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RESEARCH ARTICLE

Protective effects of nanoparticulate coenzyme Q_{10} and curcumin on inflammatory markers and lipid metabolism in streptozotocin-induced diabetic rats: a possible remedy to diabetic complications

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Abstract Diabetes and its complications have been linked to increased levels of free radicals and systemic pro-inflammatory cytokines and to an altered lipid profile. Coenzyme Q_{10} and curcumin are potent antioxidants and anti-inflammatory agents but are underutilized clinically because of their poor bioavailability when administered orally. We have recently developed poly(D,L-lactic-*co*-glycolic acid)-based nanoparticles in which we have encapsulated coenzyme Q_{10} and curcumin to increase the oral bioavailability and therapeutic efficacy of the antioxidant molecules. These formulations when tested in streptozotocin-induced diabetic rats demonstrated protective effects on inflammatory markers as well as lipid metabolism. Coenzyme Q_{10} nanoparticulates reduced only C-reactive protein levels, whereas curcumin nanoparticles reduced levels of C-reactive protein, interleukin-6 and tumor necrosis factor-α. Administration of both nanoparticulates resulted in significant reductions of plasma triglycerides and total cholesterol and an increase in high-density lipoprotein cholesterol. Together, these data indicate the promise of coenzyme Q_{10} and curcumin in diabetes when delivered through nanoparticulate formulations.

Keywords Antioxidants · Diabetic dyslipidemia · Inflammation · Oral delivery

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Introduction

Vascular inflammation and cardiovascular disease are the leading causes of morbidity and mortality in the diabetic population [1]. Oxidative stress has been correlated to inflammatory markers [2] and hyperlipidemia [3], promoting atherosclerosis and subsequent cardiovascular disease. Reactive oxygen species can activate nuclear factor-κB and mediate nuclear factor-κB-dependent mitogenic and cytotoxic signals of cytokines [4]. Levels of the widely recognized inflammatory mediators, tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP) are known to be elevated in experimental diabetes [5] and in diabetic populations [6]. Antioxidants and antioxidant defense mechanisms, which are inherent in cells and tissues, can inhibit inflammation by intervening in the pathways mediated by reactive oxygen species. However, in diabetic patients the antioxidant defense system weakens due to reduced levels of antioxidants such as glutathione [7]. Glycation of antioxidative enzymes during hyperglycemia impairs cellular antioxidant defense mechanisms, leading to the development of oxidative stress and progression of complications [8]. Antioxidant status has a major role on the rate of low-density lipoprotein (LDL) oxidation and on atherogenicity in humans [9]. Apart from oxidation of LDL, activity of lipoprotein lipase, an enzyme involved in lipid metabolism has been positively correlated with total antioxidant capacity and the atherogenic index has been negatively correlated with this capacity in rats [10].

Coenzyme Q_{10} (CoQ_{10}) is a strong antioxidant that is an important component in the mitochondrial electron trans-

port chain. CoQ_{10} is gaining importance in the treatment of cardiovascular diseases, neurodegenerative disorders and diabetes [11]. Although CoQ_{10} has many applications poor bioavailability as a consequence of poor aqueous solubility and high molecular weight remains a limitation [12]. To improve the oral bioavailability of CoQ_{10}, several new approaches have been considered and tested in animals or humans [13]. Micro- and nanoparticle preparations are the most important approaches being investigated these days to improve bioavailability and many types of micro- and nanoparticles of CoQ_{10} have been formulated for this purpose [14–16]. The poly(D,L-lactic-*co*-glycolic acid) (PLGA) nanoparticles, encapsulated with CoQ_{10}, prepared for the present study are biocompatible and biodegradable using the stabilizer didodecyl dimethyl ammonium bromide (DMAB) which gives smaller particles (around 100 nm in diameter).

Curcumin (the active ingredient of the spice turmeric), on the other hand, is a natural plant-derived antioxidant mainly found in the rhizomes of *Curcuma longa*. Curcumin is also known to possess various pharmacological applications in cardiovascular diseases, neurodegenerative disorders and diabetes. After oral administration, only very low amounts of curcumin reach the systemic circulation; thus curcumin shows poor activity in clinical trials. Clinical development of oral forms of curcumin has been hampered because of its very low bioavailability, which is attributed to its poor solubility, instability and tendency to undergo first-pass metabolism [17]. Curcumin decomposes when exposed to sunlight, in ethanolic and methanolic extracts, in neutral-basic pH conditions, and as a solid [18]. The present study employs curcumin-encapsulated PLGA nanoparticles using poly(vinyl alcohol) (PVA) as the stabilizer, which were previously been reported to improve the oral bioavailability of curcumin [19].

Nanoparticles are being investigated for oral delivery of many challenging molecules that are limited in their use because of their poor biopharmaceutical and pharmacokinetic properties. Entrapment of active ingredients in polymeric nanoparticles results in improved stability [20], uptake [21], and distribution profiles [22]. Nanoparticles, upon oral administration, are believed to be absorbed intact [23] and to circulate for extended periods in the blood releasing the entrapped agent in a sustained fashion, resulting in dose reduction [19]. In our previous studies, CoQ_{10} nanoparticles showed improved antioxidant and antihyperlipidemic activities over a suspension formulation in experimental diabetes [24], and curcumin nanoparticles showed improved oral bioavailability in normal rats [19]. Based on the advantages of the polymeric nanoparticles, the current research program aims to evaluate the nanoparticulate forms of CoQ_{10} and curcumin in a rat model of diabetes by measuring the inflammatory markers and lipid levels in the blood.

Materials and methods

Materials

PLGA (Resomer R503H; MW 35–40 kDa) was purchased from Boehringer Ingelheim, (Ingelheim, Germany). PVA (MW 30–70 kDa), DMAB, and ethyl acetate were purchased from Sigma-Aldrich (Poole, UK). High-performance liquid chromatography-grade methanol, ethanol, and acetonitrile were procured from J.T. Baker (now Avantor Performance materials, Phillipsburg, NJ). Curcumin and CoQ_{10} were gift samples from Indsaff, Punjab, India, and Tishcon Corp., Westbury, NY, respectively.

Preparation of CoQ_{10} and curcumin nanoparticles

Nanoparticles loaded with CoQ_{10} or curcumin were prepared by a modified emulsion–diffusion–evaporation method, previously reported [25]. Briefly, CoQ_{10} (10 mg) or curcumin (7.5 mg) and PLGA (50 mg) were dissolved in 2.5 ml of ethyl acetate and stirred at 1,000 rpm for 30 min under room temperature to obtain a homogeneous solution. Either PVA (50 mg) or DMAB (50 mg), used as a stabilizer, was dissolved in 5 ml distilled water. The organic phase containing the active ingredient and PLGA was then added in a drop-wise manner to the stabilizer solution during homogenization. Homogenization was continued for 5 min at 15,000 rpm. After this step, the emulsion was transferred to 20 ml water to facilitate diffusion and was stirred overnight to ensure the complete evaporation of the organic solvent. After the evaporation step was complete, the nanoparticle solution was centrifuged at 15,000×*g* for 15 min to separate free active ingredient and any unbound stabilizer in the solution. The supernatant was separated and the pellet was redispersed in 20 ml water. The CoQ_{10} nanoparticles were stabilized with DMAB and curcumin nanoparticles with PVA.

Size and zeta potential

All nanoparticles were characterized using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, UK) for size (average of five measurements for one batch) and zeta potential (average of 20 measurements for one batch). The size given by the Zetasizer is the measure of hydrodynamic diameter based on the Brownian motion of the nanoparticles. The average of three batch measurements was expressed as mean±standard deviation.

Transmission electron microscopy

The morphology of the prepared CoQ_{10} and curcumin was analyzed using transmission electron microscopy (LEO 912 Omega, Zeiss, Cambridge, UK). Carbon-coated 200-mesh copper grids (Polysciences, Inc., Eppelheim, Germany) were glow discharged, and specimens in distilled water were dried down to a thin layer onto the hydrophilic support film. Next, 20 μl of 1% aqueous methylamine vanadate (NanoVan, Nanoprobes, Yaphank, NY) stain was applied and the mixture dried down immediately with filter paper to remove excess liquid. The dried specimens were imaged with a LEO 912 energy filtering transmission electron microscope at 120 kV. Contrast-enhanced, zero-loss energy-filtered digital images were recorded with a 14-bit/2K charge-coupled device camera (Proscan Electronische Systeme, Germany).

Entrapment efficiency

Entrapment efficiency (EE) of both CoQ_{10} and curcumin nanoparticles was calculated by measuring the amount of free active agent in the supernatant and subtracting it from the initial amount loaded. EE was determined by centrifuging the nanoparticles loaded with the active ingredient at 15,000×*g* for 15 min and separating the supernatant. The supernatant was analyzed for free CoQ_{10} and curcumin. CoQ_{10} was analyzed using high-performance liquid chromatography. Separation was achieved using a reversed phase C18 column (Hypersil GOLD 15×4.6 mm, 5 μm, Thermo Scientific, Loughborough, UK) fitted with a guard column (Hypersil 10×4 mm Thermo Scientific, Loughborough, UK). A ratio of 9:1 parts of ethanol and methanol was used as the mobile phase at a flow rate of 1 ml/min with ultraviolet detection at 275 nm. Curcumin was detected using a fluorescence spectrophotometer (Cary Eclipse, Varian Ltd, UK) at an excitation wavelength of 420 nm and emission wavelength of 530 nm. The EE was calculated using the following formula.

$$\mathrm{EE}(\%) = \frac{(\text{Initial amount} - \text{amount in the supernatant}) \times 100}{\text{Amount of active agent initially added}}$$

Effect of CoQ_{10} and curcumin nanoparticles in experimental diabetes

Diabetes was induced in male Sprague Dawley rats (250–300 g; n=6 per group). All animal experiments were performed according to a project license under the Animals (Scientific Procedures) Act 1986 (UK). Upon receiving, all rats were allowed to stabilize for 15 days and were housed under 12-h dark–light cycles with access to food and water ad libitum. Animals were divided into six groups; group I comprised normal animals (NC); animals in groups II–VI were made diabetic by a single intraperitoneal injection of 55 mg/kg streptozotocin (STZ) in 10 mM ice-cold citrate buffer adjusted to pH 4.5. Only the rats with plasma glucose levels >250 mg/dl after 48 h of diabetes induction were considered diabetic and were used in the study. Blood samples were collected by the tail prick method, and glucose levels were estimated using the Accu-Check Aviva Nano® Glucometer (Roche Diagnostics, Mannheim, Germany). From the 16th day of diabetes induction, group II (STZ) diabetic animals received vehicle, group III (STZ +CoN) received 100 mg/kg/day CoQ_{10} nanoparticles, group IV (STZ+CuN) received 100 mg/kg/day curcumin nanoparticles, group V (STZ+BD) received blank PLGA nanoparticles stabilized with DMAB, and group VI (STZ+BP) received blank PLGA nanoparticles stabilized with PVA. All formulations were given orally using an oral gavage needle. After 31 days from the diabetes induction, animals were sacrificed and blood was collected. Blood samples were centrifuged at 10,000 rpm for 5 min, and plasma was separated and stored at −20°C until further analysis. Evaluation of treatment efficacy was carried out by assaying the plasma levels of inflammatory and lipid markers in the blood samples comprised of IL-6, CRP, TNF-α, triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C). These parameters were estimated using their respective assay kits. Rat TNF-α kits were purchased from Assay Designs (now part of Enzo Life Sciences, Inc., Farmingdale, NY), rat IL-6 assay kits from R&D Systems (Abingdon, UK) and rat CRP assay kits from BD Biosciences Pharmingen (San Diego, CA). TG, TC, and HDL-C quantitation kits were purchased from Source BioScience Autogen (Nottingham, UK). The results were expressed as graphs. Statistical analysis was carried out using one-way analysis of variance followed by comparison of the STZ group to other groups using Holm-Sidak method and Sigma Stat (Systat Software, Inc., San Jose, CA).

Results

Nanoparticles with encapsulated CoQ_{10} and curcumin have been prepared successfully using the emulsion diffusion evaporation method. The particle Z-average sizes of CoQ_{10}- and curcumin-encapsulated nanoparticles were 115±12 and 237±6 nm, respectively (Fig. 1). The electron micrographs indicated the spherical shape of all nanoparticles (Fig. 1). The zeta potential values (at pH ~6) were 75.4±9.5 mV for CoQ_{10}-loaded nanoparticles and −10.8±1.9 mV for curcumin-loaded nanoparticles. The EEs of CoQ_{10} and curcumin nanoparticles were determined to be 70±3% and

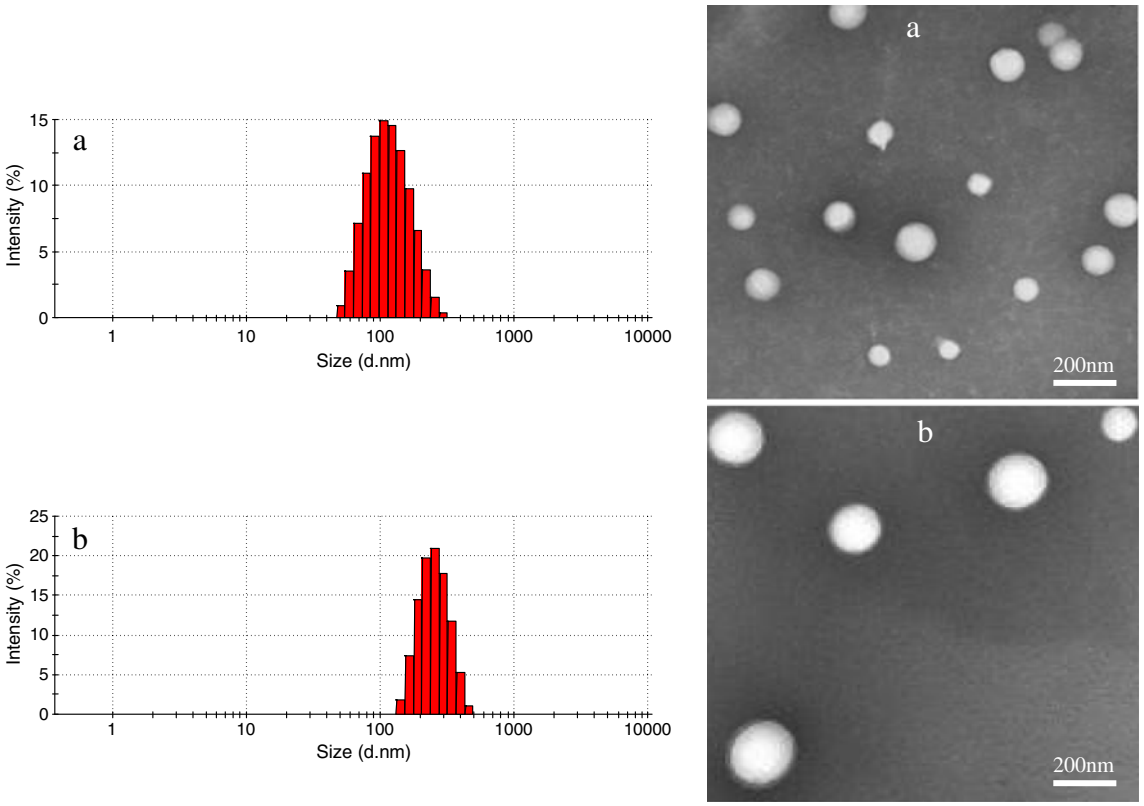

Fig. 1 Size distribution (*left panel*) and transmission electron microscopy images (*right panel*) of **a** CoQ_{10} nanoparticles and **b** curcumin nanoparticles

66±3%, respectively, with 20% *w/w* and 15% *w/w* initial loading of the polymer PLGA. Both CoQ_{10} and curcumin nanoparticles were studied using a 100 mg/kg dose of the active ingredient. Based on the initial loading and the EE, the polymer content associated with curcumin was higher than that of CoQ_{10}.

At the end of the experimental period, IL-6 and CRP levels in the STZ group were not significantly different from those of the normal group (Figs. 2 and 3). After 1 month, STZ rats were found to have elevated levels of TNF-α (Fig. 4), plasma TG (Fig. 5), and TC (Fig.6) in comparison with the NC rats. HDL-C levels were significantly decreased in the STZ group in comparison to the NC group (Fig. 7).

Although IL-6 and CRP levels were not significantly raised in the STZ group in comparison with the normal group, CRP levels were significantly lower in the STZ +CoN group, and both IL-6 and CRP levels were lower in STZ+CuN group compared with the STZ group (Figs. 2 and 3). TNF-α levels were significantly lower in the STZ +CuN group (but not the STZ+CoN group) in comparison with the STZ rats at the end of the study (Fig. 4). After 15 days of treatment with CoQ_{10} and curcumin nanoparticles, plasma TG and TC levels were reduced significantly in the STZ+CoN and STZ+CuN groups, in comparison with the STZ group (Figs. 5 and 6). Plasma HDL-C levels were significantly higher in the STZ+CoN and STZ+CuN groups in comparison with the STZ group (Fig. 7). Diabetic rats treated with blank nanoparticles, stabilized with either DMAB (STZ+BD group) or PVA (STZ+BP group), showed similar levels as the diabetic control (STZ) showing no effect of the blank nanoparticles on all the measured parameters (Figs. 2, 3, 4, 5, 6, and 7).

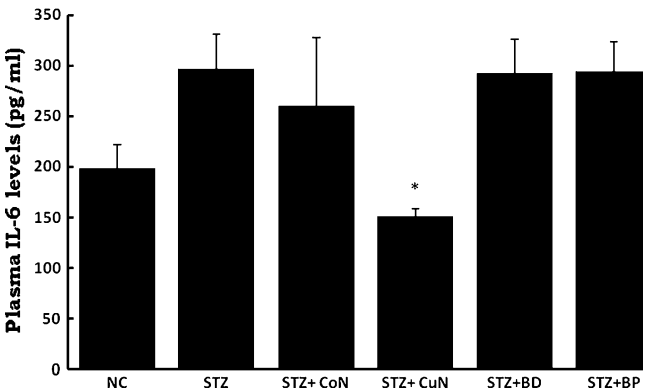

Fig. 2 Plasma interleukin-6 levels. *NC* non-diabetic negative control, *STZ* diabetic positive control developed by injecting STZ, *STZ+CoN* CoQ_{10} nanoparticle-treated diabetic rats, *STZ+CuN* curcumin nanoparticle-treated diabetic rats, *STZ+BD* DMAB stabilized blank nanoparticle-treated diabetic rats, *STZ+BP* PVA stabilized blank nanoparticle-treated diabetic rats. Values are depicted as mean±S.E.M. *$p<0.05$ vs. STZ group

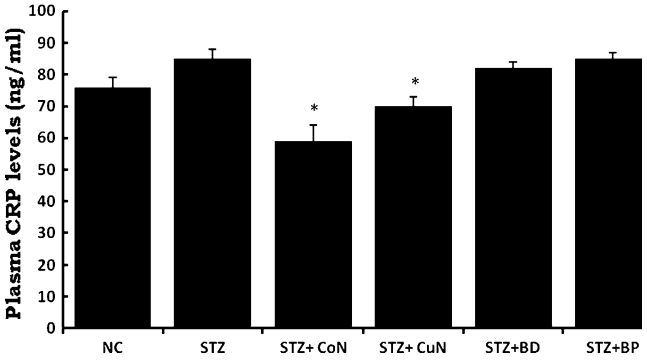

Fig. 3 Plasma C-reactive protein levels. *NC* non-diabetic negative control, *STZ* diabetic positive control developed by injecting STZ, *STZ+CoN* CoQ_{10} nanoparticle-treated diabetic rats, *STZ+CuN* curcumin nanoparticle-treated diabetic rats, *STZ+BD* DMAB stabilized blank nanoparticle-treated diabetic rats, *STZ+BP* PVA stabilized blank nanoparticle-treated diabetic rats. Values are depicted as mean±S.E.M. *$p<0.05$ vs. STZ group

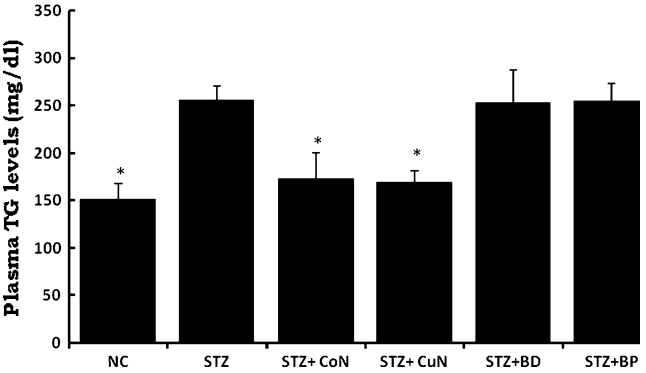

Fig. 5 Plasma triglyceride levels. *NC* non-diabetic negative control, *STZ* diabetic positive control developed by injecting STZ, *STZ+CoN* CoQ_{10} nanoparticle-treated diabetic rats, *STZ+CuN* curcumin nanoparticle-treated diabetic rats, *STZ+BD* DMAB stabilized blank nanoparticle-treated diabetic rats, *STZ+BP* PVA stabilized blank nanoparticle-treated diabetic rats. Values are depicted as mean±S.E.M. *$p<0.05$ vs. STZ group

Discussion

Coenzyme Q_{10}- and curcumin-loaded nanoparticles

The present study illustrates the use of a novel delivery strategy using polymer nanoparticles for peroral administration of CoQ_{10} and curcumin, which otherwise are poorly bioavailable [26, 27]. Owing to this shortcoming, these compounds were tested by a non-oral route of administration [26, 28]; however, the oral route is preferred for such compounds until therapeutic efficacy and doses are established. The simplest approach is to use oil-based formulations of CoQ_{10} and curcumin [13, 27]; these result in only slight increases in bioavailability [12], but show better efficacy than suspension formulations. Although the nanoparticle approach is not new for delivering drugs, it is new for antioxidants and is slowly gaining importance. Polymer nanoparticles offered enhanced uptake of CoQ_{10} in comparison to the solubilized form of a commercial CoQ_{10} formulation in studies of in situ uptake in rodents [14]. Curcumin nanoparticles showed a ninefold increase in bioavailability compared with curcumin plus the absorption enhancer piperine [19]. Together, these results paved the way for evaluating these formulations in a disease model.

The smaller size of the CoQ_{10} nanoparticles in comparison to curcumin nanoparticles can be attributed to the characteristics of the stabilizers used (Fig. 1). DMAB was found to be superior to PVA in minimizing interfacial tension [29]. However, not all compounds are compatible with DMAB; in the case of curcumin, it was not possible to

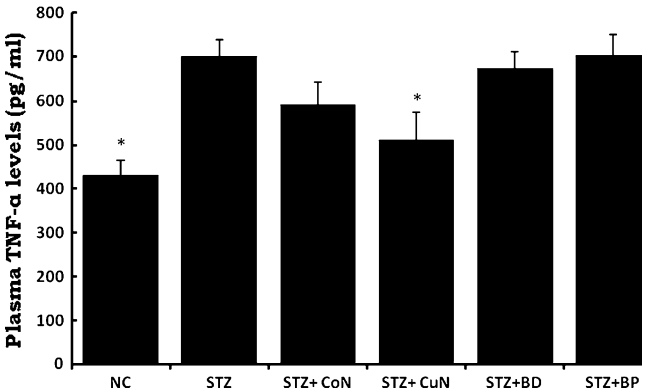

Fig. 4 Plasma TNF-α levels. *NC* non-diabetic negative control, *STZ* diabetic positive control developed by injecting STZ, *STZ+CoN* CoQ_{10} nanoparticle-treated diabetic rats, *STZ+CuN* curcumin nanoparticle-treated diabetic rats, *STZ+BD* DMAB stabilized blank nanoparticle-treated diabetic rats, *STZ+BP* PVA stabilized blank nanoparticle-treated diabetic rats. Values are depicted as mean±S.E.M. *$p<0.05$ vs. STZ group

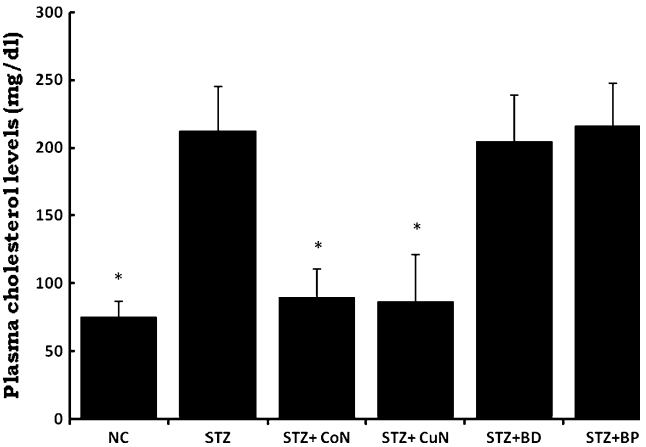

Fig. 6 Plasma cholesterol levels. *NC* non-diabetic negative control, *STZ* diabetic positive control developed by injecting STZ, *STZ+CoN* CoQ_{10} nanoparticle-treated diabetic rats, *STZ+CuN* curcumin nanoparticle-treated diabetic rats, *STZ+BD* DMAB stabilized blank nanoparticle-treated diabetic rats, *STZ+BP* PVA stabilized blank nanoparticle-treated diabetic rats. Values are depicted as mean±S.E.M. *$p<0.05$ vs. STZ group

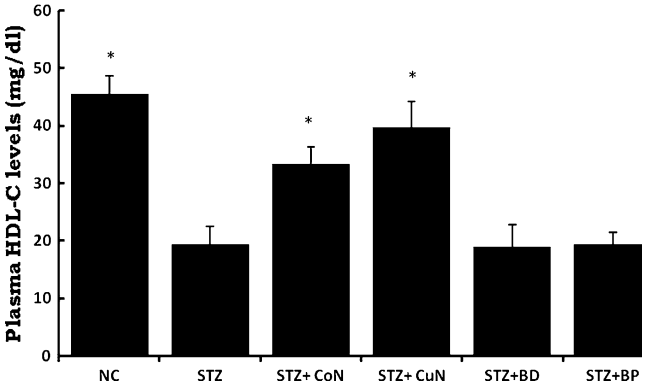

Fig. 7 Plasma HDL levels. *NC* non-diabetic negative control, *STZ* diabetic positive control developed by injecting STZ, *STZ+CoN* CoQ_{10} nanoparticle-treated diabetic rats, *STZ+CuN* curcumin nanoparticle-treated diabetic rats, *STZ+BD* DMAB stabilized blank nanoparticle-treated diabetic rats, *STZ+BP* PVA stabilized blank nanoparticle-treated diabetic rats. Values are depicted as mean±S.E.M. *$p<0.05$ vs. STZ group

formulate the particles with DMAB because of the precipitation of curcumin during the nanoparticle preparation process in the presence of DMAB. Nanoparticles with curcumin loading more than 15% *w/w* resulted in larger size distributions of the nanoparticles because of the limited solubility of curcumin in ethyl acetate, which is used as the organic phase in the nanoparticle preparation process. Other researchers have also encountered similar problem when initial loading of curcumin more than 17% *w/w* was attempted while preparing curcumin-loaded poly (ε-caprolactone) nanofibers [30]. The differences in EEs of CoQ_{10} and curcumin nanoparticles can be attributed to the properties of the stabilizers used and the antioxidant physicochemical properties per se. CoQ_{10} is highly soluble, but curcumin has limited solubility in ethyl acetate.

The stabilizer is believed to form a coat around the particle [31] and thus to exert a major effect on the surface charge. The zeta potential of CoQ_{10} nanoparticles was positive and that of curcumin nanoparticles was negative, depending on the stabilizer's characteristics. Owing to its cationic nature, DMAB gives a high positive charge to the nanoparticles [32]. PVA, on the other hand, is a polymer without any charge and gives the nanoparticles a slight negative charge.

Protective effects of CoQ_{10} and curcumin nanoparticles in experimental diabetes

Oxidative stress is correlated to the increase in inflammation and dyslipidemia in humans [2, 3]. Increased oxidative stress is also associated with the increased oxidation of LDL in humans [9]. Together, oxidative stress, inflammation and dyslipidemia work in a complex way to foster the rapid development of atherosclerosis leading to cardiovascular disease in diabetes. The variable increase in inflammatory markers in diabetic rats is perhaps due to the short duration of the present study (i.e., 4 weeks); however, with longer durations (7 weeks or more) after diabetes induction, IL-6 and CRP levels also increase significantly in experimental diabetes [5, 27]. The development of diabetes in humans is more closely mimicked by rodent models using feeding of high-fat diet and injection of a low dose STZ. The use of a rodent model developed this way can be an effective tool in pharmacological screening of therapeutic agents [33, 34].

CoQ_{10} showed anti-inflammatory activity only in terms of CRP, although these levels were not elevated significantly in diabetic rats (Fig. 3). CoQ_{10} has been shown to possess anti-inflammatory activity in other disease models with systemic inflammation, for example it lowered elevated CRP levels in a model of hypertensive rats developing metabolic syndrome [35] and in a mouse model of diet-induced obesity [36]. IL-6 and TNF-α levels were slightly reduced with CoQ_{10} nanoparticle treatment; however, these levels were not significantly different from those of untreated diabetic rats (Fig. 4), which was also the case with the supplementation of CoQ_{10} in humans [37]. The anti-inflammatory effect of CoQ_{10} was shown to be independent of its activity on lipid peroxidation in obese mice [36].

Our results are in agreement with reports in the literature in which curcumin has shown its strong anti-inflammatory effects against TNF-α and IL-6 release in a diabetic rat model as well as in cultured monocytes subjected to high levels of glucose [27]. The inhibitory effect of curcumin on TNF-α and IL-6 release was found to be dose dependent in cell cultures. The studies conducted in diabetic animals employed a similar dose of curcumin as in our study (100 mg/kg). It was proposed that the anti-inflammatory effects of curcumin can be mediated through both oxidative stress-dependent (through generation of reactive oxygen species) and independent pathways (through induction of GSH) [38, 39].

In the present study, CoQ_{10}- or curcumin-encapsulated nanoparticles showed statistically significant reductions in plasma TG and TC levels and increased the HDL-C levels in diabetic rats (Figs. 5, 6, and 7). We recently developed co-encapsulated antioxidant nanoparticles that use CoQ_{10} and ellagic acid together for synergistic effects and the evaluation involved the measurement of lipid peroxidation, plasma insulin, glucose, and TG levels in diabetic rats [24]. CoQ_{10} alone nanoparticle-treated group in that study, which was used as control, showed decreases in TG and TC [24]. In humans 150 mg/kg CoQ_{10} in combination with fenofibrate increased the effect of fenofibrate in lowering the massive hypertriglyceridemia [40]. The lipid-lowering effects of CoQ_{10} can be attributed

to its direct effect on mitochondria to increase fatty acid oxidation, an antioxidant effect that could decrease oxidative stress, and/or a direct vascular effect that might have led to increased lipolysis of TG-rich lipoproteins [26, 40]. The literature supports the notion that curcumin is effective against hyperlipidemia developed in the STZ-induced diabetic rats [41] and in hamsters fed a high-fat diet [42]. The hypolipidemic action of dietary curcumin is believed to be mediated by the increase in activity of hepatic cholesterol-7a-hydroxylase suggesting a higher rate of cholesterol catabolism [41]. Blank nanoparticles were used as controls in the present study and showed similar results to that of the STZ group in all the parameters studied, suggesting that the polymer PLGA and stabilizers DMAB and PVA had no significant effect on any of the parameters studied.

Conclusion

This study describes the protective effects of nanoparticulate coenzyme Q_{10} and curcumin on inflammatory markers and lipid metabolism, which can be beneficial in diabetic conditions. Curcumin nanoparticles were effective on all the inflammatory markers studied, such as CRP, IL-6, and TNF-α, whereas CoQ_{10} nanoparticles reduced only CRP levels. Plasma TG, TC, and HDL-C were normalized by both CoQ_{10} and curcumin nanoparticles.

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