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Possible Synergistic Effect and Antioxidant Properties of Chitosan Nanoparticles and Quercetin against Carbon Tetrachloride-Induce Hepatotoxicity in Rats

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Abstract

This study was conducted to prepare chitosan nanoparticles (CNPs), to determine their properties and to evaluate the synergistic protective role of CNPs alone or in combination with quercetin (Q) against oxidative stress and hepatotoxicity in rats. Female Sprague-Dawley rats were divided into 12 groups (7 rats/group) and were maintained on their respective diet for 3 weeks as follow: control group, the group treated with CCl_4 (100 mg/kg b.w twice a week); the groups received CNPs at low and high doses (140 and 280 mg/kg b.w); the group received Q (50 mg/kg b.w); the groups received CNPs at the low or high doses plus Q and the groups treated with CCl_4 plus Q and/or CNPs at the two tested doses. Blood and liver samples were collected at the end of experiment period for biochemical and histological studies. The results indicated that chitosan showed deacetylation degree of 17.5% and 19.2% and the molar mass average of monomer was 168.35 g/mol and 169.1 g/mol by UV and IR methods respectively. The particle size of CNPs was around 100 nm with a rough surface. The *in vivo* results revealed that CCl_4 induced biochemical and histological changes typical to those reported in the literature. Animals treated with CNPs at the two tested doses alone or in combination with Q were comparable to the control. CNPs alone or plus Q succeeded to induce significant improvements in the biochemical parameters and histological picture of the liver in rats treated with CCl_4. This improvement was in dose-dependent manner for CNPs and was

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more pronounced in the group treated with the high dose plus Q. It could be concluded that both CNPs and Q could induce protection against hepatotoxicity. Consequently, CNPs was a promise candidate as drug delivery in liver diseases treatments.

Keywords

Nanochitosan, Quercetin, Liver, Antioxidant, Oxidative Stress

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third most common cause of cancer-related death [1]. It is representing 90% of primary liver cancer worldwide and its incidence is dramatically increasing in the United States, Western Europe and Japan [2] [3]. In the last two decades, no significant improvement in the survival rate of patients with HCC and till now no available drug is able to prevent or reduce tumor spread and/or recurrence [4]. According to Xu *et al.* [5], chemotherapy and radiotherapy are avoided in the treatment of this disease due to associated liver toxicity.

Chitosan is a natural polysaccharide consisting of varying amounts of (1-4)-glycosidic bonds linking N-acetyl-2-amino-2-deoxy-Dglucopyranose (glucosamine) and 2-amino-2-deoxy-D-glucopyranose (N-acetyl-gluamine) [6]. Chitosan has attracted much attention as a biomedical material, as it exhibits a wide variety of biological activities, such as antitumor activities [7]-[9], immunostimulating effects [10], antiallergic effects [11], hemostatic agent [12], anticoagulant effects [13] [14] hypocholesterolemic effects [15], antiinflammatorty activities [16], free radical scavenging activities [17], antimicrobial effects [18], antibacterial effects [19] [20] wound-healing effects [21] [22], antifungal activities [23] [24] and antiviral activity [25]. However, because of its high molecular weight and water-insolubility, the applications of chitosan are severely limited. As a solution, nanoparticle formulation provides a plausible pharmaceutical basis for enhancing oral bioavailability and therapeutic efficacy of chitosan and other drugs that are poorly soluble [26]. Chitosan nanoparticles (CNPs) exhibit more superior activities than chitosan and have been reported to have heightened immune-enhancing effect, anticancer activity and antimicrobial activity than those of chitosan. In addition, nanoparticles possess a stronger curvature of the surface, compared to large particles; this produces more dissolution pressure with a corresponding increase in saturation solubility [27]. The increased saturation solubility, in turn, favors an increase in concentration gradient between intestinal epithelial cells and the mesenteric circulation beneath.

Quercetin (Q) is one of the most abundant flavonoids in the human diet, which is found in fruits and vegetables such as blueberries, onions, broccoli and leek [28] [29]. It is well documented that Q has broad bioactivity, such as anti-proliferative and anticancer properties [30] [31], anti-fibrotic [32] [33], anti-coagulative [34], anti-bacterial [35], anti-atherogenic [36] [37], anti-hypertensive [38] [39] and anti-inflammatory capacities [40]-[43]. However, the antioxidant effects of quercetin have been closely linked with the potential generation of reactive pro-oxidant intermediates resulting in mutagen and genotoxicant [44]. In an *in vitro* study, quercetin has showed positive mutagenic activity in most standard strains of *Salmonella typhimurium* [45] [46]. Moreover, In *Escherichia coli*, quercetin has been shown to induce SOS activity, reverse mutations, and DNA single strand breaks with or without metabolic activation [46]-[49]. These positive mutagenic and genotoxic responses in bacterial test systems have been confirmed in eukaryotic cells, including yeast [50] and mammalian cells included mouse, hamster, rat and human [51]-[55], in which sister chromatid exchanges, chromosomal aberrations, unscheduled DNA synthesis (UDS), and micronucleus formation are among some of the endpoints evaluated. However, in a study for a 2-year rodent carcinogenicity bioassay conducted by the National Toxicology Program (NTP), these genotoxic effects have not confirmed suggesting the safety of quercetin for oral consumption in humans [52] [56] [57]. These results are supported by Utesch *et al.* [58] who demonstrate that quercetin is not genotoxic *in vivo* following oral exposure.

Many serious problems are associated with the therapeutic use of poorly water-soluble drugs. This includes poor absorption and bioavailability upon oral administration, embolization of blood vessels from intravenous injection of the water-insoluble drug because of drug precipitation, and local tissue toxicity and low systemic drug bioavailability [59]. Consequently, the aims of the current study are to prepare chitosan nanoparticles

(CNPs), to study the characterization of CNPs and to evaluate the protective role of CNPs alone or in combination with quercetin against oxidative stress and hepatotoxicity induced by carbon tetrachloride (CCl_4) in rat.

2. Materials and Methods

2.1. Chemicals and Kits

Chitosan was purchased from Sigma-Aldrich (France). Quercetin extract was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Carbon tetrachloride (CCl_4) was supplied by Merck/Schuchardt (Darmstadt, Germany). Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) kits were purchased from Spectrum-diagnostics Co. (Cairo, Egypt). Alkaline phosphatase (ALP), glutathione peroxidase (GPx), superoxide dismutase (SOD), lipid peroxidation (MDA), catalase, alpha fetoprotein (AFP) and carcinoembrionic antigen (CEA) kits were purchased from Biodiagnostic Co. (Giza, Egypt). All other chemicals used throughout the experiments were of the highest analytical grade available.

2.2. Characterization of Chitosan

The acetylation degree of chitosan (DA%) was determined by UV spectrophotometric as described previously [60]. The molar mass average of chitosan monomer and the concentration of free amino groups in chitosan were measured according to the method described by Aljawish *et al.* [61]. The molecular weight of chitosan was evaluated using size exclusion chromatography with multi-angle laser light scattering detectors (SEC-MALLS) [62]. All measurements were carried out at least in triplicate.

2.3. Preparation of Chitosan Nanoparticles (CNPs)

Twenty mg chitosan was dissolved in 40 ml of 2.0% (v/v) acetic acid. A 20 ml of 0.75 mg/ml sodium tripolyphosphate was dropped slowly with stirring. CNPs as a suspension were collected and stored in deionised water. Supernatant was discarded and CNPs were air dried for further use and analysis [63].

2.4. Determination of Particles size and Morphology of CNPs

Size and morphology of CNPs were analyzed using Nanotrac analyzer 6Hx4Wx15D, Model-Nanotrac 150 with a measuring range of 0.8 - 6500 nm. CNPS were cut into pieces of various sizes and wiped with a thin gold-palladium layer by a sputter coater unit (UG-microtech, UCK field, UK) and the morphology of nanoparticles was analyzed with a Cambridge stereoscan 440 scanning electron microscope (SEM, Leica, Cambridge, UK). Atomic force microscopy (AFM) was used for visualization of CNPs rough nature and AFM imaging was performed using CONTR, EZ-2-AFM analysis.

2.5. Experimental Animals

Three-month old female Sprague-Dawley rats (100 - 150 g each) were purchased from Animal House Colony, National Research Centre, Dokki, Giza, Egypt. Animals were maintained on standard lab diet (protein: 160.4; fat: 36.3; fiber: 41 g/kg, namely 12.1 MJ of metabolized energy) purchased from Meladco Feed Co. (Aubor City, Cairo, Egypt) and housed in filter-top polycarbonate cages in a room free from any source of chemical contamination, artificially illuminated (12 h dark/light cycle) and thermally controlled (25˚C ± 1˚C) at the Animal House Lab., National Research Centre, Dokki, Cairo, Egypt. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre, Dokki, Giza, Egypt and the National Institute of Health (NIH publication 86 - 23 revised 1985).

2.6. Experimental Design

Animals were divided into twelve groups (7 rats/group) and were maintained on their respective diet and treated orally for 3 weeks as follow: group 1, normal control animals fed on basal diet and received 0.5 ml saline solution; Group 2, animals treated orally with CCl_4 suspended in corn oil (1.0 ml/kg b.w.) twice a week; Groups 3 and 4, animals treated daily with low and high dose of chitosan nanoparticles CNPs (140 and 280 mg/kg b.w.) respectively susbended in saline solution; Group 5, animals treated daily with quercetin (50 mg/kg b.w.) in saline solution; Groups 6 and 7, animals treated orally with quercetin plus low and high dose of CNPs respectively;

Groups 8 and 9, animals treated orally with CCl_4 plus the low and high dose of CNPs respectively; Group 10, animals treated orally with quercetin plus CCl_4; Groups 11 and 12, animals treated orally CCl_4 plus quercetin and low and high dose of CNPs respectively.

The animals were observed daily for signs of toxicity during the experimental period. At the end of the treatment period (*i.e.* day 21), all animals were fasted for 12 h, then blood samples were collected from the retro-orbital venous plexus under diethyl ether anesthesia. Sera were separated using cooling centrifugation and stored at −20˚C until analysis. The sera were used for the determination of ALT, AST, ALP, CEA, and AFP according to the kits instructions using spectrophotometer.

After the collections of blood samples, animals were sacrificed and samples of the right lobe of the liver of each animal were dissected, weighed and were homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate according to Lin *et al.* [64]. This homogenate was centrifuged at 1700 rpm and 40˚C for 10 min; the supernatant was stored at −70˚C until analysis. This supernatant (20%) was used for the colometricaly determination of hepatic lipid peroxidation expressed as n mol malondialdehyde (MDA)/g liver tissue according to the kits instructions. The homogenate was further diluted with phosphate buffer solution to give 2% and 0.5% dilutions for the colometricaly determination of hepatic glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD) activities, respecetively according to the kits instructions. Other samples of liver were hydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin. Sections of 5 µm thick were cut and stained with hematoxylin and eosin (H & E) for histological examination and Masson's trichrome for connective tissue namely collagen evaluation [65]. The area percentage of collagen fibers were measured by image analyzer system.

2.7. Statistical Analysis

All data for biochemicall analysis and the numerical values of area% of collagen were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System [66]. The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio [67]. All statements of significance were based on probability of $P \leq 0.05$.

3. Results

The characterization results of the chitosan showed a deacetylation degree of 87.5% ± 0.5%. The molar mass average of monomer was 169.35 ± 0.3 g/mol and the molecular weight of chitosan was 90 ± 5 kDa. The results also revealed that the concentration of free amino groups was 0.048 ± 0.002 mol/L.

The scanning electron microscopy (SEM) images of CNPs in the nanorod form are presented in **Figure 1**. CNPs were analyzed for its particle size and distribution using Nanotrac analyzer. The results indicated that the minimum particles size of CNPs was around 100 nm and distribution in the range of 100 - 400 nm. The Atomic force microscopy (AFM) topography of CNPs indicating the rough nature of the surface, due to the rod shaped CNPs (**Figure 1**).

The *in vivo* biochemical results indicated that the treatment with CCl_4 showed a significant increase in AST, ALT, ALP, CEA and AFP (**Table 1**) and oxidative stress markers (MDA) accompanied with a significant decrease in antioxidant enzymes (GPx, SOD and catalase) in liver (**Table 2**). However, treatment with CNPs at the low dose (CNPs-LD) or the high dose (CNPs-HD) showed a significant decrease in AST, ALT, ALP, CEA and AFP except in the group treated with CNPs-HD and AFP in the group received CNP-D when compared to the control group. Whereas, animals treated with quercetin (Q) alone or plus CNPs at the two tested doses were comparable to the control group in the ALT, AST and ALP except ALP in the group received Q plus CNPs-HD which showed a significant decrease compared to the control group. Moreover, these groups showed a significant decrease in the tumor markers indices AFP and CEA except AFP in the group received CNPs-HD which was found to be in the normal range of the control group. ALT in animals treated with CCl_4 and Q or Q plus CNPs-LD were comparable to the control group whereas; ALT, AST and ALP in animals treated with CCl_4 plus Q and CNPs-HD were comparable to the control group. In general, the companied treatment with Q and CNPs at the two tested doses succeeded to induce a significant improvement in liver function parameters. On the other hand, AFP and CEA in the groups treated with CCl_4 plus Q alone or Q plus CNPs-LLD or CNPs-HD were significantly decreased compared to the control group except CEA in the group treated with CCl_4 plus CNPs-LD which was comparable to the control. It is of interest to mention that the decrease in AFP level was more pro-

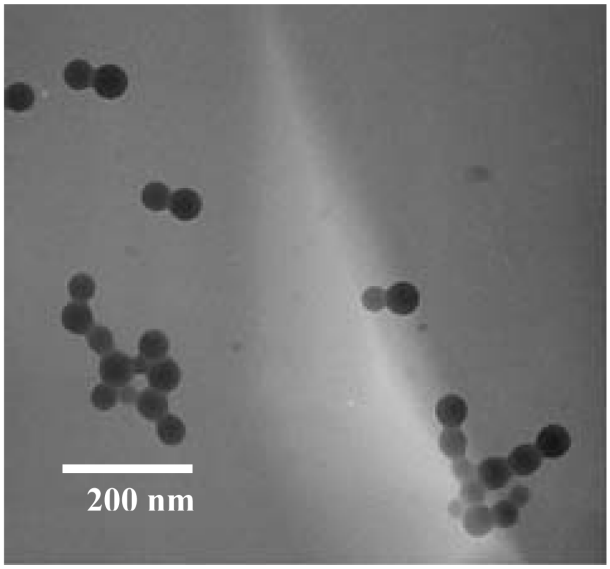

Figure 1. Scanning electron microscopy of chitosan nanoparticles (CNPs).

Table 1. Effect of chitosan nanoparticles (CNPs) and Quercetin (Q) on the liver enzymes and serum tumor markers in rats treated with CCl_4.

Parameter Groups	AST (U/L)	ALT (U/L)	ALP (U/L)	CEA (ng/ml)	AFP (ng/ml)
Control	63.71 ± 1.48^{a}	67.29 ± 2.49^{a}	155.86 ± 2.76^{a}	12.78 ± 1.09^{a}	6.14 ± 1.17^{a}
CCl_4	94.29 ± 2.15^{d}	212.57 ± 5.11^{c}	188.71 ± 1.04^{d}	24.47 ± 1.13^{d}	12.16 ± 0.28^{c}
CNPs-LD	61.00 ± 2.73^{a}	64.43 ± 3.59^{a}	151.57 ± 2.26^{b}	5.15 ± 0.70^{b}	5.14 ± 0.91^{a}
CNPs-HD	57.43 ± 1.56^{a}	65.14 ± 3.71^{a}	155.29 ± 2.86^{b}	5.19 ± 0.83^{b}	5.46 ± 0.42^{a}
Q	59.43 ± 0.75^{a}	70.57 ± 4.59^{a}	153.71 ± 2.00^{a}	5.49 ± 0.93^{b}	5.60 ± 1.00^{a}
CNPs-LD + Q	64.71 ± 1.19^{a}	70.29 ± 4.08^{a}	158.86 ± 1.99^{a}	3.91 ± 0.59^{c}	5.57 ± 0.90^{a}
CNPs-HD + Q	56.57 ± 1.84^{a}	70.71 ± 2.87^{a}	153.43 ± 3.57^{a}	4.30 ± 0.38^{c}	5.07 ± 0.9^{a}
CNPs-LD + CCl_4	79.50 ± 0.56^{e}	88.5 ± 3.80^{d}	150.00 ± 1.51^{e}	13.45 ± 0.95^{a}	2.74 ± 0.65^{d}
CNPs-HD + CCl_4	76.75 ± 0.84^{e}	73.63 ± 3.68^{e}	161.38 ± 1.85^{a}	6.37 ± 2.14^{b}	1.82 ± 0.33^{e}
Q + CCl_4	76.80 ± 1.46^{e}	68.8 ± 2.92^{a}	165.00 ± 1.64^{f}	5.35 ± 1.05^{b}	2.41 ± 0.37^{d}
CNPs-LD + Q + CCl_4	73.56 ± 0.82^{e}	67.78 ± 3.50^{a}	146.00 ± 2.55^{b}	6.37 ± 0.86^{b}	2.74 ± 0.45^{d}
CNPs-HD + Q + CCl_4	60.86 ± 1.24^{a}	69.14 ± 2.91^{a}	160.29 ± 1.34^{a}	5.81 ± 0.56^{b}	3.00 ± 0.45^{b}

Within each column, means superscript with different letter are significantly different ($P \leq 0.05$). Data presented as means ± SE. CNPs-LD: low dose of chitosan nanoparticles. CNPs-HD: high dose of chitosan nanoparticles.

nounced in the animals treated with CCl_4 plus CNPs-HD, but least pronounced in the animals treated with CCl_4 plus Q and CNPs-HD (**Table 1**).

The current study revealed that animals treated with CCl_4 alone showed a significant decrease in hepatic GPx, SOD and catalase accompanied with a significant increase in MDA. However, animals treated with Q and/or CNPs at the two tested doses showed a significant increase in the level of hepatic GPx, catalase and SOD and did not affect MDA level compared to the control group (**Table 2**). This increase n the antioxidant parameters was more prounanced in the groups received the combined treatment with Q plus CNPs at the two doses. The combined treatment with CCl_4 plus Q and CNPs-LD or CNPs-HD resulted in a significant improvement in the

Table 2. Effect of chitosan nanoparticles (CNPs) and Quercetin (Q) on antioxidant enzymes and MDA in rat treated with CCl_4.

Parameter Groups	GPx (unit/mg protein)	SOD (unit/mg protein)	Catalase (unit/mg protein)	MDA (nmol/g tissue)
Control	283.42 ± 11.11^{a}	255.21 ± 12.60^{a}	3.55 ± 0.13^{a}	109.02 ± 2.20^{a}
CCl_4	174.72 ± 12.00^{f}	118.75 ± 13.54^{c}	1.64 ± 0.11^{c}	148.37 ± 1.96^{b}
CNPs-LD	451.67 ± 20.20^{b}	266.52 ± 12.79^{a}	4.85 ± 0.02^{b}	111.97 ± 1.30^{a}
CNPs-HD	778.14 ± 42.45^{c}	344.20 ± 13.37^{b}	4.84 ± 0.09^{b}	116.56 ± 0.42^{a}
Q	389.07 ± 42.45^{d}	340.18 ± 14.89^{b}	4.95 ± 0.20^{b}	105.73 ± 1.36^{a}
CNPs-LD + Q	691.41 ± 36.77^{e}	349.22 ± 16.80^{b}	4.95 ± 0.10^{b}	106.19 ± 0.77^{a}
CNPs-HD + Q	629.3 ± 19.11^{e}	357.59 ± 23.79^{b}	4.96 ± 0.10^{b}	105.86 ± 1.22^{a}
CNPs-LD + CCl_4	445.66 ± 18.87^{b}	352.73 ± 14.32^{b}	4.51 ± 0.40^{b}	119.24 ± 2.06^{c}
CNPs-HD + CCl_4	513.44 ± 38.30^{g}	362.5 ± 10.98^{b}	4.85 ± 0.01^{b}	122.03 ± 1.22^{c}
Q + CCl_4	453.91 ± 41.01^{b}	354.38 ± 10.88^{b}	4.96 ± 0.20^{b}	120.15 ± 1.94^{c}
CNPs-LD + Q + CCl_4	529.71 ± 23.59^{g}	355.21 ± 12.44^{b}	4.93 ± 0.20^{b}	115.89 ± 6.83^{a}
CNPs-HD + Q + CCl_4	738.16 ± 21.97^{c}	354.69 ± 11.56^{b}	4.94 ± 0.21^{b}	95.36 ± 1.82^{d}

Within each column, means superscript with different letter are significantly different ($P \leq 0.05$). Data presented as means ± SE. CNPs-LD: low dose of chitosan nanoparticles. CNPs-HD: high dose of chitosan nanoparticles.

antioxidant enzyme activities and MDA level toward the control level.

The hepatic MDA level in group treated with Q and/or CNPs at the two tested doses were comparable to the control group. Treatment with CNPs at the low dose plus quercetin effectively normalized the level of hepatic MDA in groups treated with CCl_4. On the other hand, treatment with the high dose of CNPs plus quercetin resulted in a significant increase in hepatic MDA compared to the control group in CCl_4-treated animals. Although the single treatment with quercetin or nanochitosan at the two tested doses in animals treated with CCl_4 resulted in a significant increase in the level of hepatic MDA towards the control value, it did not normalize the level of hepatic MDA (**Table 2**). This improvement was more pronounced in the group treated with Q plus CNPs-HD.

The biochemical results of the current study were further confirmed by the histological examination of the liver tissue. The microscopic examination of the control group and those treated with CNPs-LD, CNPs-HD, Q, CNPs-LD plus Q or CNPs-HD plus Q showed normal lobular architecture with central veins and radiating hepatic cords (**Figure 2(a)**). The liver of animals treated with CCl_4 alone showed severe pathological changes included multi focal of fibrosis and cirrhosis together with extensive centrilobular necrosis, cell death, ballooning, thickening in blood vessels, fatty droplets, lymphocytes inflammation, bile ducts proliferation and dense fibrous tissue were well marked (**Figures 2(b)-(d)**). The liver of animals treated with CCl_4 plus CNPs-LD (**Figure 3(a)**), CNPs-HD (**Figure 3(b)**), Q (**Figure 3(c)**), CNPs-LD plus Q (**Figure 3(d)**) or CNPs-HD plus Q (**Figure 3(e)**) showed marked decrease in hepatocytes degenerative changes induced by CCl_4. There are featuers of mitotic cells and marked decrease in collagen fibril, steatosis and inflammation, the improvements in hepatocytes morphology are clarify around the center vein than that in portal vein.

Masson's trichrome stained tissues of control rats showed no fibrosis and a fine liner of collagen around the central vein (**Figure 4(a)**). The liver sections of animals treated with CCl_4 alone showed the fibrous septa and cirrhosis as a bundle of collagen fibrils extended from the central vein and portal tract and dissecting the parenchyma (**Figure 4(b)**). The liver section in rats treated with CNPs-LD, CNPs-HD, Q, CNPs-LD plus Q or CNPs-HD plus Q showed moderate amount of fibrosis and collagen around the portal area (**Figure 4(c)**). However, the liver sections in the animals treated with CCl_4 plus Q or CNPs at the two tested doses alone or in combination showed a reduction in collagen fibers and inflammatory cells (**Figure 4(d)**)

The image analysis (**Figure 5**) indicated a significant increase in area percentage of collagen fibers in the group treated with CCL_4 alone compared to the control group. Whereas, a significant improvement in hepatic parenchyma and a marked decreased in connective tissues and collagen fibers were observed in the groups

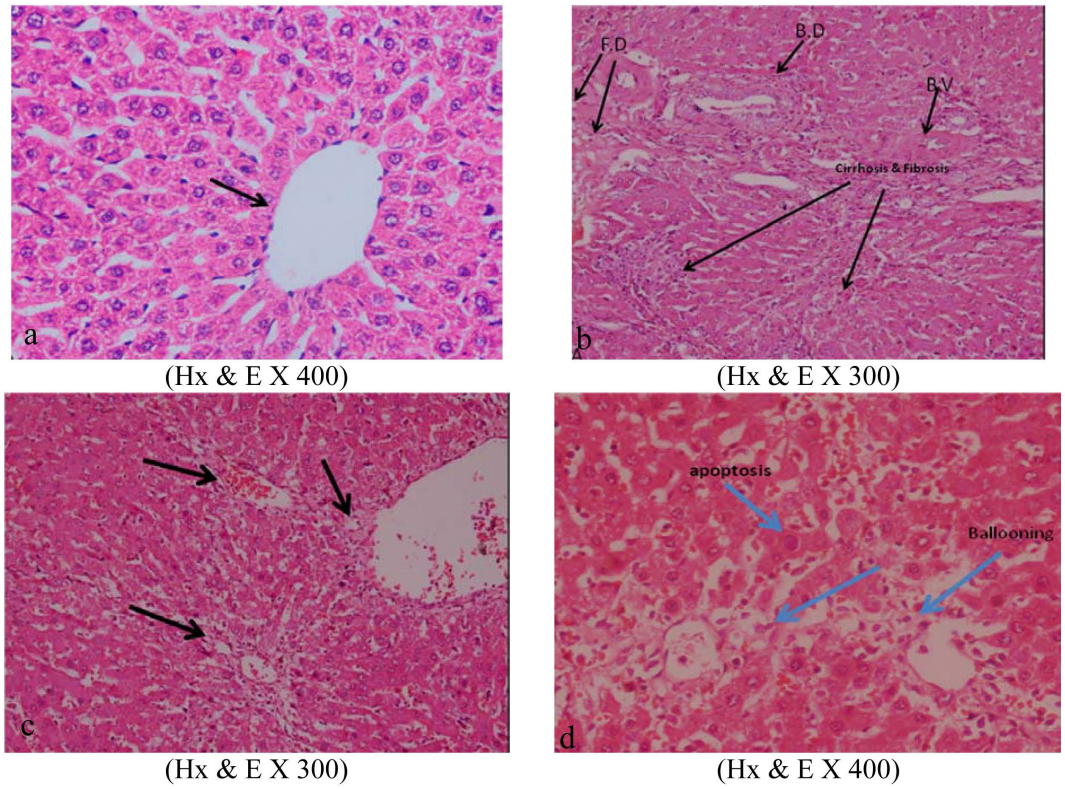

Figure 2. Photomicrographs of liver sections of (a) the control group and those treated with CNPs-LD, CNPs-HD, Q, CNPs-LD plus Q or CNPs-HD plus Q showing normal histological appearance, (b, c) the group treated with CCl_4 alone showing marked fibrosis and cirrhosis together with extensive centrilobular necrosis, cell death, ballooning, thickening in blood vessels, fatty droplets, lymphocytes inflammation and bile ducts proliferation, (d) High magnification of the liver section in rats treated with CCl_4 alone showing cellular apoptosis and ballooning damage. (Q: Quercetin; CNPs-LD: chitosan nanoparticle-low dose; CNPs-HD: chitosan nanoparticle-high dose).

treated with CCl_4 plus Q or CNPs at the two tested doses alone or in combination. On the other hand, no significant differences were observed between the control group and the groups treated with Q, Q plus CNPs-LD, Q plus CNPs-HD and Q plus CCl_4 (**Figure 5**).

4. Discussion

The characterization of chitosan showed that it has low molecular weight with high degree of deacetylation. This resulted in an increase of positive charges due to free amino groups and consequently facilitates the coupling process with other molecules such as quercetin. The *in vivo* study revealed that the protective role of chitosan nanoparticle alone or in combination with quercetin against oxidative stress and hepatotoxicity induced by carbon tetrachloride in rat model was evaluated. The selected doses of CCl_4, chitosan nanoparticles and quercetin were literature based [68]-[70], respectively). In the current study, treatment with CCl_4 induced severe hepatotoxicity and oxidative stress as well as histological changes typical to those reported in the literature. Animals treated with CCl_4 showed a significant increase in liver function enzymes (ALT, AST and ALP), tumor markers (CEA and AFP) and oxidative stress markers (MDA) accompanied with a significant decrease in antioxidant enzymes (GPx, SOD and catalase) in liver tissue. Elevation of ALT and AST reflects generalized damage to hepatocytes while elevation of ALP reflects cholestasis [71]. The elevated levels of ALT, AST, ALP reported in the current study in animals treated with CCl_4, indicated severe hepatic parenchymal cell injury and were in agreement with the previous reports [72]-[75]. Moreover, CCl_4 was found to induce oxidative stress in hepatic tissue leading to accumulation of free radicals and consequently decreased activities of hepatic antioxidant enzymes; SOD, CAT and GPx with increased level of MDA indicating the oxidative damage to the hepatic tissue caused by CCl_4 [76]-[78].

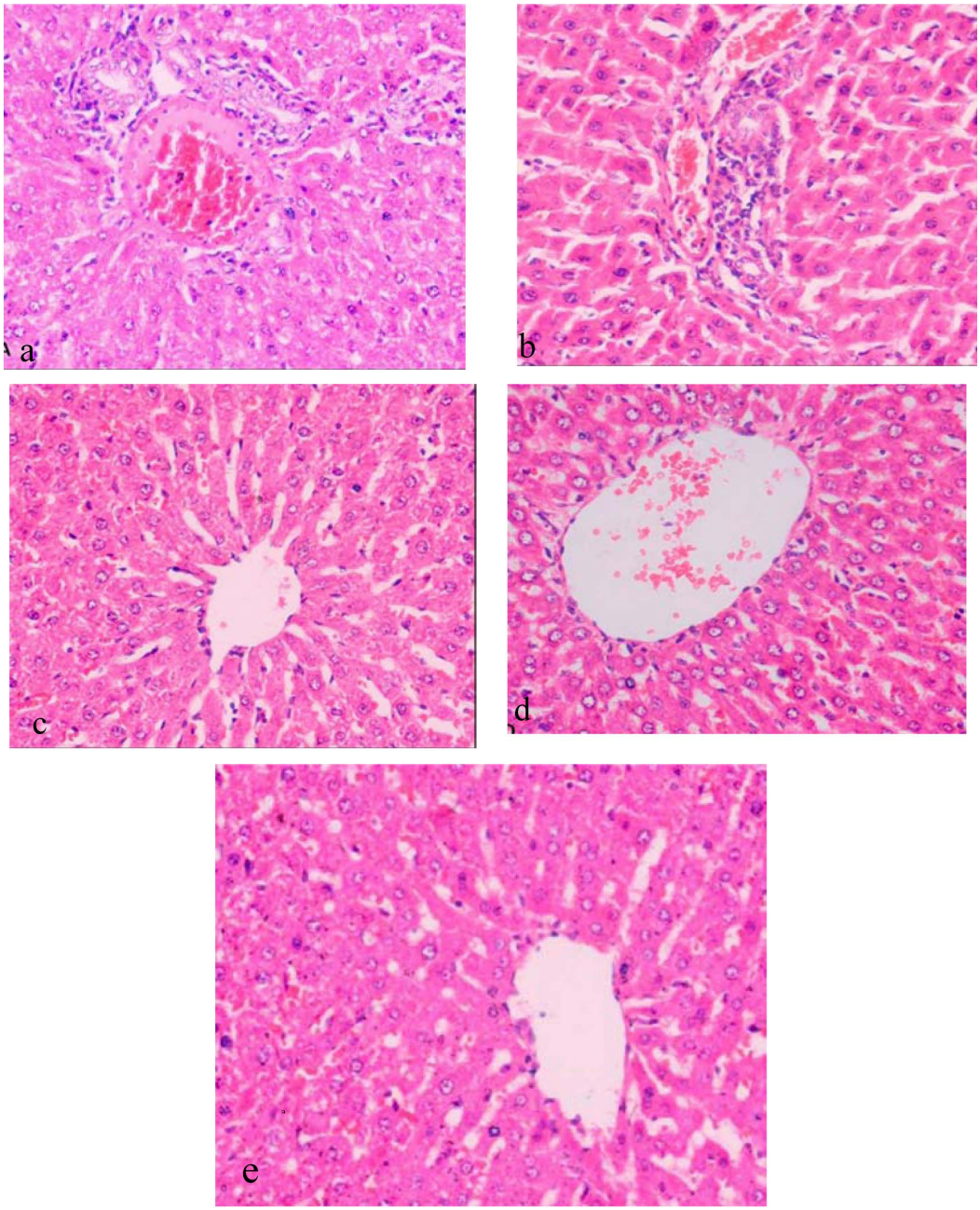

Figure 3. Photomicrographs of liver sections of (a) the group treated with CCl_4 plus NC-LD; (b) the group treated with CCl_4 plus NC-HD; (c) the group treated with CCl_4 plus Q; (d) the group treated with CCl_4 plus CNPs-LD and Q and (e) of the group treated with CCl_4 plus CNPs-HD and Q, showing marked decrease in hepatocytes degenerative changes induced by CCl_4. There are features of mitotic cells and marked decrease in collagen fibril, steatosis and inflammation, the improvement in hepatocytes morphology is clarify around the center vein than that in portal vein. Q: Quercetin; CNPs-LD: chitosan nanoparticle-low dose; CNPs-HD: chitosan nanoparticle-high dose) (H× & E × 400).

Carcinoembryonic antigen (CEA) is considered specific biomarkers for liver cancer. Therefore, elevated serum CEA level reported herein is associated with liver metastasis [79]. Input of CEA into the circulation is controlled by production rate of the tumor; its location and stage, its size, differentiation and vascularity and the presence or absence of distant metastases [80] [81]. However, alpha feto protein (AFP) is the principal in fetal plasma in early gestation is subsequently present at very low levels (<25 mg/L). It increases in hepatocellular carcinoma (HCC) and other liver diseases like chronic hepatitis, in regeneration phase of acute hepatitis and in

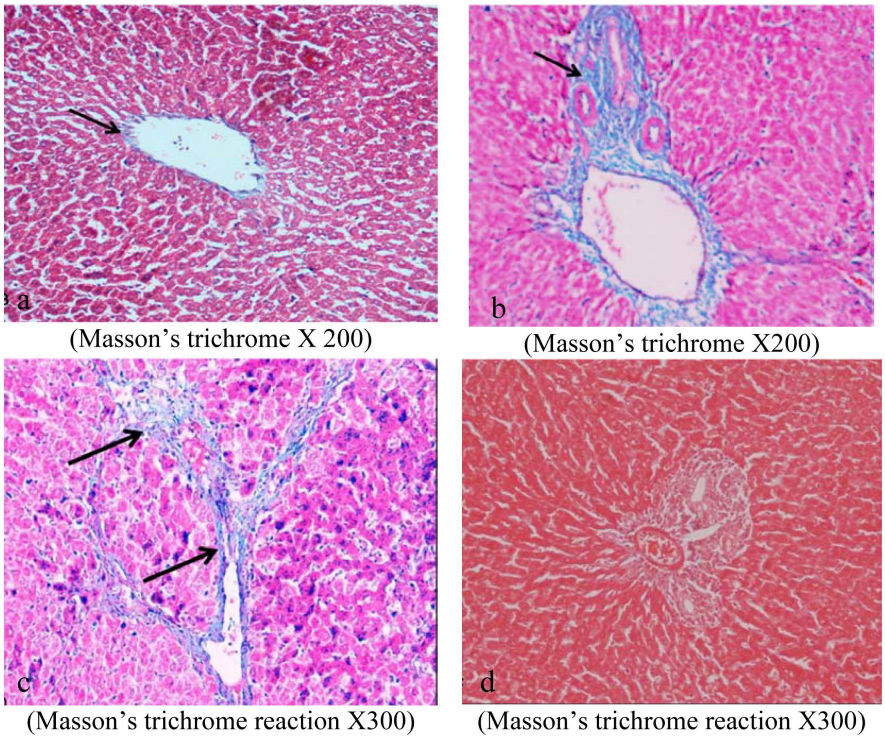

Figure 4. Photomicrographs of liver sections of (a) control rats showing no fibrosis investigated and a fine liner of collagen around the central vein; (b) rats treated with CNPs-LD, CNPs-HD, Q, CNPs-LD + Q or CNPs-HD + Q showing moderate amount of fibrosis and collagen around the portal area; (c) rats treated with CCl_4 alone showing the fibrous septa and cirrhosis are investigated as a bundle of collagen fibrils extended from the central vein and portal tract and dissecting the parenchyma and (d) rats treated with CCl_4 plus CNPs-LD, CCl_4 plus CNPs-HD, CCl_4 plus Q, CCl_4 plus CNPs-LD and Q and CCl_4 plus CNPs-HD and Q showing a reduction in collagen fibers and inflammatory cells. (Q: Quercetin; CNPs-LD: chitosan nanoparticle-low dose; CNPs-HD: chitosan nanoparticle-high dose).

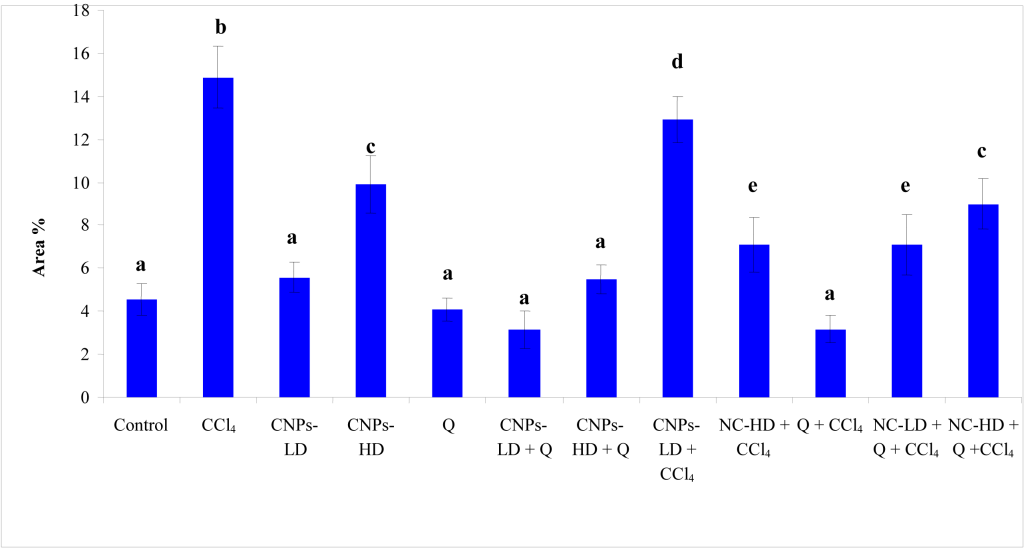

Figure 5. Effect of different treatments on the area percentage of collagen in liver tissues.

hepatic metastasis [82]. The elevated levels of CEA and AFP reported in the current study after the administration of CCl_4 were indicative of liver injury and alteration of tumor response by CCl_4 and were in accordance with the results of previous reports [83] [84]. Lipid peroxidation (LP) is one of the main manifestations of oxid-

ative damage and it has been found to play an important role in the toxicity and carcinogenicity. The increase in hepatic MDA level reported in the current study revealed the increase in LP resulting in tissue damage and failure of the antioxidant defense mechanisms to prevent the formation of excessive free radicals [73] [84]-[86]. The biochemical results of the current study were further confirmed by the histopathological study which showed that liver tissue more or less have significant changes in the histological pictures in CCl_4-intoxicated rats typical to those reported in the literature [73] [84] [87].

As a solution for the water-insolubility problem of chitosan, nanoparticle formulation provides a plausible pharmaceutical basis for enhancing oral bioavailability and therapeutic efficacy of chitosan and other drugs that are poorly soluble [26]. In addition, nanoparticles possess a stronger curvature of the surface, compared to large particles; this produces more dissolution pressure with a corresponding increase in saturation solubility [27]. The increased saturation solubility, in turn, favors an increase in concentration gradient between intestinal epithelial cells and the mesenteric circulation beneath. Consequently, chitosan nanoparticles (CNPs) could exhibit more superior activities than chitosan due to their small size and quantum size effect. Not surprisingly, CNPs have been reported to have heightened immune-enhancing effect [88], anticancer activity [89], antimutagenic activity [90] and antimicrobial activity [91] than those of CS. Nevertheless, other biological activities are unknown, such as the antioxidant activity of CNPs, which has received less attention. In the present study, animals treated with CCl_4 plus CNPs at the two tested doses showed significant improvements in the hepatic toxicity biomarkers, antioxidant enzymes, oxidative stress markers, tumor markers as well as the significant improvement in the histological picture of the liver. These findings are in harmony with the findings of Wen *et al.* [92] who reported a protective role of CNPs with a mean diameter of 83.66 nm against H_2O_2-induced RAW-264.7 cell injury through restoring the activities of endogenous antioxidants (SOD, GPx and CAT), along with enhancement of their gene expression.

Although no scientific report has been published so far describing the protective role of CNPs against CCL_4 induced hepatotoxicity and oxidative stress in liver tissues, the hepatoprotective effect of chitosan has been documented in several reports; for instance, Jeon *et al.* [93] investigated the antioxidative effect of chitosan on chronic CCl_4-induced hepatic injury in rats and showed that chitosan has strong antioxidative effects, which decrease TBARS production and increase antioxidant enzyme (catalase and SOD) activities during chronic CCl_4 induced lipid peroxidation. In the same concern, Subhapradha *et al.* [94] reported the hepatoprotective effect of *β*-Chitosan from Gladius of *Sepioteuthis lessoniana* against CCl_4-induced oxidative stress in rats. These authors showed that, in addition to normalizing the oxidative stress markers, which is attributed to the antoxidant properties of chitosan, the normalization of plasma AST and ALT levels in *β*-chitosan and CCl_4-treated rats indicates that *β*-chitosan may stabilize the cell membrane and may prevent a leakage of intracellular enzymes into the blood. Thus, the overall hepatoprotective effect of chitosan is probably due to a counteraction of free radicals by its antioxidant nature and/or to its ability to inhibit lipid accumulation by its antilipidemic property [95] [96]. Santhosh *et al.* [97] showed that co-treatment with chitosan may prevent antitubercular drugs-induced hepatotoxicity in rats. Moreover, chitosan was also effective against TCDD-induced hepatotoxicity which is persistent and highly toxic environmental pollutants [98]. Furthermore, chitosan was proved to be hepatoprotective against oxidative damage induced by radiotherapy [99].

In the current study, treatment with quercetin and CNPs at the two tested doses succeeded to improve the antioxidant capacity of the body and reduce the oxidative stress as indicated by the increase in antioxidant enzymes (CAT, SOD, GPx) and decrease of MDA levels. Treatment with quercetin plus CNPs to the intoxicated rats could markedly suppress the high serum level of liver biochemical parameters (ALT, AST, ALP) as well as decrease the increased levels of tumor markers (AFP and CEA). Moreover, these protective agents succeeded to improve the histological changes resulted in liver tissue of CCl_4-treated rats. The hepatoprotective and antioxidant properties of quercetin are supported by the findings of Pavanato *et al.* [100] who found that administration of quercetin to rats significantly improved all the hepatic toxicity biomarkers in CCl_4-induced liver fibrosis in addition to improve liver histology. In the same concern, Pilkhwal *et al.* [101] showed that quercetin attenuated LPS-induced hepatotoxicity and oxidative stress. On the other hand, Kebieche *et al.* [102] reported that quercetin ameliorated the altered enzyme levels and protected rat liver against chemicals- or drugs-induced hepatotoxicity and Utesch *et al.* [58] demonstrated that quercetin administration did induce any genotoxic effects *in vivo* following oral exposure. Padma *et al.* [103] investigated the protective effect of quercetin against lindane-induced hepatotoxicity and suggested that the protective effect of quercetin on oxidative damage was attributable to its free radical scavenging action and antioxidant nature. Furthermore, Abo-Salem *et al.* [104] showed that

treatment with quercetin decreased the acrylonitrile-induced elevation in biochemical parameters and was effective in structural improvement of liver. Moreover, Nabavi *et al.* [105] showed that the administration of quercetin prior to sodium fluoride intoxication prevents hepatotoxicity and oxidative stress in rat liver, probably because of its antioxidant effect. Furthermore, the recent study by Tang *et al.* [106] showed quercetin dose dependently induced HO-1(rate-limiting enzymes of heme catabolism), HO-1 upregulation may limit the CYP2E1 induction by ethanol through depleting heme pool. Moreover, HO-1 metabolite CO mediated the beneficial effect of quercetin against ethanol hepatotoxicity through directly inactivating CYP2E1. It is of interest to mention that beside the protective role of CNPs alone, it enhanced the antioxidant effect of quercetin and overcome the problem associated with the poor absorption and bioavailability of quercetin upon oral administration suggested that it is a good candidate as drug delivery.

In this concern, Torresa *et al.* [107] synthesized chitosan-flavonoid conjugate by covalent enzymatic chitosan modification with quercetin; the quercetin-modified chitosan to enhancement of the antioxidant and antimicrobial properties and retained thermal degradability. As for safety, although nanonization is an effective method to improve the bioavailabitity of chitosan and enhance its antioxidant effects, attention should be paid concerning the particle size; *i.e.* nanoparticles that are extremely minute in size can disrupt the normal function of the basic biological structures.

5. Conclusion

It could be concluded that the both CNPs and quercetin succeeded to induce hepatoprotection against CCl_4-induced toxicity. Hepatoprotection occurred in a dose-dependant fashion by CNPs. The combined treatment with quercetin plus the high dose of CNPs had the most effective antioxidative and protective effects against CCl_4-induced damage in liver.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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