCl_4 plus CMC1 group meanwhile; the mean MnPCEs in CCl_4 plus CMC2-treated group reduced to reach 10.4. Moreover, CMC alone did not induce any significant differences in the frequency of MnPCEs compared to control group.

3.4. Percentages of DNA fragmentation in the liver tissue

The current results showed that CCl_4 induced apoptotic DNA fragmentation in mice liver on agarose gel **(Fig 5)**. Animals treated with CCl_4 revealed necrosis where the DNA breakdown was random and led to irregular-length DNA fragments with an indistinct pattern on gel electrophoresis (lanes 3&4). No apoptotic bands were observed in animals given the combined treatments of CCl_4 and CMC (lanes 6 and 7). Moreover, the administration of CMC at the two doses did not induce any differences from the control group (lanes 2 & 5). On the other hand, the results of DNA fragmentation in CCl_4-induced apoptotic changes in the liver are presented in **Table (3)** and indicated that the percentage of DNA fragmentation

Table (2): Micronucleus test and comet assay in bone marrow of mice treated with CCl_4 alone or in combination with CMC

Treatments	PCEs %	No. Mn-PCEs	Mn-PCEs Mean ± S.E.	% of cells showing comet tails	Comet tail length M ± SE
Control	52.2	21	4.2 ± 0.8^{d}	1.4	1.52 ± 0.22^{d}
CMC1	53.1	19	3.8 ± 0.66^{d}	0.8	1.06 ± 0.05^{d}
CMC2	55.4	23	4.3 ± 0.37^{d}	1.0	0.98 ± 0.04^{d}
CCl_4	41.4	156	31.2 ± 1.77^{a}	15.0	6.9 ± 0.28^{a}
CMC1 +CCl_4	48.0	93	18.6 ± 1.63^{b}	9.2	3.95 ± 0.2^{b}
CMC2 +CCl_4	50.0	52	10.4 ± 1.6^{c}	6.2	2.49 ± 0.20^{c}

Five mice were used in each group. Means with different superscripts (a, b, c, d) between groups in the same column are significantly different at $P<0.05$.

significantly increased in the group treated with CCl_4 compared to the control group. Treatment with either CMC1 or CMC2 showed a decrease in the percentage of DNA fragmentation. However, mice treated with CCl_4 and received either CMC1 or CMC2 showed a significant improvement in the percentage of DNA fragmentation towards the control values

3.5. Comet assay

DNA damage assayed using fluorescence microscopy in individual cells in the animals treated with CCl_4 showed the comet tail which indicated that cellular DNA was fragmented due to apoptotic change. The percentages of the tailing cells calculated in each tested group for bone marrow cells and hepatocytes are presented in **Tables (2 & 3).** It is clear that CCl_4 increased the percentage of cells with comet tail to 15% and the mean tail length to 6.9 ± 0.28 in bone marrow cells **Table (2).** However, in hepatocytes, the percentage of cells with comet tail was increased to 23.4% and the mean tail length was also increased to 9.87 ± 0.38 in hepatocytes **Table (3).** The percentage of cells with comet tail and the mean of tail length in the groups treated with CMC at the two tested doses were comparable to the control group. On the other hand, animals treated with CCl_4 and CMC1 showed a decrease in the percentage of cells with comet tail to 9.2% and 12.4% and the mean of tail length to 3.95 ± 0.2 and 6.54 ± 0.21 for bone marrow and hepatocytes respectively. It is of interest to mention that a further reduction was observed in the percentage of cells with comet tail and the mean of tail length in the group treated with CCl4 and CMC2 in both bone marrow and hepatocytes since these values recorded 6.2% for cells with comet tail and 2.49 ± 0.20 for the mean of tail length in bone marrow however; the percentage of cells with comet tail recorded 5.4% and the mean of tail length recorded 3.17 ± 0.22 in the hepatocytes.

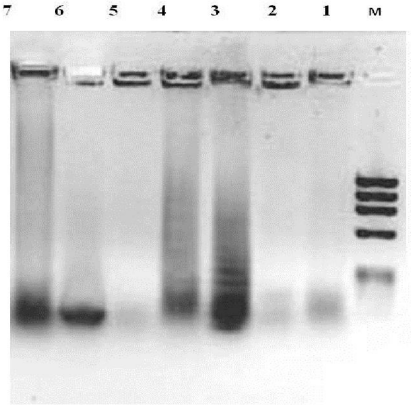

Fig 5. Agarose gel electrophoresis of DNA extracted from liver of mice after treatment, lane M: molecular marker (Φx174 DNA HaeIII digest), lane 1: control, lane 2: CMC1, lanes 3& 4: CCl_4, lane 5: CMC2, lane 6: CMC2 + CCl_4 and lane 7: CMC1+CCl_4

Table (3) DNA fragmentation and Comet assay in liver of mice treated with CCl_4 alone or in combination with CMC

Treatments	DNA fragmentation %	% of changes	% of cells showing comet tails	Comet tail length M ± SE
Control	6.4	0	1.6	0.94 ± 0.08^d
CMC1	5.8	- 0.6	0.8	0.84 ± 0.03^d
CMC2	6.0	- 0.4	0.8	0.82 ± 0.04^d
CCl_4	39.7	+ 33.3	23.4	9.87 ± 0.38^a
CMC1 +CCl_4	26.3	+ 19.9	12.4	6.54 ± 0.21^b
CMC2 +CCl_4	12.9	+ 6.5	5.4	3.17 ± 0.22^c

Five mice were used in each group. Means with different superscripts (a, b, c, d) between groups in the same column are significantly different at P<0.05.

Table (4): Number and percentage of the different types of chromosomal aberrations and frequency of sister chromatid exchanges in mouse bone marrow cells after treatment with CCL_4 plus CMC

Treatments	No. and (%) of metaphases with				Total chromosomal aberrations		No. of abnormal metaphases[a]	Inhibition %	No. and % ( ) of different types of SCE's/chromosome				Total No. of SCE's[b]	SCE's/cells[c] Mean ± S.E.	Inhibition %
	Gap	Frag.	Break	Del.	Including gaps Mean ± S.E.	Excluding gaps Mean ± S.E.			Single	Double	Triple	Quadruple			
Control	10 (2.0)	9 (1.8)	4 (0.8)	0	4.6 ± 0.4	2.6 ± 0.2	23	--	766 (9.5)	48 (0.6)	1 (0.01)	-	865	4.32 ± 0.25	
CMC1	12 (2.4)	8 (1.6)	2 (0.4)	0	4.4 ± 0.4	2.0 ± 0.3	22	--	855 (10.6)	48 (0.6)	1 (0.01)	-	954	4.77 ± 0.46	
CMC2	9 (1.8)	10 (2.0)	2 (0.4)	0	4.2 ± 0.2	2.4 ± 0.2	21	--	759 (9.4)	43 (0.5)	3 (0.03)	-	854	4.27 ± 0.23	
CCL_4	31 (6.2)	68 (13.6)	15 (3.0)	3 (0.6)	23.4 ± 0.7**	17.2 ± 0.6**	117	--	1657 (20.7)	271 (3.3)	26 (0.3)	9 (0.11)	2313	11.56 ± 0.44**	
CMC1+ CCl_4	28 (5.6)	40 (8.0)	9 (1.8)	2 (0.4)	15.8± 0.66♦♦	10.2 ± 0.5♦♦	79	40.6	1138 (14.2)	124 (1.55)	9 (0.11)	2 (0.02)	1421	7.1 ± 0.3♦♦	38.58
CMC2 + CCl_4	19 (3.8)	29 (5.8)	7 (1.4)	0	11.0± 0.86♦♦	7.2 ± 0.4♦♦	55	58.1	967 (12.0)	60 (0.75)	6 (0.07)	-	1105	5.52 ± 0.24♦♦	52.24

a. The total number of scored metaphases is 500 (5 animals / group). Frag. = fragment, Del. = deletion
** Significant at 0.01 level (t-test) comparing to control (non-treated). ♦♦ Significant ♦♦ Significant at 0.01 level (t-test) comparing to treatment b. The total number of chromosomes is 8000 c. The total number of scored metaphases is 200 (5 animals / group)

3.6. Chromosomal aberrations and sister chromatid exchanges (SCE's) in bone marrow cells

The frequencies of SCE's/cell induced by CMC were not significantly different compared to the control group. Animals treated with CMC three weeks prior to CCl_4 showed a significant decrease in the mean percentage of SCE's/cell induced by CCL_4 alone and recorded 11.56 ± 0.44 in CCL_4-treated group and 7.1 ± 0.3 and 5.52 ± 0.24 in the groups pre-treated with CMC at the two tested doses respectively **Table (4)**. Furthermore, the percentage of inhibitory index increased from 38.58% with low dose to 52.24% with the high dose of CMC **Table (4)** and **Fig (6)**. Data presented in **Table (4)** showed the number and percentage of different chromosomal aberrations induced in different groups. These data revealed that both the low and high doses of CMC significantly ($p<0.01$) reduce the percentages of aberrant cells compared to the control group in a dose dependent manner. Moreover, CMC succeeded to block the chromosomal aberrations induced by CCL_4 and the percentage of reduction reached 40.6 and 58.1% after treatment with CMC1 and CMC2 respectively

4. Discussion

In the current study, we evaluated the protective role of CMC against CCl4-induced apoptosis and genetic alterations via the determination of caspas-3, expressions of apoptosis related genes such as bcl-2 and bax ,DNA fragmentation, comet formation, sister chromatid exchanges and chromosomal aberrations in mice.

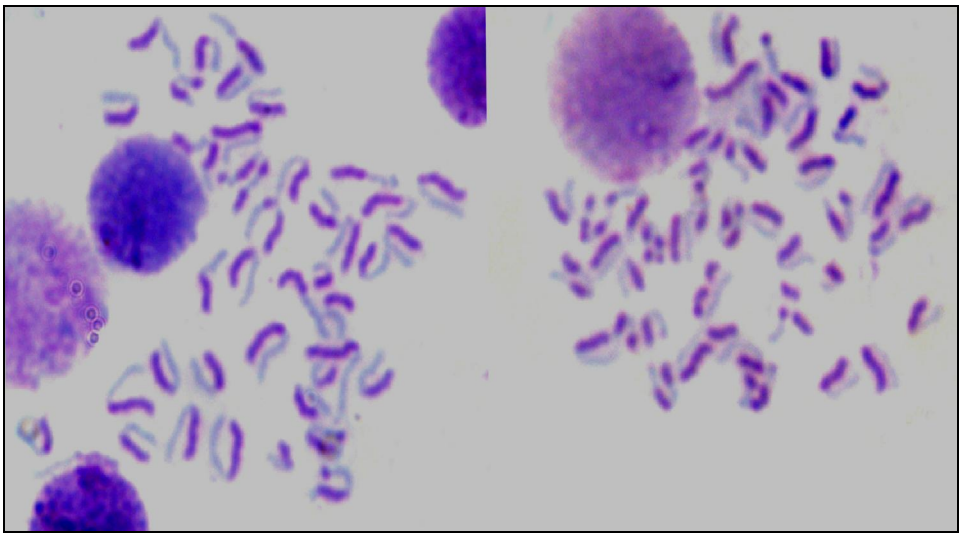

Fig. (6). Sister Chromatid exchanges in bone marrow cells of mouse treated with CCl_4 (right) or CCl_4 plus CMC (left).

The selective dose of CCl4 and CMC were literature based (Singab et al., 2005; and Khodagholi et al., 2010). It is well documented that the toxicity of CCl4 is thought to involve two phases; first, CCl4 metabolization by cytochrome P450 in the hepatocytes produces the highly reactive CCl3-radical, which leads to lipid peroxidation and membrane damage. The second step is a Kupffer cell mainly related inflammatory response. Kupffer cells are activated by free radicals and secrete cytokines that attract and activate neutrophils. Neutrophils themselves release reactive oxygen intermediates (ROIs), thereby enhancing the liver injury (Louis et al., 1998). Excess ROI, a condition referred to as oxidative stress, is considered to be a major contributor to cell injury, although many studies have shown that higher levels of ROIs can also activate specific genetic programs in various cells (Fredovich, 1978; McCord and Fredovich, 1978; Bartosz, 2009). In the current study, CCl4 significantly increased caspase 3 activity and a pro-apoptotic gene (bax) expression as well as decreased anti-apoptotic gene (Bcl-2) gene expression. The significant high level of bax expression found in liver of CCl4-treated mice indicated that these cells are susceptible to apoptosis. In this concern, Masson et al., (2000) reported that the proapoptotic proteins Bad and Bax were significantly higher in liver cirrhosis induced by CCl4 and apoptosis takes place in liver during CCl4-induced cirrhosis. The results revealed that treatment with CCl4 resulted in a significant increase in micronucleus (MN) formation in bone marrow cells which represents fragments of the chromosome or whole chromosomes resulting from clastogenic or aneugenic events (Savage, 1989; Fenech and Morley, 1989) since micronucleus formation is the very early steps of chromatin condensation due to apoptosis (Melntleres et al., 2001).

Apoptosis lead to DNA damage as indicated by DNA fragmentation and comet formation reported in the current study since a 39.7 % enhancement of DNA fragmentation in liver of mice treated with CCl4 compared to the control group. Similar result were observed by Lee et al., (2010) who reported that CCl4 induced hepatocyte DNA fragmentation and cytosolic caspase-3 and caspase-8 activity in rats. Moreover, CCl4 induced DNA strand breaks in hepatocytes and in bone marrow cells measured by single cell gel electrophoresis through the increase in comet tail length in CCl4-treated group compared to control group. Similar result noticed by Vanitha et al., (2007) who reported that CCl4 induced toxicity by comet formation in rats. Moreover, CCl4 increased chromosomal aberrations and SCE's in bone marrow which arise from DNA breaks and reversion of broken fragments at almost homologous loci after their exchange between the two sister chromatids of the same chromosome (Latt et al., 1981) and hence their formation is dependent on the S-phase of the cell cycle (Kato, 1977) or on DNA replication processes (Painter, 1980; Lasne et al., 1984). SEC's is widely used as a reliable and

sensitive indicator of chromosome (DNA) instability, since the SCE patterns can reveal general genome instability (Wilcoskey and Rynard, 1990; Kang et al., 1997). The present results demonstrated a significant elevation of SCE's/cell in CCl4-treated mice compared to control group. The in vivo chromosomal aberration is one of the most important bioassays for monitoring the genotoxicity of environmental chemicals (Tucker and Preston, 1996). Previous reports indicated that CCl4 induced SCE's and chromosomal aberrations in peripheral lymphocytes of sheep (Dianovsky and Ivikova, 2001) and bone marrow of mice (Abou Gabal et al., 2007) which clarifies that CCl4 has the ability to induce chromosomal aberrations in bone marrow cells.

CMC is a way for conversion of COS into a water-soluble form. CMC has many unique chemical, physical and biological properties such as low toxicity, biocompatibility and good ability to form films, fibres and hydrogels (Muzzarelli, 1988; Sun et al., 2008). Consequently, it has been extensively used in many biomedical fields such as a moisture-retention agent, a bactericide, in wound dressings, as artificial bone and skin, in blood anticoagulants and as a component in the drug delivery matrices (Janvikul and Thavornyutikarn, 2003; Liu et al., 2007).

Several molecular weight (MW) COS were tested as a dietary supplement (Gades and Stern, 2005; Kaats et al., 2006). High MW COS would be expected to inhibit the absorption of certain lipids and bile acids. However, low MW COS would be predicted to absorb such substances, but would also be expected to show increased antioxidant effects. Anraku et al., (2009) showed that the administration of low MW COS to human volunteers strongly inhibited the oxidation of human serum albumin (HSA) in vivo. The antioxidant properties of low MW COS are substantial, whereas high MW COS was found to be much less effective in terms of antioxidant properties (Tomida et al., 2009).

According to Xue et al., (1998) and Chiang et al., (2000), low MW chitosan can be absorbed from the intestinal tract and subsequently shows a number of additional bioactivities such as antitumor, cholesterol-lowering, immunostimulating, antidiabetic, antimicrobial, and antioxidant effects, etc., in both the systemic circulation and the intestinal tract. During these biological events, the property of particular interest for this study is the antioxidant activity of COS (Xue et al., 1998; Chiang et al., 2000). In the current study, CMC was found to improve liver injury, prevent apoptosis and protect cells from damaging effects of oxygen radicals. Moreover, CMC did not only prevent oxidative injury in bone marrow and liver cells, but also potently interfere with apoptosis and genotoxicity due to attenuated exogenous oxidative stress. Similar, results were reported by Koo et al., (2002) who indicated that COS able to protect against apoptosis in human astrocytoma cells (CCF-STTG1) induced by serum starvation. Moreover, Liu et al., (2010) stated that COS is not only reversed the decrease of cell viability and proliferation activity, but ameliorated nuclear chromatin damage in H2O2-induced HUVECs.

In the present study, treatment with CMC resulted in a significant reduction in all tested parameters which increased as a results of free radicals generation produced by CCl4 including caspase-3 activities, DNA fragmentation in liver, comet formation in liver and bone marrow, micronucleus (MnPCEs), frequencies of SCE's, total chromosomal aberrations in bone marrow, over expression in bax and down expression in Bcl-2. Several reports indicated that COS enhanced the resistance to the effects of oxidative stress and increased the plasma total antioxidant radical trapping capacity (Wayner et al., 1987). Moreover, Anraku et al. (2011) reported that COS reduces the levels of pro-oxidants such as cholesterol and uremic toxins in the gastrointestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation. Thus, CMC has the potential ability to act as a protein antioxidant, since oxidative stress is an important pathogenic factor in CCl4 toxicity. Moreover, the reducing power properties of CMC are generally associated with the presence of reductions, which have been shown to exert antioxidant action by breaking the free radicals' chain by donating a hydrogen atom (Duh et al., 1999) and/or radical scavenging mechanisms of substituting carboxymethyl group (Sun et al., 2008).

In conclusion

The current work revealed a significant correlation between caspase-3 activities and the expression of bax. Meanwhile CMC decreased caspase-3 activities and bax gene expression induced by CCl4. This may be indicated that bax may participate in the apoptosis by regulating caspase-3 and may indicate a close relationship between these two proteins in apoptosis. On the other hand, CMC increased Bcl-2 expression and indicated that Bcl-2 may play a pivotal role in the regulation of hepatic cell apoptosis and indicated that CMC treatment substantially prevents CCl4-induced genotoxicity and apoptosis in the bone marrow and liver of mice.

5. References

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