Original Article

Targeting Haemostatic Derangement and Endothelial Dysfunction in Dyslipidemic Rabbits: Focus on Turmeric versus Ginger Extract

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ABSTRACT

Objective: To compare between the effect of curcuminoids and ginger extract on hypercoagulability, endothelial dysfunction and some ischemic markers in dyslipidemic rabbits.
Methods: 24 male rabbits were randomly allocated into normal control (NC) group, dyslipidemic group fed atherogenic diet for 6 weeks, curcuminoids and ginger groups received their extracts as well as atherogenic diet for 6 weeks.
Results: curcuminoids and ginger extract significantly decreased serum plasminogen activator inhibitor-1 (PAI-1), prothrombin fragment (1+2) (PF1+2), endothelin-1 (ET-1), ischemia modified albumin (IMA), plasma fibrinogen (FIB) while nitric oxide (NO) showed a significant increase as compared to dyslipidemic group. Ginger extract exerted more pronounced effect than curcumionoids regarding serum PAI-1 and PF1+2. Curcuminoids, however demonstrated better effect regarding NO, ET-1 and IMA as compared to ginger extract which may refer to their antioxidant potential. Both extracts demonstrated a similar effect on plasma FIB level.
Conclusion: Ginger seems to improve haemostatic abnormalities in dyslipidemic rabbits better than curcuminoids. The latter exerted similar but weaker effects than ginger, except for their remarkable antioxidant potential and improving endothelial functions. Therefore, co-supplementation of both extracts may synergize their effects in dyslipidemia-induced haemostatic and vascular complications.

Keywords: Atherogenic diet, Curcuminoids, Endothelin-1, Ginger, Ischemia modified albumin, Plasminogen activator inhibitor-1, Prothrombin fragment (1+2).

INTRODUCTION

Hyperlipidemia is a pathological condition, mostly attributed to a marked increase in plasma lipids, particularly cholesterol and triglycerides (TG). It represents an important risk factor for atherosclerosis and cardiovascular diseases (CVD)[1]. Based on the information's derived from the American Heart Association (1995), nearly 40% of the American population dies of some cardiovascular diseases, a fact that has driven intensive research on the altered inflammatory and oxidative mechanisms responsible for atherosclerosis[2].

Oxidized LDL (ox-LDL) plays a crucial role in the initiation of atherosclerotic lesions[3]. Thus, antioxidants intake may exert protective effects as observed in experimental studies[4,5]. Besides, inflammation, plaque instability, thrombosis and smooth muscle proliferation represent risk factors for atherosclerosis development[6,7]. It was assumed that certain association between thrombus formation and atherosclerosis might exist where atherosclerotic lesions arose mainly from the arrangement of intravascular fibrin deposits[8,9]. Consequently, initiation of atherogenesis and thrombosis is considered a response to endothelial cell injury or compromise[10]. Vascular endothelium is functioning as an important barrier between the blood vessel and the blood stream. Thus, it can protect from atherosclerosis. Endothelium also preserves vascular homeostasis through the production of various surface proteins, mediators, and autacoids implicated in vasomotion, coagulation, and inflammation[11]. Endothelial dysfunction can take place prior to the structural manifestations of atherosclerosis. Therefore, it can be regarded as a unique predictor for the development of atherogenesis and cardiovascular events in the future[12].

Herbs and spices like turmeric and ginger contain high concentrations of active ingredients. The major active ingredient of turmeric is curcumin, which belongs to the family of curcuminoids and has beneficial effects on various pathological conditions comprising inflammations[13], digestive disorders[14], diabetes mellitus[15], and some types of malignancies[16,17]. Moreover, curcumin reduces serum TC and LDL-C levels by up-regulating the expression of hepatic LDL receptors, blocks the oxidation of LDL[18] and increases levels of HDL-C in serum of healthy volunteers[2]. It has been reported also that ginger extract can reduce lipid profile, lipid peroxidation, and platelet aggregation in healthy individuals and patients with coronary artery diseases[19,20]. Alizadeh *et al.*[21] and Arablou *et al.*[22] found that ginger consumption significantly decreased serum TC in type 2 diabetes mellitus patients. Furthermore, it prevents cholesterol biosynthesis in rat liver[21]. Ginger can also increase HDL_2, HDL particle size and reduce atherogenesis through decreasing insulin and TG levels[22,23]. Mahluji *et al.*[24] reported that administration of 2 g of ginger per day for 2 months could reduce LDL in diabetic patients. A recent report indicated that curcuminoids and ginger exerted anti-atherogenic effects through their ability to improve lipid profile and to lower hepatic cholesterol synthesis, cholesteryl ester transfer protein (CETP) level and gene expression as well as some inflammatory biomarkers (Figs. 1-2)[25]. Accordingly, we focused here on the potential of these natural products on endothelial dysfunction and haemostatic impairment in dyslipidemic rabbits.

MATERIALS AND METHODS

Drugs and chemicals

Turmeric roots and ginger rhizomes were purchased from herbal market (Cairo, Egypt) and identified by Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University. Cholesterol powder was obtained from Winlab (Leicestershire, UK). Other chemicals were of the highest purity and purchased from Sigma Chemicals Co. (St Louis, Mo, USA).

Extraction of curcuminoids

Curcuminoids extract were obtained from Curcuma Longa (Family Zingiberaceae) according to *Piper et al.*[26]. Briefly, 2kg of turmeric roots were ground into fine powder and then soaked in 8liters 95% ethanol at room temperature 3 times. The extract was filtered and evaporated under reduced pressure at 45˚C to remove ethanol. A yellowish orange residue was obtained. The residue was loaded on silica gel column (60-120 meshes) and eluted with a mixture of (9 methanol:1 chloroform). Curcuminoids (active fraction: yellow color) was then collected and evaporated under reduced pressure by rotary evaporator to remove alcohol and stored at 2-8ºC.

Extraction of ginger

Zingiber officinale rhizomes were extracted using the method of Bhandari *et al.*[27]. 10kg of ginger rhizomes were cut into small pieces and soaked in methanol for 3 days at room temperature. After filtration, the extract was concentrated under reduced pressure. The obtained dark brown residue was then suspended in water to get total ginger extract.

Experimental design

The current study was conducted on twenty four male New Zealand white rabbits, 3-4 months old, weighing 1.75±0.25 kg, obtained from the National Research Center, Dokki, Giza, Egypt. Rabbits were fed standard chow rabbit pellets containing 18% pure protein, 2.88% pure fats, and 10.5% pure fibers. They were kept for one week for acclimatization. Rabbits were kept under environmentally controlled conditions with free access to water ad libitum. Experimental procedures were in accordance with the guidelines of the ethical committee for experimental animals handling, Faculty of Pharmacy, Zagazig University, Egypt. Eighteen rabbits were fed atherogenic diet consisting of normal rabbit pellets containing 0.2% w/w cholesterol in coconut oil[28]. Such dyslipidemic model was previously established in our laboratory[25,29].

Rabbits were then allocated into four groups (n=6/each) as following:

- Group (1): Normal control (NC) group kept on plain chow rabbit pellets.
- Group (2): Dyslipidemic group fed atherogenic diet for 6 weeks.
- Group (3): Curcuminoids group, received curcuminoids (50 mg/kg body weight/day) orally[26] concurrently with atherogenic diet for 6 weeks.
- Group (4): Ginger group, received ginger extract (200 mg/kg body weight, p.o/day)[30] along with atherogenic diet for 6 weeks.
- The rationale for using curcuminoids and total ginger extract at the aforementioned doses was based on a preliminary study carried out by our research group using several doses of these extracts which revealed that these doses achieved the optimal effect on serum lipid profile[25].

Blood sampling

At the end of the experiment, rabbits were fasted overnight. Blood samples were then collected from marginal ear veins and divided into two portions; the first one was directed to serum preparation for estimation of ischemia modified albumin (IMA),

endothelial and prethrombotic biomarkers. The second one was collected in citrated tubes for the determination of plasma fibrinogen (FIB).

Biochemical analysis

Determination of IMA

Serum IMA was analyzed colorimetrically by the method of Bar-Or *et al.*[31]. This method involved the addition of 50 µL of 0.1% cobalt chloride solution (Sigma, $CoCl_2.6H_2O$) to 200 µL of serum. The reaction mixture was gently mixed and allowed to stand for 10 min to ensure albumin-cobalt binding. 50 µl dithiothreitol (DTT) solution was then added as a color developing agent and the reaction was quenched 2 min later by the addition of 1 mL of 0.9% NaCl. The developed color was compared to serum-cobalt blank without DTT. The results are expressed as absorbance units (ABSU) at 470 nm. This assay measures the quantity of unbound cobalt left after albumin-cobalt binding. Thus, an elevated absorbance means reduced albumin-cobalt binding.

Determination of NO

The serum level of NO was measured indirectly as nitrite using Griess reagent[32]. Serum samples were diluted and deproteinized by adding 1/20th volume of $ZnSO_4$ solution to give a final concentration of 15 g/L and centrifuged at 1000g for 15 min. 1mL supernatant was mixed with 1mL Griess reagent. The reaction mixture was incubated for 10 min at room temperature. The absorbance of the developed color was measured at 540 nm. The concentration of nitrite was determined from sodium standard nitrite curve.

Determination of ET-1

Serum ET-1 was assayed by ELISA technique using kits supplied by abcam® (Cambridge, UK). Samples and standards were pipetted into ELISA plate coated with monoclonal antibodies specific for ET-1. After incubation, the plate was then washed. A solution of Horseradish Peroxidase labeled monoclonal antibodies to ET-1 was then added. After incubation and washing, chromogenic substrate solution was added and a blue color was generated. The substrate reaction was stopped by addition of a stop solution and a yellow color formed which was read at 450 nm.

Determination of PAI-1 and PF1+2

Serum PAI-1 and PF1+2 were determined by ELISA technique using Kits provided by Wuhan Eiaab Science Co., LTD "EIAaB®" (Wuhan, China) according to the method of Aziz *et al.*[33]. The assay employs the quantitative sandwich ELISA technique. Monoclonal antibodies specific for PAI-1 and PF1+2 have been pre-coated onto a microplates. Standards and samples were added to the plate wells. Enzyme-linked monoclonal antibodies specific for PAI-1 and PF1+2 were then added to the wells. The plate was then washed and a substrate solution was pipetted into the wells to develop color which is proportional to the amount of initially bound PAI-1 and PF1+2. The color development was stopped by a stop solution and the color intensity was measured at 450 nm.

Determination of FIB

Fibrinogen was determined by coagulation method[34] using BioMed-FIB kit (BioMed Diagnostics Inc., White City, OR, US). The clotting assay is a direct measurement of functional FIB which involves coagulation of fresh citrated plasma following the addition of thrombin. The coagulation time is inversely proportional to the FIB concentration. FIB content was read off directly from FIB calibration curve in mg/dl.

Statistical analysis

The values are expressed as mean ± standard deviation (SD) for six animals in each group. Statistical differences among groups were determined using one-way analysis of variance (ANOVA). Tukey–Kramer post hoc test was performed for inter-group comparisons using In Stat 2.04 statistical package (GraphPad InStat). *P*-values <0.0001, <0.001, <0.01 and <0.05, respectively were considered statistically significant. .

RESULTS

Effect of Curcuminoids and Ginger Extract on Prethrombotic, Ischemia and Endothelial Biomarkers

Curcuminoids and ginger extract intake individually induced a significant decrease in serum PAI-1, PF1+2, IMA, plasma FIB and restored the balance between NO and ET-1 in dyslipidemic rabbits ($p<0.01$). However, ginger extract provided greater improvement in haemostatic derangement than curcuminoids. On the other hand, curcuminoids produced more pronounced effect on serum IMA, NO and ET-1, thereby conferring a greater protection against endothelial dysfunction than ginger extract.

Correlations

Analysis of the combined results from all tested rabbits indicated that the serum CETP level was closely correlated with serum IMA (r = 0.9, $p<0.0001$), ET-1 (r = 0.95, $p<0.0001$), PAI-1 (r = 0.87, $p<0.0001$), PF1+2 (r = 0.66, $p<0.0001$), and plasma FIB (r = 0.92, $p<0.0001$). In contrast, it was negatively correlated with serum NO (r = -0.92, $p<0.0001$). There were strong positive correlations among serum IMA, serum ET-1 (r = 0.94, $p<0.0001$), PAI-1 (r = 0.9, $p<0.0001$), PF1+2 (r = 0.76, $p<0.0001$), and plasma FIB (r = 0.97, $p<0.0001$). On the other hand, there was a negative correlation between serum NO and IMA (r = -0.89, $p<0.0001$). Additionally, we observed a linear positive correlation among serum ET-1, IMA (r = 0.94, $p<0.0001$), PAI-1 (r = 0.83, $p<0.0001$), PF1+2 (r = 0.6, $p<0.001$), and plasma FIB (r = 0.91, $p<0.0001$). However, it was negatively correlated with serum NO (r = -0.93, $p<0.0001$). We found also a strong positive correlation among prethrombotic biomarkers PAI-1, PF1+2 (r = 0.92, $p<0.0001$), and FIB (r = 0.96, $p<0.0001$).

DISCUSSION

The present results demonstrated that curcuminoids supplementation (50 mg/kg body weight) attenuated the increase in IMA better than ginger extract (200 mg/kg body weight) in dyslipidemic rabbits. This suggests that curcuminoids possess an antioxidant effect against oxidative stress. IMA is a combined diagnostic marker accompanied with microvascular damage, abnormal lipid profile, inflammation, hyperglycemia and hypertension[35]. Supporting this notion, previous studies showed that curcumin inhibited certain molecular targets and signaling pathways involved in oxidative stress as well as inflammation[18,36]. Curcumin can also inhibit the protein glycation and lipid peroxidation induced by high glucose levels in erythrocytes as well as stress-induced activation of activator protein 1[37,38]. It is considered as a powerful inducer of heme oxygenase-1 (HO-1), a redox-sensitive inducible protein *via* regulation of nuclear factor E2-related factor 2 (Nrf2) and the antioxidant-responsive element (ARE). This confers protection against various forms of stress[39].

It has been -reported that beneficial effects of curcumin on vasoactive factors like ET-1 are mediated by inhibiting transcription factors like NF-κB[40]. In confirm, we reported that curcuminoids

produced more pronounced amelioration in ET-1 increase and NO decrease than ginger extract, suggesting an improvement in endothelial function.

Ginger has antioxidative, anti-inflammatory, and anti-carcinogenic properties[41,42]. Several active constituents including gingerols, shogaols, paradols, and gingerdiols have been recognized in ginger. Shogaols and gingerols are phenolic compounds. They contain a volatile oil that can be isolated from ginger root and providing the characteristic odor and flavor of ginger[43]. We found also that ginger extract containing 10-dehydrogingerdione, a natural CETP inhibitor, was able to suppress high cholesterol diet-induced increase in PAI-1 and PF1+2 greater than curcuminoids. Thus, the atheroprotective effect of ginger is associated with suppression of prethrombotic biomarkers. A previous study showed that 6-gingerol directly down-regulated uPA mRNA and decreased the levels of PAI-1 protein and RNA, whereas 6-shogaol-mediated down-regulation in uPA gene was associated by increased expression of PAI-1[44]. It has been shown that ginger powder supplementation concomitantly with fatty meal promotes fibrinolytic activity[45]. The early growth response (Egr-1) gene is a transcription factor that modifies the activity of PAI-1, usually associated with insulin resistance and obesity. Curcumin inhibits the expression of PAI-1 by reducing the activity of Egr-1 in obesity-related diseases[46,47]. In the same manner, pretreatment with curcumin hampered diesel exhaust particle exposure-induced increase in plasma PAI-1 and D-dimer concentrations, which are greatly involved in coronary thrombosis and arteriosclerosis[48-50], alleviating its cardiovascular effects and partially mitigating thrombosis[51]. This might be attributed to the anti-inflammatory activity of curcumin as mediated by suppression of TNF-α elevation. This cytokine is a-potent agonist for PAI-1 expression and plays an essential role in the regulation of PAI-1 in various diseases. In a mouse endoxemia model, TNF-α contributed to the lipopoly-saccharide-induced PAI-1 expression[52]. During obesity the increased levels of PAI-1 and TNF-α act as regulatory factors of PAI-1 expression in adipose tissue[49]. In concordance, it has been reported that TNF-α triggers PAI-1 expression in human subcutaneous adipose tissue[53,54]. These findings are in line with the study of Budinger *et al.*[55] where up-regulation of PAI-1 was diminished upon treatment with a TNF-α inhibitor. It was found also that curcumin prevented thrombin activation of prothrombin in a dose-dependent manner. Preincubation of HUVECs with curcumin also blocked FVIIa activation of FX. Therefore, curcumin could prevent the generation of thrombin or FXa where the methoxy group in curcumin positively regulated these functions[56]. Besides, curcumin administration at 500 mg level twice daily to human subjects with atherosclerosis also significantly reduced the levels of plasma FIB that is greatly involved in the formation of thrombi and related heart attacks and stroke, decreasing therefore cardiovascular risk[57].

CONCLUSION

Supplementation with turmeric or ginger extract mitigates dyslipidemia-induced hypercoagulability and vascular dysfunction by suppressing oxidative stress and inflammation. Owing to the numerous active constituents in turmeric and ginger, further investigations are needed to underline the molecular mechanisms responsible for their vascular beneficial effects in dyslipidemic patients. Their combination should be studied in the future to obtain maximal benefits.

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Table 1. Effect of curcuminoids and ginger extract on prethrombotic, ischemic and endothelial biomarkers in atherogenic diet-fed rabbits

Parameter	Groups			
	NC	Dyslipidemic	Curcuminoids	Ginger
PAI-1 (ng/ml)	0.17±0.005	0.25±0.005$^{\$}$	0.18±0.005*	0.16±0.004^{*a}
PF1+2 (pmol/L)	15.83±1.11	22.67±1.38$^{\$}$	14±0.79*	10.82±1.38^{*a}
FIB (mg/dl)	31.67±4.8	352.67±10.75$^{\$}$	71.17±5.98*	67.5±5.5*
IMA (ABSU)	0.28±0.02	0.83 ±0.1$^{\$}$	0.37±0.02*	0.42±0.04^{*a}
Serum NO (µmol/L)	52.05±4.68	16.75±2.37$^{\$}$	37.67±2.54*	33.37±2.4^{*a}
Serum ET-1 (pg/ml)	4.91±0.98	22.83±2.5$^{\$}$	11.8±0.73*	12.9±0.66^{*a}

PAI-1 (plasminogen activator inhibitor 1), PF1+2 (prothrombin fragment 1 + 2), FIB (fibrinogen), IMA (ischemia modified albumin), NO (nitric oxide), ET-1 (endothelin-1). Data are shown as the mean ± SD of 6 rabbits/group. $^{\$}$Significantly different from NC group ($P< 0.0001$), *significantly different from Dyslipidemic group ($P < 0.0001$), ^{a}significantly different from Curcuminoids group ($P< 0.01$).

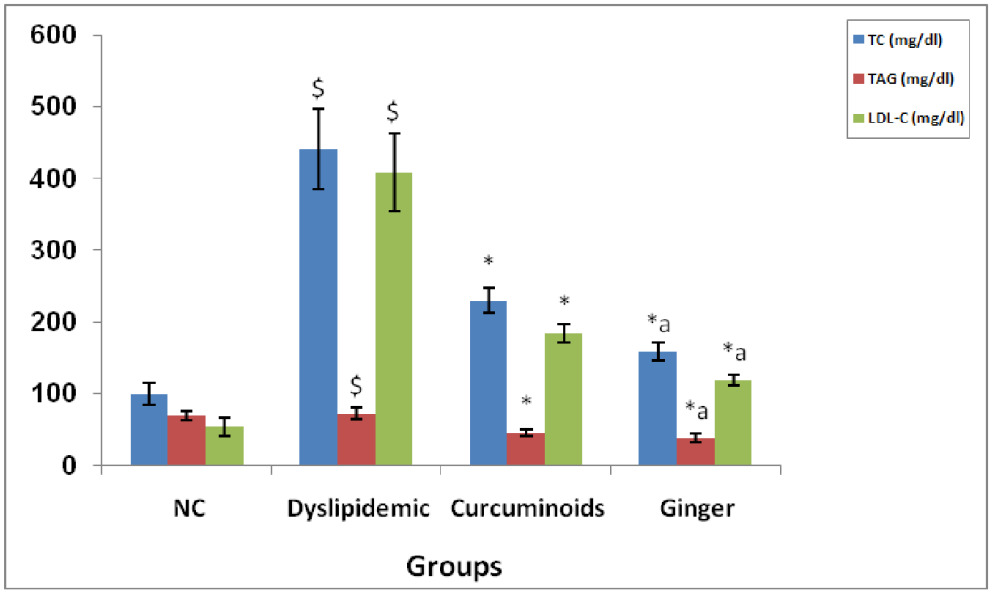

Figure 1. Effect of curcuminoids (50 mg/kg/day) or ginger extract (200 mg/kg/day) treatment for 6 weeks on serum lipid profile in rabbits fed an atherogenic diet. Dyslipidemic group were fed high-cholesterol-diet for 6 weeks without any drug treatment

Data are shown as mean ± SD (n=6). $ significant differences from NC group at $p<0.0001$, *significant differences between treated groups and Dyslipidemic group at $p<0.0001$ and asignificant differences from curcuminoids group at $p<0.05$[25].

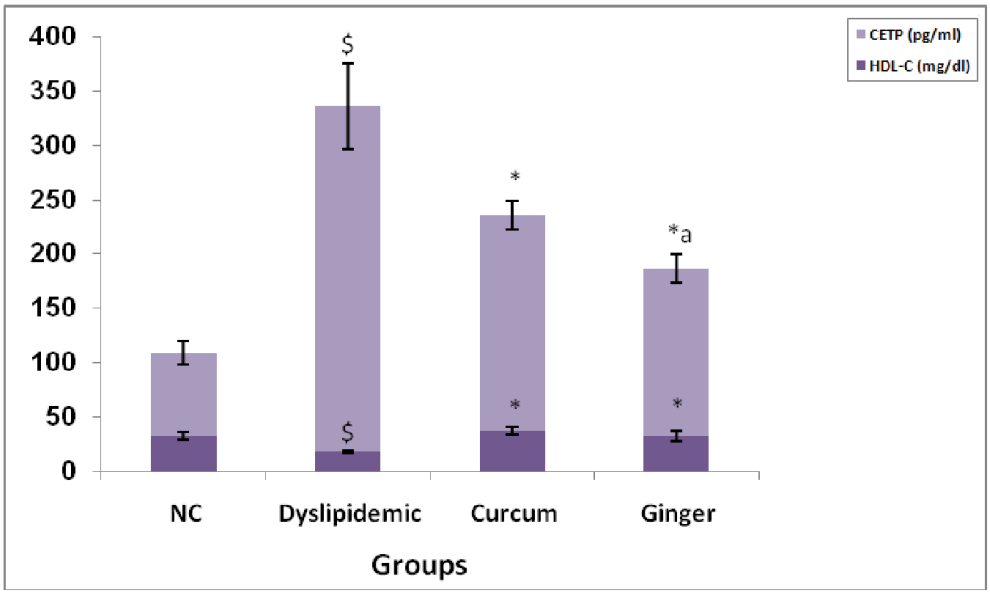

Figure 2. Effect of curcuminoids (50 mg/kg/day) or ginger extract (200 mg/kg/day) treatment for 6 weeks on serum HDL-C and CETP levels in rabbits fed an atherogenic diet. Dyslipidemic group were fed high-cholesterol-diet for 6 weeks without any drug treatment

Data are shown as mean ± SD (n=6). $ significant differences from NC group at $p<0.0001$, *significant differences between treated groups and Dyslipidemic group at $p<0.0001$ and a significant differences from curcuminoids group at $p<0.0001$[25].