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Short Communication

Dietary supplementation with an extract of lycopene-rich tomatoes does not reduce atherosclerosis in Watanabe Heritable Hyperlipidemic rabbits

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Tomatoes are rich in lycopene and other carotenoids which have shown beneficial effects on CVD in epidemiological and intervention studies. In the present study the effect of an extract of lycopene-rich tomatoes, Lyc-O-Mato® on atherosclerosis was studied in Watanabe Heritable Hyperlipidemic rabbits. The rabbits were fed a control diet, a control diet supplemented with the tomato extract or a control diet supplemented with a mixture of plant oils for 16 weeks. Lycopene was detected only in plasma of rabbits receiving tomato extract. The tomato extract had no effect on cholesterol and triacylglycerol levels measured in total plasma, lipoprotein fractions and on aortic atherosclerosis evaluated biochemically and by microscopy. Oxidation of lipids in unfractionated plasma also was unaffected by the intake of tomato extract. In conclusion, the tomato extract increased plasma levels of lycopene in rabbits, but had no effect on hypercholesterolaemia, oxidation of plasma lipids or aortic atherosclerosis.

Atherosclerosis: Lycopene: Phytoene: β-Carotene: Cholesterol

Some epidemiological studies have shown inverse correlation between lycopene consumption (notably through tomatoes) and incidence of CHD, a sequela of atherosclerosis (Rao, 2002; Rissanen *et al.* 2003). These results, together with an understanding of the contribution of excessive free radical generation in the onset of the disease led to the hypothesis that high intake of dietary antioxidants – including tomato-derived carotenoids such as lycopene – might play a preventive role in the development of atherosclerotic disease (Visioli *et al.* 2003). Lycopene, the acylic form of β-carotene, is one of the major carotenoids in the Western diet. It accounts for about 50 % of carotenoids in human serum (Willcox *et al.* 2003). The oxidation-protective effect of lycopene and tomatoes has been shown in both human and animal studies (Agarwal & Rao, 1998; Suganuma & Inakuma, 1999; Breinholt *et al.* 2000; Hadley *et al.* 2003) but negative results have also been reported (Shaish *et al.* 1995; Briviba *et al.* 2004). However, the oxidation-protective effects of the compounds are not necessarily indicative of anti-atherosclerotic effects (Shaish *et al.* 1995; Jacobsson *et al.* 2004) as several mechanisms contribute to disease development (Stocker & Keaney, 2004). Human studies have reported an inverse correlation between lycopene and atherosclerosis (Gianetti *et al.* 2002; Rissanen *et al.* 2003). Results from animal studies on the effects of carotenoids on atherosclerosis are contradictory (Shaish *et al.* 1995; Jacobsson *et al.* 2004; Li *et al.* 2004; Zheng *et al.* 2005, 2006). To elucidate the postulated preventive role of lycopene on atherosclerosis a study in Watanabe Heritable Hyperlipidemic (WHHL) rabbits, a model of human familiar hypercholesterolaemia (Havel *et al.* 1989), was designed. Previously we have used the model for drug and dietary interventions (Mortensen *et al.* 2001; Nielsen *et al.* 2005). In the present study the WHHL rabbits were fed a diet with added Lyc-O-Mato®, an extract of lycopene-rich tomatoes, for 16 weeks. The end-points in the present study were plasma lipid oxidation, changes in plasma lipoproteins and aortic atherosclerosis evaluated biochemically and by light microscopy.

Materials and methods

Animals

Animal experiments and housing procedures were performed in accordance with the Danish Animal Experimentation act on a licence granted by the Ministry of Legal Affairs and the Convention ETS 123 of the Council of Europe.

Sixty-five homozygous male WHHL rabbits with mean plasma cholesterol of 23·51 (SD 3·05) mM, mean triacylglycerols of 5·46 (SD 2·01) mM and mean body weight of 1·40

Abbreviations: WHHL, Watanabe Heritable Hyperlipidemic.

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(SD 0·23) kg at 7 weeks of age were obtained from our own breeding colony. A previous study has shown that the aortas are free from lesions at this age (Hansen *et al.* 1994). The study and all procedures were approved by the Danish Animal Experimental Inspectorate.

Experimental design

The rabbits were stratified to three groups based on plasma cholesterol and triacylglycerol concentrations, body weight and litter. From the seventh week of age group I (control, *n* 22) received daily 100 g standard diet (No. 2113; Altromin International, Lage, Germany). Group II (*n* 22) received 100 g standard diet with added 0·25 % Lyc-O-Mato® 6 %, an extract of lycopene-rich tomatoes (LycoRed Ltd, Beer-Sheva, Israel, supplied by Chr. Hansen A/S, Hørsholm, Denmark) containing 6 % lycopene (about 15 mg lycopene/100 g diet), 2 % tocopherols, 0·6 % phytoene, 0·5 % phytofluene, 0·2 % β-carotene, 0·6 % phytosterols and 10 % phospholipids. Furthermore, the Lyc-O-Mato® product (further referred to as tomato extract) contained 77 % tomato oil determined according to Leth *et al.* (2003) and therefore group III (oil control, *n* 21) received a standard diet supplemented with 0·19 % (w/w) of a plant oil mixture selected to mimic the fatty acid composition of the tomato extract (Table 1): 14 % (w/w) linseed oil (Nybrogaard, Vildbjerg, Denmark), 21 % (w/w) coconut oil (Dragsbæk A/S, Thisted, Denmark), 30 % (w/w) grape seed oil (Foodline Fællesindkøb A/S, Brøndby, Denmark) and 35 % (w/w) soya oil (Inco Denmark, Copenhagen, Denmark). All rabbits had free access to tap water. The feed intake was recorded daily and body weight was recorded weekly.

After 16 weeks of treatment the rabbits were killed by intravenous injection of pentobarbital (100 mg/kg body weight) into the marginal ear vein and sampling was performed as described elsewhere (Mortensen *et al.* 2001).

Sampling and analysis of blood and aortic atherosclerosis

Blood samples were collected in heparin tubes from the marginal ear vein of unanaesthetized animals fasted overnight. Plasma cholesterol and triacylglycerols were measured prior to treatment and monthly thereafter using an automatic analyser (Hitachi 912; Roche Diagnostics, Mannheim, Germany). At termination the concentration of cholesterol and triacylglycerols in lipoproteins was determined. Lipoproteins were separated into four fractions by single density gradient ultracentrifugation for 18 h at 21 °C (Terpstra *et al.* 1981).

Oxidation of lipids in unfractionated plasma (oxidation lag time) was determined in groups I and II as previously described (Mayer *et al.* 2001) with some modifications. Plasma (100 μl) and fluorescent marker DPHPC (5 μl; Chemica Technologies, Bend, OR, USA) were incubated for 5 h under argon at 37°C; 15 μl of this mixture were added to 210 μl PBS and the reaction was started by adding 30 μl 150 mM 2,2′-azobis(2-methylpropionamidine)dihydrochloride. The time-dependent decrease in fluorescence intensity was followed using a Wallac 1420 multilabel counter (Perkin Elmer, Life Sciences, Allerød, Denmark). The inter-day variation for a control plasma sample was 9 %.

Table 1. Body weight, feed intake, and dose of oils and the test compound*
(Mean values and standard deviations)

	Diet					
	Group I: control		Group II: Lyc-O-Mato®		Group III: plant oil	
	Mean	SD	Mean	SD	Mean	SD
No. of animals	22		22		21	
Body weight (kg)						
Initial†	1·18	0·26	1·16	0·24	1·19	0·23
At termination†	2·82	0·15	2·75	0·20	2·76	0·20
Relative feed intake (g/kg body wt per d)†	47·1	5·6	47·4	3·1	47·9	4·7
Dose of oil (mg/kg body wt per d)‡	–		91·3	5·9	92·2	9·1
Fatty acid composition of diet (%)						
Saturated	20·1		22·2		21·9	
MUFA	25·8		17·5		17·8	
PUFA	54·1		60·4		60·3	
Dose of test compound (mg/kg body wt per d)						
Lycopene	–		7·1	0·5	–	
Tocopherols	–		2·4	0·2	–	
Other carotenoids§			1·5	0·1		
Lycopene in plasma (nM)‖						
Week 8 of treatment	ND		76·8	44·3	ND	
Week 16 of treatment	ND		76·4	32·5	ND	

ND, not detectable.
* For details of procedures and diets, see p. 7.
† There were no significant differences in group means (ANOVA with Duncan's multiple range test).
‡ Oil dose in group II was Lyc-O-Mato® and in group III it was a mixture of plant oil mixed to mimic the fatty acid composition of Lyc-O-Mato®.
§ Phytoene, phytofluene and β-carotene.
‖ Four animals in each group.

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The lycopene concentration in plasma collected at weeks 8 and 16 from four randomly chosen animals from each group was determined by HPLC (Waters, Milford, MA, USA) (Breinholt *et al.* 2000).

Aortic atherosclerosis was evaluated biochemically (cholesterol content in aortic tissue expressed as μmol cholesterol/mg wet weight) and microscopically at the level of the first intercostal arteries by point counting (intima:media ratio and area of intima in mm^2) as described previously (Mortensen *et al.* 2001).

Statistics

All data are expressed as means and standard deviations. Data were tested for normal distribution by Shapiro-Wilks test and for homogeneity of variance by standardized residuals plot. When necessary, logarithmic transformations were performed. Normally distributed data were analysed by ANOVA followed by Duncan's test or *t* test and data not normally distributed were analysed by Wilcoxon test. The effects with $P<0{\cdot}05$ were considered statistically significant. All statistical analyses were performed using SAS (SAS Institute, Cary, NC, USA).

Results

Initial and terminal body weights and feed intake were similar in all three groups (Table 1). Lycopene was only detected in plasma from group II and similar levels were found at weeks 8 and 16. Carotenoids were not detected in plasma from the other groups. No effect of the treatment on clinical appearance was observed in any of the rabbits.

There were no significant differences between the groups in all recorded parameters (Table 2). Levels of cholesterol and triacylglycerols in total plasma over time and between the groups were similar. Also levels of cholesterol and triacylglycerols in lipoproteins were comparable between the groups. Plasma lipid resistance to oxidation, measured as lag time, was similar in groups I and II. Aortic atherosclerosis evaluated biochemically and microscopically did not differ between the three groups.

Table 2. Cholesterol and triacylglycerols in plasma and in lipoproteins, plasma lipid oxidation and aortic atherosclerosis*

(Mean values and standard deviations)

	Diet					
	Group I: control		Group II: Lyc-O-Mato®		Group III: plant oil	
	Mean	SD	Mean	SD	Mean	SD
No. of animals	22		22		21	
Cholesterol in plasma (mM)†						
Week 0 of treatment	23·5	3·2	23·5	3·1	23·4	3·0
Week 8 of treatment	24·4	4·1	25·3	3·7	24·3	3·4
Week 16 of treatment	24·9	5·0	25·0	4·9	24·7	5·2
Triacylglycerols in plasma (mM)†						
Week 0 of treatment	5·69	2·21	5·47	2·07	5·21	1·80
Week 8 of treatment	4·15	1·61	4·61	2·45	3·90	1·75
Week 16 of treatment	5·18	2·41	5·47	3·61	5·15	2·10
Cholesterol in lipoproteins (mM)†						
Week 16 of treatment						
HDL fraction	0·26	0·14	0·34	0·17	0·31	0·16
LDL fraction	14·0	4·2	13·3	2·75	13·1	3·0
IDL fraction	4·32	1·14	4·76	1·32	4·22	1·14
VLDL fraction	9·01	3·26	8·25	4·91	7·45	2·65
Triacylglycerols in lipoproteins (mM)†						
Week 16 of treatment						
HDL fraction	0·079	0·057	0·092	0·080	0·10	0·06
LDL fraction	2·06	0·65	2·04	0·89	2·25	0·73
IDL fraction	0·71	0·36	0·82	0·54	0·86	0·48
VLDL fraction	1·98	1·19	2·19	2·31	2·06	1·43
Lipid oxidation lag time (min)‡						
Week 16 of treatment	86·8	25·7	91·4	39·6	–	
Cholesterol (μmol/mg wet weight)†						
Ascending aorta	63·9	21·8	63·2	22·7	58·5	20·0
Thoracic aorta	52·2	26·8	49·9	22·8	49·5	26·6
Abdominal aorta	47·7	25·9	43·9	18·0	45·6	23·5
Aortic lesions						
No. of animals without lesions	1		0		4	
Intima:media ratio§	0·29	0·28	0·40	0·43	0·36	0·50
Area of intima (mm^2)§	0·14	0·12	0·15	0·14	0·15	0·21

IDL, intermediate-density lipoprotein.

* For details of procedures and diets, see p. 8.

† There were no significant differences in group means (ANOVA with Duncan's multiple range test).

‡ There were no significant differences in group means (*t* test).

§ There were no significant differences in group means (Wilcoxon test).

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Discussion

The present study is, to our knowledge, the first to report the effect of lycopene on the development of spontaneous hyperlipidaemia and atherosclerosis in WHHL rabbits. The lycopene dose of 15 mg/d (7·1 (SD 0·5) mg/kg body weight per d) in the present study was selected to mimic a high but realistic human daily intake (Dragsted *et al.* 2004), considering the metabolic rate difference between the two species. However, the plasma lycopene concentration obtained in the rabbits was about 10-fold lower than in human subjects receiving a diet enriched in fruits and vegetables (Dragsted *et al.* 2004).

As control for the high content of fatty acids in the Lyc-O-Mato® extract used as the lycopene source in the present study, we introduced a second control group receiving a mixture of plant oils with a similar fatty acid profile as the one found in the tomato extract. The oil supplementation had, however, no effects on any of the measured parameters.

Several animal and epidemiological studies have been conducted to elucidate possible beneficial effects of tomatoes or carotenoids on CVD. Some of these studies have shown positive effects of tomato compounds, but others have failed to show effects. In the present study, no protective effect of the tomato extract was found on the development of atherosclerosis. All measured parameters were unaffected by the treatment. Similarly, the carotenoids, astaxanthin and α-tocopherol did not prevent atherosclerosis in WHHL rabbits (Jacobsson *et al.* 2004). In contrast, β-carotene (but not vitamin E) (Shaish *et al.* 1995), β-carotene and vitamin E (Sulli *et al.* 1998) and carotenoid crocetin (Zheng *et al.* 2006) have been shown to have anti-atherogenic effects in normal (and initially healthy) rabbits, which became hypercholesterolaemic due to an atherogenic diet. Hypercholesterolaemia and atherosclerosis in WHHL rabbits are genetically conditioned. Therefore the lack of effect in the present and previous studies in WHHL rabbits (Jacobsson *et al.* 2004) may be related to genetic origin of atherosclerosis in this model.

Human intervention studies report contradictory effects of carotenoids on oxidative biomarkers related to atherosclerosis. Reduced lipid oxidation has been reported in healthy human subjects after a few weeks with a daily intake of tomato products (Bub *et al.* 2000; Hadley *et al.* 2003; Visioli *et al.* 2003). In contrast, lycopene from watermelon and tomato juice did not affect the antioxidant or cholesterol status in middle-aged subjects (Collins *et al.* 2004), although the lycopene dose and dosage time were comparable to those in the previous report (Visioli *et al.* 2003). However, the BMI and age of the human subjects were different in the two studies. Visioli *et al.* (2003) used young female volunteers (age 22–38 years, BMI 18–24), while Collins *et al.* (2004) used middle-aged volunteers with a much higher BMI (five men with mean age of 49 years and mean BMI of 26; five women with mean age of 51 years and mean BMI of 29). The human data suggesting a possible anti-atherogenic effect of lycopene are limited to two studies reporting an inverse association between carotid atherosclerosis and blood lycopene concentrations (Gianetti *et al.* 2002; Rissanen *et al.* 2003).

The available literature data combined with the present results lead us to hypothesize that the disease-preventive effects of lycopene may be absent whenever the onset of atherosclerosis is genetically conditioned or the disease has been established in the arterial tissue. More studies in healthy subjects or animals compared to individuals at increased risk are however warranted to confirm this hypothesis. Furthermore, one should be cautious in extrapolating from the results in the present study to the human situation because of differences in lipid metabolism between the two species (Ha & Barter, 1982; Havel *et al.* 1989) and because of the higher activity in rabbit than in man of paraoxonase, an enzyme whose polymorphism has been associated with reduced lipid peroxidation in healthy young men on a diet supplemented with tomato juice (Bub *et al.* 2005).

In conclusion, dietary supplementation with Lyc-O-Mato® resulted in elevated plasma concentrations of lycopene in WHHL rabbits. However, no effects of the treatment were observed on hypercholesterolaemia, oxidation of plasma lipids or aortic atherosclerosis in WHHL rabbits. Further studies investigating the dose-dependent effect of lycopene in other animal models and in man are required to elucidate the effects of lycopene on the development of atherosclerosis.

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