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Hypolipidemic and antioxidant activities of thymoquinone and limonene in atherogenic suspension fed rats

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ABSTRACT

The hypolipidemic and antioxidant actions of thymoquinone (TQ) and limonene (LMN) were investigated by giving 1 ml of 10 mg TQ or 200 mg LMN suspension, by gavage in two equal doses (morning and evening) of 0.5 ml each for 30 days, in rats, fed an atherogenic suspension. These compounds effectively ameliorated all the altered cardiovascular risk parameters via a reduction in HMG-CoA reductase activity, along with an increase in arylesterase activity. The compounds significantly blocked the shift in buoyancy from less atherogenic lb-LDL to highly atherogenic sd-LDL, restoring the percent distribution of LDL-C and apoB into sd-LDL and lb-LDL to near normal levels. These compounds also blocked basal and maximal formation of CD and malondialdehyde, and lengthened the lag times of LDL, sd-LDL and lb-LDL in the order TQ > LMN. Our results strongly suggest an important therapeutic use of test compounds, especially TQ, in the prevention of cardiovascular disease risks parameters.

1. Introduction

Hypercholesterolemia is one of the most crucial risk factors for atherosclerosis and subsequent cardiovascular disease (CVD) (Steinberg, 2002). Diets high in total and saturated fats, and cholesterol have been shown to cause significant elevations in plasma cholesterol, while diets low in these constituents cause a decrease in cholesterol levels in humans (Schaefer, Levy, Ernst, Van Sant, & Brewer, 1981). Feeding animals with cholesterol has often been used to elevate serum or tissue cholesterol levels to study the aetiology of hypercholesterolemia-related metabolic disturbances (Bocan, 1998). Feeding a diet rich in cholesterol and saturated fat is associated with enhanced production of free radicals, oxidative stress and hypercholesterolemia (Bulur et al., 1995; Stehbens, 1986). On the other hand, cholesterol-induced oxidative stress is one of the factors that links hypercholesterolemia with atherogenesis (Halliwell, 1996), and is known to produce vascular atherosclerotic lesion and increased oxidative stress in several tissues (Balkan et al., 2002), including the development of atherosclerosis in the vascular wall through the formation of reactive oxygen species (ROS) (Byon et al., 2008; Shi, Haberland, Jien, Shih, & Lusis, 2000). There are several reports indicating that hypercholesterolemia is associated with a significant increase in plasma TG, TC, VLDL-C and LDL-C with a decrease in antiatherogenic HDL-C and its components HDL_2-C and HDL_3-C (Bouderbala, Lamri-Senhadji, Prost, Lacaille-Dubois, & Bouchenak, 2008; Chenni et al., 2007; Lee et al., 2007). Several lines of research have established that increased plasma LDL-C and decreased HDL-C levels have been directly associated with the development of premature CVD. There has been increasing evidence of an association between TG and increased risk of CVD (Austin, Hokanson, & Edwards, 1998; Stampfer et al., 1996), a risk that is especially high in subjects with low HDL-C (Castelli, 1992).

Lipoprotein profiles that are relatively rich in small dense (sd-) LDL are associated with up to threefold increase in coronary heart disease (CHD) risk among patients than those mainly consist of large buoyant (lb-) LDL particles (Austin, King, Vranizan, & Krauss, 1990; Austin et al., 1988; Stampfer et al., 1996). Furthermore, both the prevalence and concentration of more proatherogenic sd-LDL subpopulation were significantly increased in subjects with various types of hyperlipidemia such as hyperLDLcholesterolemia, hypertriglyceridemia, combined hyperlipidemia and chylomicronemia, hyperlipidemia with CHD, type 2 diabetic patients with CHD and CHD alone (Hirano et al., 2004; Koba et al., 2006; St-Pierre et al., 2003). Oxidative stress-linked hypercholesterolemia and oxidative modification of LDL have been postulated to play a pivotal role in initiation of the atherosclerotic process (Bentley et al., 2002; Steinberg & Witztum, 2002). Oxidation of LDL has been implicated at both the early and late stages of the pathogenesis of atherosclerosis, during which plaque rupture leads to further clinical events. Attack of ROS against biomembranes or lipoproteins leads to oxidative destruction of polyunsaturated fatty acids by a process called lipid peroxidation, of which malondialdehyde (MDA) is a product and is therefore an indicator of oxidative stress in cells

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and tissues. This increased atherogenecity of LDL is associated with a preponderance of sd-LDL subpopulation that is more prone to oxidative modification than lb-LDL (Chancharme et al., 1999). Relative to lb-LDL, sd-LDL subpopulation with a much greater tendency to become oxidised might thus be more likely to participate in proatherogenic events in the vessel wall of humans with hyperlipidemia, similar to atherogenic lipoprotein phenotype pattern B subjects (Chait, Brazg, Tribble, & Krauss, 1993; Tribble et al., 2001).

During the last several years, experiments have been performed on natural antioxidant compounds in order to attenuate oxidative stress-induced pathogenesis of diseases. These experiments have also dealt with the use of natural antioxidants to alleviate atherosclerosis induced by lipidemic-oxidative stress. Dietary intake of antioxidants could also inhibit oxidation of LDL and thereby reduce the risk factors for CVD (Agarwal & Rao, 1998; Meyer, Yi, Pearson, Waterhouse, & Frankel, 1997; Vinson, Teufel, & Wu, 2004). Thymoquinone (TQ), a principal constituent of the volatile oil of *Nigella sativa* (NS) seeds, has been shown to produce multiple health beneficial activities, which include antihistaminic, antibacterial, antihypertensive, hypoglycaemic, antiinflammatory, immunopotentiating (Ali & Blunden, 2003; Kanter, Coskun, & Uysal, 2006) and antiarthritic actions (Budancamanak et al., 2006). Recently, Nader, El-Agamy, and Suddek (2010) showed hypolipidemic and antioxidant properties of TQ in cholesterol fed rabbits. Woo et al. (2011) reported that TQ has antcarcinogenic activity by increasing PPAR-γ activity and down-regulating the expression of the genes for Bcl-2, Bcl-xL and survivin in breast cancer cells. On the other hand, limonene (LMN), also a component of NS seeds has been shown to exhibit antioxidant (Lado, Then, Varga, Szoke, & Szentmihalyi, 2004; van Lieshout, Posner, Woodard, & Peters, 1998), hypocholesterolemic (Qureshi, Mangels, Din, & Elson, 1988) and anticarcinogenic properties (Nakaizumi, Baba, Uehara, Iishi, & Tatsuta, 1997). Park, Lee, Yaoyao, Jun, and Lee (2011) reported that LMN is an agonistic ligand for adenosine A2A receptors, responsible for sedative effects. In the present study, we have initially investigated the putative protective efficacy of TQ and LMN on plasma atherogenic TG, VLD-C, LDL-C, atheroprotective HDL-C, and its subfractions, HDL_2-C and HDL_3-C, and HDL-linked arylesterase antioxidant enzyme activity levels, including cholesterol and apoB contents of LDL and its density subfractions, sd-LDL and lb-LDL, as well as the percent share of LDL cholesterol and apoB in highly atherogenic sd-LDL and less atherogenic lb-LDL in the presence of lipidemic-oxidative stress, induced in rats by feeding an atherogenic suspension. In addition, in these stressed-hyperlipidemic animals, the antioxidant protection of the above test compounds afforded to LDL, sd-LDL and lb-LDL, from *in vivo*, and Cu^{2+}-catalysed *in vitro* oxidation was examined.

2. Materials and methods

2.1. Materials

Thymoquinone, limonene and phenyl acetate were purchased from Sigma–Aldrich Inc., USA. Thiobarbituric acid and hydroxylamine hydrochloride were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Phosphotungstic acid was purchased from Loba Chemie (Mumbai, India). HMG-CoA was purchased from Pharmacia, USA, while mevalonolactone was procured from United States Bioch. Corp., USA. Sodium dodecyl sulphate was purchased from Bio-Rad Laboratories, USA. Triglycerides assay kit was purchased from Autospan Kit SPAN Diagnostics (New Delhi, India).

2.2. Animals and treatments

The study protocol for animal studies was approved by the Board of Studies of the Biochemistry Department and the ethics committee of J.N. Medical College, A.M.U, Aligarh. Male Wistar albino rats, weighing 180–210 g, from an inbred colony maintained by the central animal facility of J.N. Medical College, were utilised in this study. Animals had free access to standard rat chow and water. For the induction of hyperlipidemia, animals in experimental groups received an atherogenic diet for 30 days, which consists of a suspension (w/v) of 0.5% cholesterol, 3% coconut oil and 0.25% cholic acid, prepared by mixing in a Potter–Elvehjem homogenizer. One millilitre of this atherogenic suspension containing 5 mg cholesterol, 30 mg coconut oil and 2.5 mg cholic acid was given orally to each rat on daily basis by using intragastric intubation in two divided doses (morning and evening) of 0.5 ml each. Five normolipidemic rats in control group (NLP-C) were administered 0.5 ml of saline twice daily. Kanter (2008) used 50 mg TQ/kg body weight of male Wistar rat/day (i.e. 10 mg/rat/day), orally to treat experimental diabetic neuropathy. To treat gastric injury, El-Abhar, Abdallah, and Saleh (2003) have taken four different doses of TQ at 5, 20, 50 and 100 mg/kg body weight of male Wistar rat/day (i.e. 1, 4, 10, 20 mg/rat/day), orally. High doses of TQ (50 and 100 mg) were significantly effective. On the other hand, van Lieshout et al. (1998) took lab chow supplemented with 10,000 ppm LMN *ad libitum* (i.e. 200 mg LMN/male Wistar rat/day) for the treatment of cancer. On above rationales, 1% TQ and 20% LMN suspensions were prepared by dissolving in DMSO (12.5%) and then homogenizing with saline. Prior to administration of atherogenic suspension, four rats in hyperlipidemic TQ (HLP-TQ) and hyperlipidemic LMN (HLP-LMN), groups received 1 ml of above saline suspension containing 10 mg of TQ or 200 mg of LMN, by using intragastric intubation in two equal doses (morning and evening) of 0.5 ml each for 30 days, while four rats in hyperlipidemic control (HLP-C) group, with no drug intervention, received 0.5 ml of saline containing 12.5% DMSO, before the administration of 0.5 ml of atherogenic suspension twice daily for 30 days.

2.3. Collection of blood and separation of plasma

At the conclusion of the study, overnight fasted rats in each group were anaesthetized and blood was drawn from cardiac puncture, collected in heparinized tubes, mixed gently by inversion 2–3 times and incubated at 4 °C for 2–3 h. Plasma was separated from blood by centrifugation at 2500 rpm for 30 min, aliquoted and stored at 4 °C.

2.4. Fractionation of plasma lipoproteins

The precipitation method described by Wieland and Seidel (1989) was used for the isolation of plasma LDL. The method for the isolation of sd-LDL and lb-LDL from isolated LDL is based on the two-step procedure of Hirano et al. (2004), which employs quantification of sd-LDL from serum by heparin-Mg^{2+} precipitation. In our modified method of sd-LDL and lb-LDL isolation, 0.1 ml of precipitation reagent containing 15 IU of heparin and 90 mM $MgCl_2$ was added to 0.1 ml of isolated LDL sample as described above. After mixing, the sample was incubated at 37 °C for 10 min. Next, each sample was incubated in an ice-bath for 15 min and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant containing sd-LDL was carefully removed and saved. The pellet containing lb-LDL was dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9% NaCl. Appropriate aliquots of LDL, sd-LDL and lb-LDL fractions were used for the analysis of their cholesterol and apoB content.

For the isolation of plasma VLDL pellicles, the method of Bachorik and Albers (1986) was used. The method of Kostner (1976) was employed for the isolation of plasma HDL, while the HDL_3 subfraction of HDL was isolated according to the procedure of Bachorik and Albers (1986).