Interactive effects of increased intake of saturated fat and cholesterol on atherosclerosis in the Japanese quail (*Coturnix japonica*)

Yvonne V. Yuan[1]*, David D. Kitts[1]† and David V. Godin[2]

[1]*Department of Food Science, Faculty of Agricultural Sciences, University of British Columbia, 6650 NW Marine Drive, Vancouver, British Columbia, Canada V6T 1Z4*

[2]*Department of Pharmacology and Therapeutics, Faculty of Medicine, University of British Columbia, 2176 Health Sciences Mall, Vancouver, British Columbia, Canada V6T 1Z3*

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Increasing the energy value of diets with dietary fat, particularly fats rich in saturated fatty acids, can result in the elevation of plasma total and lipoprotein cholesterol. In the present study, experimental diets were designed to examine the effects of increasing the energy content of diets with a saturated fat source and cholesterol in a non-purified diet on hyperlipoproteinaemia and aortic plaque composition in the atherosclerosis-susceptible Japanese quail (*Coturnix japonica*) model of human atherosclerosis. Commercial poultry diets containing two levels (i.e. 60 or 120 g/kg) of beef tallow as the primary source of saturated fat were balanced for endogenous cholesterol or supplemented with cholesterol (i.e. 0·5 or 5·0 g/kg) and fed to quail for 9 weeks to examine the effects on whole plasma, lipoprotein and aortic plaque lipid composition in relation to aortic plaque formation. Hypercholesterolaemia ($P < 0{\cdot}001$) was confirmed in birds fed on high-cholesterol (HC) diets only. An interaction ($P = 0{\cdot}05$) between dietary cholesterol and fat intake level was observed for plasma triacylglycerols (TG) and was specific to changes observed in VLDL composition. Diet-induced changes in lipoprotein total cholesterol, TG and phospholipid composition were greatest in the portomicron and VLDL fractions in birds fed on atherogenic diets. Hyperlipoproteinaemia induced by the 60 g/kg added beef tallow–HC diet resulted in significant ($P < 0{\cdot}001$) aortic plaque deposition, which was further enhanced in birds fed on the 120 g/kg beef tallow–HC diet. Quail fed on 120 g/kg beef tallow–HC diets exhibited the most severe aortic plaque formation, with marked increases in aortic tissue cholesterol content and quantifiable amounts of several cholesterol oxides (5,6α-epoxy-5α-cholesterol, 7β-hydroxycholesterol, cholestanetriol, 7-ketocholesterol and 25-hydroxycholesterol). In summary, hyperlipoproteinaemia associated with HC diets with a greater proportion of energy from saturated fat produced a combined effect in altering plasma and lipoprotein lipid composition as well as aortic tissue cholesterol and cholesterol oxide content in the Japanese quail.

Atherosclerosis: Japanese quail: Lipoproteins: Cholesterol

Evidence from epidemiological (Lipid Research Clinic Program, 1984; Castelli, 1986), clinical (Schaefer *et al.* 1981; Mustad *et al.* 1997) and animal (Fernandez & McNamara, 1991, 1994; Nishina *et al.* 1993; Yuan *et al.* 1997) studies have indicated a strong association between hyperlipoproteinaemia and the intake level, source and cholesterol content of dietary fat. Common to findings from both animal (Lin *et al.* 1992; Mott *et al.* 1992) and human (Shepherd *et al.* 1980; Mattson & Grundy, 1985; Kris-Etherton *et al.* 1993) studies have been the elevation of LDL-cholesterol levels and apparent reduction of LDL receptors (Mustad *et al.* 1997) observed in subjects fed on diets rich in saturated fat and/or cholesterol. Experiments conducted in both the monkey and gerbil have indicated the importance of the relative 14 : 0 to 18 : 2*n*-6 fatty acid ratio in particular, as well as dietary cholesterol, in producing hypercholesterolaemia (Khosla & Hayes, 1993; Pronczuk *et al.* 1994). These studies have also indicated that the presence of cholesterol in the diet can produce a further increase in plasma cholesterol associated specifically with 16 : 0, which does not occur when cholesterol is absent from the diet. Feeding cholesterol alone (Ohtani *et al.* 1990), or

Abbreviations: apo, apolipoprotein; GC–MS, gas chromatography – mass spectroscopy; HC, high cholesterol; P : S, polyunsaturated : saturated fatty acid ratio; PL, phospholipid; TC, total cholesterol; TG, triacylglycerol; TS diet, Turkey Starter diet.

***Present address:** Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, 150 College Street, Toronto, ON, Canada M5S 3E2.

†**Corresponding author:** Dr David Kitts, fax +1 604 822 3959, email ddkitts@unixg.ubc.ca

in combination with saturated fat, also suppresses LDL receptors in rodents (Spady & Dietschy, 1988). Modifying animal fat sources by removing endogenous sterols or blending with vegetable or oilseed lipids rich in 18 : 2*n*-6 have been successful in neutralizing the hypercholesterolaemic potential of animal fats containing saturated fatty acids (Hunt *et al.* 1992; Seppanen-Laakso *et al.* 1992; Sundram *et al.* 1995; Hayes *et al.* 1995). Moreover, the putative role of hypertriacylglycerolaemia as an independent risk factor in the development of heart disease (Castelli, 1986; Austin, 1991) may also involve an interaction between cholesterol and dietary fat intake. For example, dietary cholesterol has been shown to reduce fatty acid oxidation, which in turn increases the levels of hepatic and plasma triacylglycerols (TG) (Fungwe *et al.* 1993). Despite the interactive effects of dietary fat and cholesterol on plasma lipids (Lin *et al.* 1992), only a few studies have attempted to examine the significance of this interaction on lipoprotein composition and the severity of atherosclerosis (Mott *et al.* 1992; Nishina *et al.* 1993).

Animal models such as the atherosclerosis-susceptible White Carneau pigeon (*Columba livia*), Japanese quail (*Coturnix japonica*) and the apolipoprotein (apo)-E null mutant mouse have been valuable in studying diet-induced development of atherogenesis as associated with hyperlipoproteinaemia and aortic plaque development (Clarkson *et al.* 1962; Nishina *et al.* 1993; Godin *et al.* 1994). Feeding cholesterol- and cholic acid-supplemented diets has been shown to accelerate the formation of aortic plaque in the apo-E null mutant mouse (Nishina *et al.* 1993) and quail (Yuan *et al.* 1997), thus indicating the requirement for hypercholesterolaemia for accelerated atherosclerotic plaque development. Recently, we have confirmed the findings of other workers (Radcliffe & Tramposch, 1988; Godin *et al.* 1994) that the Japanese quail also develops hypercholesterolaemia and atherosclerotic plaque when fed on diets containing a moderate level of varying fat sources supplemented with cholesterol, and have extended these studies by describing the changes in lipoprotein composition of hypercholesterolaemic birds (Yuan *et al.* 1997). Despite the predominance of HDL in the Japanese quail compared with the predominance of LDL in man, the composition and histopathological characteristics of aortic plaque have been noted to be similar to the situation in human atherosclerosis (Shih *et al.* 1983).

The purpose of the present study was to use the atherosclerosis-susceptible Japanese quail model to examine further both the independent and interactive effects of increased energy consumption from a saturated fat source (e.g. beef tallow) with varying levels of cholesterol, on plasma and lipoprotein lipid composition and the development and composition of atherosclerotic plaque.

Materials and methods

Animals and diets

Sixty, 6-week-old male atherosclerosis-susceptible Japanese quail (*Coturnix japonica*; University of British Columbia Quail Genetic Resource Centre, Vancouver, BC, Canada) were randomly assigned to one of five dietary treatment groups (*n* 12 in each group) consisting of a reference group (diet A) fed on a crumbled commercial Turkey Starter (TS) diet containing 54 g beef tallow/kg diet (Otter Co-op, Aldergrove, BC, Canada), two groups fed on TS supplemented with 6·0 g beef tallow/kg diet, to make a total of 60 g beef tallow/kg diet (by calculation), containing either a low (0·5 g/kg diet; diet B) or high (5·0 g/kg diet; diet C) level of cholesterol, and two groups fed on TS supplemented with 66 g beef tallow/kg diet to make a total of 120 g beef tallow/kg diet (by calculation), containing either a low (0·5 g/kg diet; diet D) or high (5·0 g/kg diet; diet E) level of cholesterol. Diets were formulated to have reduced polyunsaturated : saturated fatty acid (P : S) ratios, but to have similar *n*-6 : *n*-3 ratios through the addition of the supplementary beef tallow.

The compositions of the commercial TS diets supplemented with beef tallow and cholesterol are summarized in Table 1. The basal TS diet was supplemented with beef tallow (Cargill Foods, High River, AB, Canada), which was slowly liquefied over gentle heat (10–15 min, 45–50°) and cholesterol and cholic acid (2 : 1) mixed in to ensure uniform distribution in the crumbled commercial diet following thorough mixing. The additional dietary fat and sterols were thoroughly mixed into diets in an aluminium mixing bowl using a Hobart mixer. After mixing, individual diets were stored in doubled, dark plastic bags in a walk-in freezer (−15°) throughout the experimental study. A sample of each experimental diet was taken for analysis of component fatty acids, gross energy and DM content. Diets were isonitrogenous, with diets A, B and C containing comparable levels of energy (17·21–17·97 MJ/kg; Table 1), and diets D and E containing a greater total energy (18·76–18·83 MJ/kg) due to the higher fat content.

Gross energy determination of diets

The gross energy contents of the reference diet A and experimental diets B to E were determined by bomb calorimetry and corrected for the dry weight of the diet (Miller & Payne, 1959).

Fatty acid analysis of diets

The fatty acid compositions of the reference diet A and supplemented experimental diets B, C, D and E as determined by GC are summarized in Table 2. Samples were extracted according to the method of Folch *et al.* (1957), methylated with BF_3 (Nwokolo & Kitts, 1988) and analysed for component fatty acids using a Varian Model 3700 GC equipped with a 60 m × 0·53 mm i.d. capillary column coated with 0·25 μm Supelcowax 10 (Supelco, Bellefont, PA, USA). A 17 : 0 fatty acid internal standard (Supelco) was included in the fatty acid analyses. Supplementation of the TS diet with beef tallow resulted in only slight increases in the overall saturated fatty acid (16 : 0 and 18 : 0) content, a relatively larger increase in monounsaturated fatty acid (primarily 18 : 1*n*-9) and a decrease in polyunsaturated fatty acid (18 : 2*n*-6 and 18 : 3*n*-3) content in diets D and E (Table 2). These changes in fatty acid composition of diets D and E resulted in a reduction in P : S ratios, but similar *n*-6 : *n*-3 ratios compared with the reference TS diet (diet A) and low-tallow diets B and C.

Table 1. Composition and energy content of experimental diets*

Supplemented component (g/kg)	Diets†				
	A	B	C	D	E
Beef tallow‡					
Naturally present	54·0	54·0	54·0	54·0	54·0
Added	0	6·0	6·0	66·0	66·0
Total	54·0	60·0	60·0	120·0	120·0
Cholesterol					
Naturally present	0·066	0·066	0·066	0·066	0·066
Added from beef tallow	–	0·008	0·008	0·081	0·081
Added from crystalline sources§	–	0·426	4·926	0·353	4·853
Total	0·066	0·5	5·0	0·5	5·0
Cholic acid§	–	0·25	2·5	0·25	2·5
Crude lipid	100·0	103·0	103·0	161·0	161·0
Gross energy (MJ/kg)	17·21	17·62	17·97	18·76	18·83

* Composition of reference diet A (Turkey Starter; Otter Co-op, Aldergrove, British Columbia, Canada) (g/kg diet): lucerne meal 15·0, maize 150, distiller's grain 25·0, fish meal 50·0, lime 47·0, meat meal 75·0, pellet binder 15·0, multiphos 10·0, NaCl 2·0, soyabean meal 274, beef tallow 54·0, wheat 278, vitamins and minerals 5·0, methionine 0·25. The vitamin mixture supplied the following (mg/kg diet): thiamine hydrochloride 1·0, riboflavin 5·0, niacin 75, pantothenic acid 10, pyridoxine hydrochloride 3·0, choline 402, pteroylmonoglutamic acid 1·0, D-biotin 0·1, cyanocobalamin 0·0012, menadione sodium bisulfite 1·6, D-calcium pantothenate 300, retinyl palmitate 40, cholecalciferol 8 μg, DL-α-tocopherol acetate 120. The mineral mixture supplied the following (mg/kg diet): K_2HPO_4 10600, NaH_2PO_4 2100, $MgSO_4.7H_2O$ 1600, NaCl 4600, ferric citrate 90, KI 30, $MnSO_4.H_2O$ 150, $ZnCl_2$ 20, $CuSO_4.5H_2O$ 10.

† Diets B, C, D and E are diet A with supplemented components. Cholesterol and cholic acid were added to liquefied beef tallow before mixing in with crumbled diet A.

‡ Cargill Foods, High River, AB, Canada.

§ ICN Biochemicals, Inc., Cleveland, OH, USA.

Animal feeding studies

Quail were housed in heated brooder cages (0·9 m long, 0·46 m wide) with a single treatment group per cage. Birds had free access to feed and distilled deionized water. A 14 h dark–10 h light cycle was maintained in the room. Daily replacement of diets minimized exposure of birds to oxidized dietary lipids. Animals were cared for in accordance with the principles of the *Guide to the Care of Experimental Animals* (Canadian Council of Animal Care, 1993). Following 9 weeks on their respective diets, birds were killed at 09.00 hours. After decapitation, trunk blood was collected in chilled heparinized tubes and centrifuged (1000 ***g***; 5 min at 4°) for separation of plasma. Liver tissue was dissected, rinsed in 50 mM–Tris 0·1 mM-EDTA buffer, pH 7·6 and weighed before determination of total cholesterol (TC) and TG content (Boehringer Mannheim, Laval, PQ, Canada). Aortic tissue (the brachycephalic arteries to their bifurcations and the aorta to the iliac branching) was removed and opened lengthwise for examination of the presence of lesions on the lumen surface using a 10–30× dissecting microscope. Aortic lesions were assigned a score on a scale from 0 to 4 according to Shih *et al.* (1983), Godin *et al.* (1994) and Yuan *et al.* (1997). Scores were assigned by two independent investigators, in a blind fashion, according to the following scale: 0 = clean surface; 1 = ≤5 plaques; 2 = 6–20 plaques and an affected area less than 50 %; 3 = >20 plaques with an affected area greater than 50 %; 4 = massive atheromas observed. Following completion of scoring, aortic tissue was placed into chilled 50 mM–Tris 0·1 mM-EDTA buffer, pH 7·6 before determination of cholesterol oxides by GC with confirmation by mass spectrometry (GC–MS). Additional birds from each treatment group were used to provide aortic tissue specimens for scanning electron microscopy evaluation of aortic lumen wall morphology following plaque scoring as described earlier.

Table 2. Fatty acid profile of experimental diets

Fatty acid (g/100 g total fatty acids)	Diets*				
	A	B	C	D	E
Saturated					
Lauric (12 : 0)	0·1	0·1	0·1	0·1	0·1
Myristic (14 : 0)	1·5	1·6	1·6	2·2	2·2
Palmitic (16 : 0)	21·0	21·0	21·0	22·0	22·0
Stearic (18 : 0)	9·8	9·7	9·7	10·8	10·8
Arachidic (20 : 0)	0·3	0·3	0·3	0·2	0·2
Behenic (22 : 0)	1·2	1·3	1·3	1·0	1·0
Monounsaturated					
Myristoleic (14 : 1)	0·2	0·2	0·2	0·4	0·4
Palmitoleic (16 : 1)	2·9	3·0	3·2	3·3	3·3
Oleic (18 : 1*n*-9)	37·9	37·5	37·5	40·1	40·1
Eicosenoic (20 : 1)	0·9	1·4	1·4	0·9	0·9
Polyunsaturated					
Linoleic (18 : 2*n*-6)	20·0	19·4	19·4	15·0	15·0
Linolenic (18 : 3*n*-3)	2·3	2·4	2·4	1·8	1·8
Arachidonic (20 : 4*n*-6)	0·2	0·2	0·2	0·2	0·2
Total saturates	33·9	34·0	34·0	36·3	36·3
Total unsaturates	64·4	64·1	64·3	61·7	61·7
P : S†	0·66	0·65	0·65	0·47	0·47
n-6 : *n*-3‡	8·8	8·2	8·2	8·4	8·4

* Diet A, Turkey Starter (TS); B, TS + 0·5 g cholesterol/kg diet; C, TS + 5·0 g cholesterol/kg diet; D, TS + 66 g beef tallow/kg diet + 0·5 g cholesterol/kg diet; E, TS + 66 g beef tallow/kg diet + 5·0 g cholesterol/kg diet.

† Polyunsaturated : saturated fatty acid ratio.

‡ *n*-6 : *n*-3 Polyunsaturated fatty acid ratio.

Plasma and lipoprotein lipid analyses

Plasma TC (Siedel *et al.* 1983),TG (Ziegenhorn, 1975),

phospholipid (PL; Takayama *et al.* 1977) and protein (Bradford, 1976) were determined using standard methods (Boehringer Mannheim). Lipoproteins were separated from whole plasma by density gradient ultracentrifugation (Terpstra *et al.* 1982). Plasma lipoprotein fractions in samples from each treatment were visualized by running a reference tube pre-stained with Sudan black in ethylene glycol. Sucrose density gradient measurements and Sudan black staining of lipoprotein fractions in individual treatment reference samples were used to confirm the density gradients used in unstained samples. Quail fed on the high-cholesterol (HC) diets exhibited a distinct lipid plug on the top ($\rho_{20}<1{\cdot}006$) of the centrifuge tube after ultracentrifugation which corresponded to the portomicron fraction. This fraction was carefully removed to prevent contamination of other fractions before their removal (Terpstra *et al.* 1982). Remaining lipoproteins were separated into five fractions at density ranges as follows: fraction 1, $1{\cdot}006 < \rho_{20} < 1{\cdot}020$; fraction 2, $1{\cdot}030 < \rho_{20} < 1{\cdot}046$; fraction 3, $1{\cdot}050 < \rho_{20} < 1{\cdot}080$; fraction 4, $1{\cdot}106 < \rho_{20} < 1{\cdot}184$; fraction 5, $\rho_{20} > 1{\cdot}21$ using an SW 40Ti rotor at 272 000 ***g*** for 22 h at 20° in a Beckman L2-65 ultracentrifuge (Beckman, Montreal, Quebec, Canada). Lipoprotein fractions were assayed for lipids and protein as previously noted for whole plasma. Preliminary analysis of lipoprotein fractions for protein content indicated that the density ranges chosen for individual lipoprotein fractions allowed the recovery of equivalent fractions from plasma of birds fed on low-cholesterol and high-cholesterol diets (Fig. 1).

Aortic cholesterol oxides

Aortic tissue cholesterol oxides were determined by GC–MS. Adhering tissue was removed from aortas before weighing and extraction of lipids according to the method of Folch *et al.* (1957). An internal standard, 5α-cholestane, was added to samples before lipid extraction. Lipid extracts were evaporated to dryness with N_2 and saponified with 1 M-KOH (in CH_3OH) overnight at room temperature. The saponified samples were extracted with diethyl ether, followed by washing with 0·5 M-KOH and distilled deionized water. The non-saponifiable material was then dried (anhydrous Na_2SO_4) before reduction of sample volume using N_2, and transferred to sample vials (Pierce Chemical Co., Rockford, IL, USA). Samples were dried

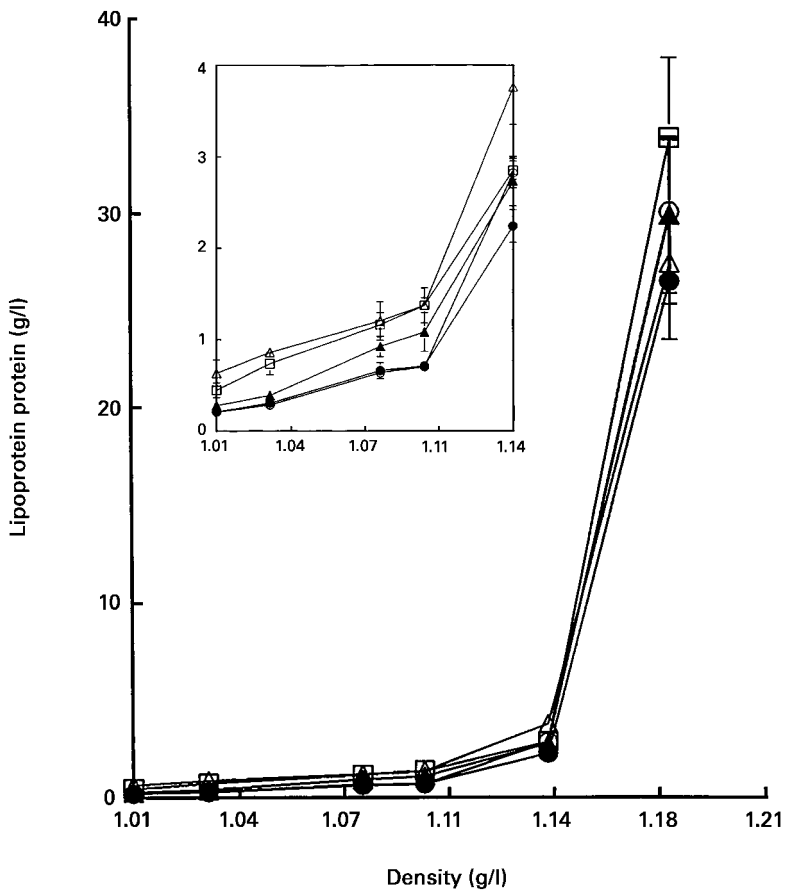

Fig. 1. Cumulative protein profiles of lipoprotein fractions recovered from plasma of atherosclerosis-susceptible Japanese quail fed on either a reference Turkey Starter diet alone (TS; diet A) or the TS diet supplemented with a low (0·5 g/kg diet) or high (5·0 g/kg diet) level of cholesterol (diets B and C) or the TS diet supplemented with additional beef tallow (66 g/kg diet) and a low (0·5 g/kg diet) or high (5·0 g/kg diet) level of cholesterol (diets D and E). (○), Diet A; (●), diet B; (△), diet C; (▲), diet D; (□), diet E.

under vacuum before solubilization in dry pyridine. Solubilized samples were derivatized (Sylon BTZ; Supelco Inc., Oakville, ON, Canada) at room temperature for 30 min. Derivatized standards (trivial name): cholest-3,5-dien-7-one, cholest-5-en-3β-ol (cholesterol), cholest-5-ene-3β,4β-diol (4β-hydroxycholesterol), cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), cholest-5ene-3β,25-diol (25-hydroxycholesterol), 5,6α-epoxy-5α-cholestan-3β-ol (cholesterol 5α,6α-epoxide), 5α-cholestan-3β,5,6β-triol (cholestanetriol), and 3β-hydroxycholest-5-ene-7-one (7-ketocholesterol; Steraloids Inc., Wilton, NH, USA) with internal standard, and samples, were analysed using a Carlo Erba GC (Carlo Erba Strumentazione, Italy) equipped with a flame ionization detector (GC-FID), and a DB-1 capillary column (15 m × 0·25 mm i.d., 0·1 μm film thickness; J & W Scientific Inc., Folsom, CA, USA). The carrier gas used was He with N_2 as the make-up gas. The injector and detector temperatures were 250° and 280° respectively, while the oven temperature was programmed from 180° to 250° at the rate of 3°/min. The identity of the cholesterol oxides was confirmed with a Kratos MS80 mass spectrometer (Ramsey, NJ, USA) coupled to a Carlo Erba GC. Cholesterol oxides were quantified after confirmation of the response linearity of each derivatized sterol.

Statistics

All data are expressed as means with their standard errors. One-way ANOVA (SPSS for Windows, SPSS Inc., Chicago, IL, USA) was used to test for differences between experimental treatments. Where differences did exist, the source of the differences at a $P \leq 0{\cdot}05$ significance level was identified by the Student–Newman–Keuls multiple range test (SPSS). Two-way multiple ANOVA (MANOVA; SPSS) was used to identify any interactions between level of dietary fat and cholesterol level. Linear regression analyses (SPSS) were also performed.

Results

Animal growth, liver weights and lipid composition

There was no significant difference in body weight gain between quail fed on the two levels of dietary fat. Final body weights of quail were not affected by levels of dietary fat or cholesterol intake (mean 135 (SE 2) g). Liver tissue weights from birds fed on HC diets (diets C and E) were significantly greater ($P < 0{\cdot}001$) than livers from birds fed on low cholesterol (LC) diets (diets B and D) or the reference TS diet (diet A; Table 3). Also, livers collected from birds fed on HC diets (diets C and E) were pale coloured in comparison with livers from animals fed on diets A, B and D. A significant interaction was recorded between dietary fat and dietary cholesterol levels with liver weight ($P = 0{\cdot}007$), as demonstrated by the greater liver tissue weights of birds fed on diet E. Quail fed on the HC diets (C and E) had greater amounts of liver cholesterol ($P = 0{\cdot}001$) compared with those fed on LC diets, as expected (Table 3). However, hepatic cholesterol content was not influenced by variations in the level of dietary fat. The TG content of livers was significantly increased ($P = 0{\cdot}01$) in quail fed on diets containing the higher level of beef tallow (diets D and E). However, dietary cholesterol level did not have any effect on liver TG content (Table 3).

Plasma composition of quail fed on reference and experimental diets

Birds fed on the reference TS diet (diet A) exhibited similar plasma TC, TG, PL and protein levels as counterparts fed on LC diets (diets B and D; Table 4). As expected, significantly greater levels of plasma TC ($P < 0{\cdot}001$) were observed in birds fed on the HC diets (C and E). Birds fed on the HC diets also exhibited significant elevations in TG ($P < 0{\cdot}001$) and PL ($P < 0{\cdot}001$) compared with birds fed on LC diets (B and D) as well as the reference diet A. A significant interaction ($P = 0{\cdot}05$) between dietary fat level and cholesterol intake was observed only with plasma TG. There was no effect of dietary fat level or cholesterol intake on plasma protein content.

Birds fed on the HC diets (diets C and E) exhibited a thick layer of lipid at $\rho_{20} < 1{\cdot}006$ on ultracentrifugation, which represented the absorptive portomicron fraction and contained large amounts of cholesterol, TG and PL (Tables 5–7). This lipoprotein fraction was undetectable in plasma

Table 3. Liver tissue weights and lipid composition in atherosclerosis-susceptible Japanese quail fed on experimental diets

(Values are means with standard errors for twelve quail)

		Diets*					ANOVA	
		A	B	C	D	E	*P* value†	
Liver wt (g/kg body wt)	Mean	15	17	22	17	28	C	<0·001
	SE	1	1	1	1	2	L	0·002
							C × L	0·007
Liver cholesterol (mmol/mg tissue wet wt)	Mean	16	13	30	14	33	C	0·001
	SE	3	1	2	2	2	L	NS
							C × L	NS
Liver triacylglycerol (mmol/mg tissue wet wt)	Mean	31	22	23	29	35	C	NS
	SE	3	1	3	2	4	L	0·01
							C × L	NS

* For details of diets, see Tables 1 and 2.
† C, cholesterol treatment effect; L, fat level treatment effect; C × L, cholesterol × fat level treatment interaction by two-way ANOVA.

Table 4. Whole plasma lipid and protein concentrations in atherosclerosis-susceptible Japanese quail fed on experimental diets

(Values are means with their standard errors for twelve quail)

Plasma component		Diets* A	B	C	D	E	ANOVA *P* value†	
Total cholesterol (mmol/l)	Mean	5·33	7·06	36·8	7·75	43·4	C	<0·001
	SE	0·32	0·38	4·9	0·36	3·5	L	NS
							C × L	NS
Triacylglycerol (mmol/l)	Mean	1·89	3·57	7·81	2·73	11·8	C	<0·001
	SE	0·19	0·76	1·51	0·38	1·8	L	NS
							C × L	0·05
Phospholipids (mmol/l)	Mean	4·95	5·02	15·7	5·19	20·7	C	<0·01
	SE	0·36	0·32	2·1	0·32	2·4	L	NS
							C × L	NS
Protein (g/l)	Mean	33·1	32·6	37·8	31·4	38·9	C	NS
	SE	2·7	4·0	3·6	4·3	3·1	L	NS
							C × L	NS

* For details of diets, see Tables 1 and 2.
† C, cholesterol treatment effect; L, fat level treatment effect; C × L, cholesterol × fat level treatment interaction by two-way ANOVA.

samples collected from quail fed on the LC diets (A, B and D). The TG and PL contents of the portomicron fraction of plasma collected from birds fed on diet C were significantly lower ($P < 0·05$) than those of birds fed on diet E. When whole plasma from experimental quail was ultracentrifuged, five lipoprotein fractions were isolated corresponding to VLDL (fraction 1), LDL (fraction 2), a lower-density HDL subclass, analogous to HDL_2 (fraction 3), a higher-density HDL subclass, analogous to HDL_3 (fraction 4) and lastly, very-high-density lipoprotein (fraction 5). The level of dietary fat fed to birds had no effect on lipoprotein TC content (Table 5). However, the TC content of lipoprotein fractions 1, 2 and 3 was enhanced ($P < 0·05$) in birds fed on HC diets (C and E) compared with birds fed on LC diets (A, B and D). The TC contents of lipoprotein fractions 4 and 5 were not affected by dietary treatment. Whole plasma TC content was positively correlated with hepatic tissue TC content (r 0·839, $P < 0·001$) and positive correlations were also observed between hepatic tissue TC content and TC levels of lipoprotein fractions 1 (r 0·819, $P < 0·001$), 2 (r 0·765, $P < 0·001$) and 3 (r 0·747, $P < 0·001$). Whole plasma TG content was positively correlated with hepatic tissue TC content (r 0·581, $P = 0·002$), but was not related to hepatic tissue TG content.

Dietary fat level did not have a significant effect on either lipoprotein TG or PL contents (Tables 6 and 7). However, the TG and PL contents of lipoprotein fractions 1 and 2 were significantly greater ($P < 0·05$) in quail fed

Table 5. Plasma lipoprotein cholesterol concentrations in atherosclerosis-susceptible Japanese quail fed on experimental diets

(Values are means with their standard errors for twelve quail)

Plasma fraction†		Diets* A	B	C	D	E
Portomicron-cholesterol (mmol/l)	Mean	–	–	12·6[a]	–	14·3[a]
	SE			4·1		3·7
Fraction 1-cholesterol (mmol/l)	Mean	0·29[a]	1·94[b]	13·0[c]	1·89[b]	15·1[c]
	SE	0·06	0·14	2·2	0·14	2·0
Fraction 2-cholesterol (mmol/l)	Mean	0·50[a]	0·84[a]	3·34[b]	0·94[a]	4·90[b]
	SE	0·10	0·06	0·98	0·07	0·53
Fraction 3-cholesterol (mmol/l)	Mean	0·65[a]	0·76[a]	1·75[b]	1·13[ab]	1·93[b]
	SE	0·04	0·19	0·53	0·10	0·24
Fraction 4-cholesterol (mmol/l)	Mean	2·44[a]	2·51[a]	3·01[a]	2·85[a]	2·92[a]
	SE	0·16	0·11	0·34	0·19	0·37
Fraction 5-cholesterol (mmol/l)	Mean	0·28[a]	0·38[a]	0·33[a]	0·41[a]	0·43[a]
	SE	0·03	0·04	0·09	0·09	0·13

[a,b,c] Mean values within a row not sharing a common superscript letter were significantly different, $P \leq 0·05$.
* For details of diets, see Tables 1 and 2.
† Fraction numbers refer to position in density gradient: portomicron, $\rho_{20} < 1·006$; fraction 1, $1·006 < \rho_{20} < 1·020$; fraction 2, $1·030 < \rho_{20} < 1·046$; fraction 3, $1·050 < \rho_{20} < 1·080$; fraction 4, $1·106 < \rho_{20} < 1·184$; fraction 5, $\rho_{20} > 1·21$. For details, see p. 92.

Table 6. Plasma lipoprotein triacylglycerol (TG) concentrations in atherosclerosis-susceptible Japanese quail fed on experimental diets

(Values are means with their standard errors for twelve quail)

		Diets*				
Plasma fraction†		A	B	C	D	E
Portomicron-TG (mmol/l)	Mean	–	–	2·89[a]	–	5·78[b]
	SE			0·76		0·93
Fraction 1-TG (mmol/l)	Mean	0·32[a]	1·14[a]	2·76[b]	0·99[a]	2·91[b]
	SE	0·04	0·22	0·78	0·26	0·77
Fraction 2-TG (mmol/l)	Mean	0·14[a]	0·28[a]	0·53[b]	0·26[a]	0·50[b]
	SE	0·03	0·02	0·11	0·03	0·11
Fraction 3-TG (mmol/l)	Mean	0·16[a]	0·29[a]	0·28[a]	0·25[a]	0·35[a]
	SE	0·01	0·06	0·09	0·04	0·04
Fraction 4-TG (mmol/l)	Mean	0·61[a]	0·89[a]	0·51[a]	0·65[a]	0·75[a]
	SE	0·02	0·08	0·08	0·07	0·08
Fraction 5-TG (mmol/l)	Mean	0·52[a]	0·62[a]	0·41[a]	0·52[a]	0·57[a]
	SE	0·04	0·05	0·06	0·05	0·06

[a,b] Mean values within a row not sharing a common superscript letter were significantly different, $P \leqslant 0{\cdot}05$.
* For details of diets, see Tables 1 and 2.
† For details of plasma fractions, see Table 5.

on HC diets (C and E) when compared with those fed on LC diets (A, B and D). Dietary treatment did not influence either the TG or PL content of lipoprotein fractions 3, 4 or 5.

Aortic plaque score and percentage area covered

Birds fed on either the TS reference diet (diet A) or the LC diets (diets B and D) did not exhibit detectable aortic plaque (Fig. 2(a)). On the other hand, feeding quail on HC diets (C and E) resulted in significant aortic plaque development ($P < 0{\cdot}001$). The severity of aortic plaque formation in animals fed on the latter diets was enhanced in quail fed on diet E compared with birds fed on diet C, as demonstrated by the significant interaction recorded ($P = 0{\cdot}02$). The percentage of aortic lumen covered by plaque from birds fed on diet E was greater than in counterparts fed on diet C, as demonstrated by the interaction recorded between dietary fat and cholesterol levels ($P = 0{\cdot}003$; Fig. 2(b)). Aortic tissue plaque score was positively correlated with plasma TC (r 0·624, $P < 0{\cdot}001$) as well as the cholesterol content of lipoprotein fractions 1 (r 0·560, $P = 0{\cdot}004$); 2 (r 0·669, $P < 0{\cdot}001$); 3 (r 0·784, $P < 0{\cdot}001$) and 4 (r 0·508, $P = 0{\cdot}011$). Similarly, the percentage of aortic tissue area

Table 7. Plasma lipoprotein phospholipid (PL) concentrations in atherosclerosis-susceptible Japanese quail fed on experimental diets

(Values are means with their standard errors for twelve quail)

		Diets*				
Plasma fraction†		A	B	C	D	E
Portomicron-PL (mmol/l)	Mean	–	–	7·56[a]	–	11·1[b]
	SE			0·52		0·9
Fraction 1-PL (mmol/l)	Mean	0·35[a]	0·53[a]	2·70[b]	0·69[a]	4·28[b]
	SE	0·03	0·08	0·70	0·12	0·84
Fraction 2-PL (mmol/l)	Mean	0·29[a]	0·12[a]	1·06[b]	0·14[a]	1·16[b]
	SE	0·02	0·02	0·36	0·01	0·14
Fraction 3-PL (mmol/l)	Mean	0·83[a]	0·64[b]	0·72[a]	0·97[a]	0·97[a]
	SE	0·10	0·14	0·15	0·17	0·08
Fraction 4-PL (mmol/l)	Mean	2·44[a]	2·84[a]	2·11[a]	2·74[a]	1·93[a]
	SE	0·17	0·14	0·26	0·11	0·30
Fraction 5-PL (mmol/l)	Mean	0·64[a]	0·43[a]	0·40[a]	0·39[a]	0·35[a]
	SE	0·06	0·05	0·06	0·03	0·04

[a,b] Mean values within a row not sharing a common superscript letter were significantly different, $P \leqslant 0{\cdot}05$.
* For details of diets, see Tables 1 and 2.
† For details of plasma fractions, see Table 5.

covered by plaque was positively related to plasma TC (r 0·578, $P < 0·001$) as well as the cholesterol content of lipoprotein fractions 1 to 3. It is noteworthy that positive, albeit weak, correlations were also recorded between whole plasma TG concentrations and aortic tissue plaque score (r 0·412, $P = 0·001$) and percentage of aortic tissue area covered by plaque (r 0·390, $P = 0·001$).

Aortic cholesterol and cholesterol oxide content

Aortas from birds fed on the reference TS diet (diet A) and the LC diets (diets B and D) were devoid of any detectable plaque on the luminal surface and exhibited low levels of cholesterol (Table 8). In all cases, the combination of the low level of tissue cholesterol and the absence of cholesterol

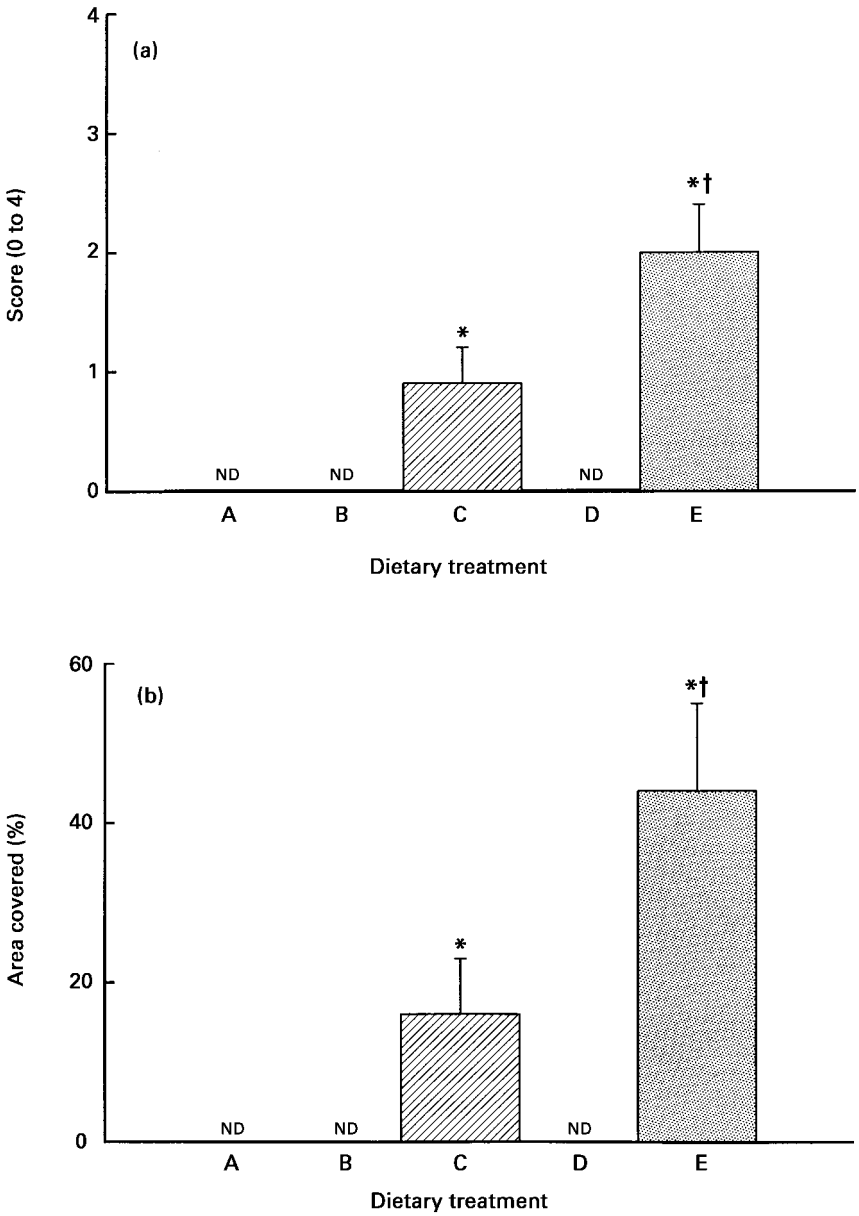

Fig. 2. (a) Aortic plaque score and (b) percentage area covered by plaque from atherosclerosis-susceptible Japanese quail fed on either a reference Turkey Starter diet alone (TS; diet A) or the TS diet supplemented with a low (0·5 g/kg diet) or high (5·0 g/kg diet) level of cholesterol (diets B and C) or the TS diet supplemented with additional beef tallow (66 g/kg diet) and a low (0·5 g/kg diet) or high (5·0 g/kg diet) level of cholesterol (diets D and E). Plaque score based on scale of 0 (not detected (ND)) = clean surface; 1 = ≤ 5 plaques, 2 = 6–20 plaques; 3 = > 20 plaques; 4 = massive atheromas seen. Values represent the mean of two judges evaluating in a double-blind protocol. Area covered (%) is the percentage of aortic epithelium covered by plaque, range 0 (ND)–100 %. Panel (a): * significant dietary cholesterol level effect ($P < 0·001$); † significant interaction between cholesterol and dietary fat level ($P = 0·02$). Panel (b): * significant dietary cholesterol level effect ($P < 0·001$); † significant interaction between cholesterol and dietary fat level ($P = 0·03$).

Table 8. Gas-chromatography–mass spectroscopic quantitation of cholesterol and cholesterol oxide content of aortic tissue from atherosclerosis-susceptible Japanese quail fed on experimental diets

		Cholesterol and cholesterol oxides (mg/g tissue)					
Dietary treatment*	Plaque score†	Cholesterol	5,6α-epoxide	7β-OH	triol	7-keto	25-OH
A	0/0/0	0·89	ND	ND	ND	ND	ND
A	0/0/0	0·68	ND	ND	ND	ND	ND
B	0/0/0	0·76	ND	ND	ND	ND	ND
B	0/0/0	0·63	ND	ND	ND	ND	ND
C	0/0/0	1·24	ND	ND	ND	ND	ND
C	0/2/0	1·69	ND	ND	ND	ND	ND
C	2/1/2	5·60	0·17	ND	ND	ND	ND
C	3/3/4	4·99	1·33	ND	ND	ND	ND
D	0/0/0	2·46	ND	ND	ND	ND	ND
D	0/0/0	2·31	ND	ND	ND	ND	ND
E	3/2/1	12·63	0·19	0·59	0·15	0·13	ND
E	4/4/4	21·62	0·21	0·99	0·30	0·08	0·38

ND, not detected; 5,6α-epoxide, 5,6α-epoxy-5α-cholesterol; 7β-OH, 7β-hydroxycholesterol; triol, cholestane-triol; 7-keto, 7-ketocholesterol; 25-OH, 25-hydroxycholesterol.
* For details of diets, see Tables 1 and 2.
† For details of plaque scoring, see p. 91. x/y/z, Individual score for each of three vessels in aortic tree.

oxides in aortic tissue of quail fed on diets A, B and D were associated with an absence of aortic plaque deposition. The aortas from quail fed on diet C contained approximately twice the amount of cholesterol compared with birds fed on diets A or B, in addition to detectable levels of a single cholesterol oxide, 5,6α-epoxy-5α-cholesterol (Table 8). The higher level of beef tallow in combination with a high level of cholesterol in diet E was associated with the presence of greater cholesterol and several cholesterol oxides, including 5,6α-epoxy-5α-cholesterol, 7β-hydroxycholesterol, cholestanetriol, 7-ketocholesterol and 25-hydroxycholesterol in aortic tissue from these animals.

Discussion

The present study describes the effects of feeding different levels of saturated fat and cholesterol on plasma and lipoprotein lipid profiles in relation to aortic tissue plaque deposition and associated cholesterol and oxysterol content in the atherosclerosis-susceptible Japanese quail. The choice of beef tallow used as the fat source in order to vary the energy content of experimental diets was based on observations of others, where feeding tallow produced an increased percentage of 14 : 0 and 16 : 0 and decreased 18 : 2*n*-6 fatty acids, specifically in plasma relative to polyunsaturated fatty acid-based diets (Faidley *et al.* 1990). In addition to altering the energy content of our experimental diets, relative differences in the proportion of saturated and polyunsaturated fatty acids were also achieved (i.e. diets A, B and C, 18 : 2*n*-6/14 : 0 value of 12·1–13·3 *v.* 6·8 for diets D and E) by supplementing diets with beef tallow.

Validation of quail animal model

The severe aortic plaque deposition obtained in quail fed on the atherogenic diets containing HC and cholic acid over a relatively short period of 9 weeks is similar to the accelerated atherogenesis observed in the apo-E deficient mouse used to examine the effect of different dietary lipids on the development of aortic lesions (Nishina *et al.* 1993). Both elevated plasma TG levels as well as the presence of TG-rich lipoproteins have also been closely associated with coronary artery disease (Patsch *et al.* 1992). Our results using the quail model extend the findings of others by showing that the diet-induced elevations in plasma TC, TG and PL and milky appearance were primarily associated with the absorptive portomicron fraction. The direct extrapolation of results obtained in this quail model to the human situation requires caution however, due to species differences in lipoprotein metabolism.

Plasma and hepatic tissue lipid response to dietary fat level

The combination of increased levels of 14 : 0 and 16 : 0 with decreased 18 : 2*n*-6 intake can further the development of hypercholesterolaemia and impaired lipoprotein metabolism in a number of animal models (Lindsey *et al.* 1990; Khosla & Hayes, 1993; Hayes *et al.* 1995). In the present study it was clear that feeding quail on LC diets supplemented with beef tallow and containing a greater proportion of energy from fat as well as a low P : S ratio was not sufficient to influence plasma lipid profiles or hepatic cholesterol content in quail. This observation could be specific to the choice of beef tallow used in this study since beef tallow is a good source of 18 : 0 and 18 : 1; two fatty acids that are not associated with a hypercholesterolaemic response relative to other saturated fats (Grundy & Denke, 1991; Fernandez & McNamara, 1994). In contrast, Fungwe *et al.* (1994) have reported an enhanced hepatic cholesterol synthesis and TG content in rats when dietary fat content increased from 5 to 20 %. However, unlike our long-term feeding study which used beef tallow to increase the fat content of diets fed to quail, Fungwe *et al.* (1994) fed maize oil, which contains a greater amount of 18 : 2*n*-6, a similar amount of 18 : 1 and approximately one-seventh the

18 : 0 content to rats for only 7 d. Taken together, these results demonstrate the importance of evaluating the specific fatty acid content of different fats in predicting species-specific changes in plasma and liver lipids, especially when cholesterol is absent or present at a very low level in the diet.

A different result was observed, however, in quail fed on tallow-supplemented diets containing high levels of cholesterol. The reduced P : S ratio and increases in 14 : 0 and 16 : 0 saturated fatty acids, relative to 18 : 2*n*-6 in diets fed to these birds, together with the higher intake of cholesterol produced dramatic hypercholesterolaemia that has been explained by others as involving an effect of cholesterol to amplify the impact of 14 : 0 and 16 : 0 in the absence of 18 : 2*n*-6 (Khosla & Hayes, 1993). Moreover, Mustad *et al.* (1996) have also shown that 18 : 2*n*-6 feeding resulted in enhanced hepatic LDL-receptor protein and mRNA levels compared with 16 : 0 when pigs were fed on high-cholesterol diets.

Effect of dietary cholesterol on plasma lipids

Dietary cholesterol has been reported either to have a minimal effect on plasma TC (Keys *et al.* 1956), or to contribute significantly to elevations in plasma cholesterol (Lipid Research Clinics Program, 1984). The presence of increased cholesterol levels in the diet will elevate serum and aortic tissue cholesterol and increase aortic atherosclerosis in the atherosclerosis-susceptible pigeon (Clarkson *et al.* 1962). In the quail model, a wide range of cholesterol supplementation levels (1·0–20 g/kg) have been used to raise plasma cholesterol concentrations and vary the temporal development and severity of aortic plaque deposition similar to that reported with human atherosclerosis (Shih *et al.* 1983; Radcliffe & Tramposch, 1988). A primary component of the hypercholesterolaemia in quail fed on HC diets was the prominent lipid-rich portomicron fraction observed in plasma collected from these birds, but not detected in counterparts fed on LC diets. Moreover, our results also associate diet-induced increases in plasma cholesterol with increases in VLDL- (fraction 1), LDL- (fraction 2) and to a lesser extent, HDL_2- (fraction 3), but not HDL_3- (fraction 4) cholesterol. In light of the fact that the liver has a central role in VLDL assembly as well as LDL catabolism, the higher VLDL- and LDL-cholesterol content of birds fed on HC diets could simply be in response to an expanded hepatic cholesterol pool. If this occurred, the hypercholesterolaemia observed in these quail could then be explained by the increased VLDL-TC, suggesting an impaired ability to clear VLDL-remnants as reflected by the enhanced LDL-TC also observed in the present study. The elevated LDL-TC in HC-fed quail is also similar to the hypercholesterolaemia reported in atherogenic pigeons, which was attributed to the expression of non-functional LDL-receptors rather than LDL-receptor down-regulation (Reagan *et al.* 1990). Similar findings have been reported in the Watanabe heritable hyperlipidaemic rabbit (Kita *et al.* 1981) and human subjects with familial hypercholesterolaemia (Thompson *et al.* 1981), both of which have defective receptor-mediated LDL clearance.

The interaction between the increased level of saturated fat from beef tallow and cholesterol supplementation of diets fed to quail observed for plasma TG levels but not cholesterol concentrations demonstrates a disparity in the metabolism of these two lipids. It is difficult to conclude that the interaction between dietary fat level and cholesterol intake for plasma TG resulted from enhanced lipogenesis, reduced fatty acid oxidation or increased secretion of VLDL, since these responses would also be expected to result in enhanced hepatic TG content (Grundy & Denke, 1990; Fungwe *et al.* 1993), which did not occur in the present study. Since a dietary treatment interaction was not observed for hepatic TG, we can only conclude that dietary fatty acids are not as effective as *de novo* synthesized fatty acids as precursors for hepatic TG synthesis (Liu *et al.* 1995).

Dietary cholesterol and aortic plaque score in quail

The severity of atherosclerosis assessed by the visual scoring method of aortic lumen appearance was confirmed by scanning electron microscopy (figures available on request). The significance of these assessments is based on the findings of previous studies which have reported that atherogenesis in the Japanese quail is characterized by a series of events involving tissue disruption and swelling, followed by the appearance of cholesterol-laden foam cells and the formation of aortic plaque with cellular proliferation and narrowing of the lumen similar to the events occurring in human atherosclerosis (Shih *et al.* 1983; Peng *et al.* 1985). Previous *in vitro* studies with tissue from atherosclerosis-susceptible pigeons have demonstrated that aortic smooth muscle cells fail to internalize LDL due to the absence of a functional LDL-receptor pathway (Randolph & St Clair, 1984). It is noteworthy that the interaction noted between dietary saturated fat level and cholesterol intake for aortic plaque score coincided with a similar treatment interaction for plasma TG levels but not for plasma TC concentrations. The absence of atherosclerotic lesions in birds fed on the LC diets confirms the requirement for a high dietary cholesterol intake which results in both hypercholesterolaemia and hypertriacylglycerolaemia and, thereby, aortic plaque development. This hypothesis is supported by the low levels of tissue cholesterol and the absence of detectable cholesterol oxides in aortic tissue from LC-fed birds. Other studies conducted in the pigeon (Clarkson *et al.* 1962) and rabbit (Kritchevsky, 1970) also reported that the extent of atherosclerosis was greatest when diets were high in cholesterol; however, the effects on plasma TG were not assessed.

Aortic tissue cholesterol and cholesterol oxide composition

The detection of a greater number of cholesterol oxide species in atherosclerotic plaque material from quail fed on diet E paralleled the increased cholesterol content of these aortic tissues which were heavily covered by plaque. The presence of small but detectable concentrations of 7β-hydroxycholesterol, cholestanetriol, 7-ketocholesterol and 25-hydroxycholesterol in aortic plaque of birds fed on diet E occurred in addition to cholesterol-5,6α-epoxide, which was also detected in quail fed on the HC–low-beef-tallow

diet (diet C). The fact that additional cholesterol oxides were present in aortic tissue collected from birds fed on diet E is further evidence of the atherogenic potential of the combination of a high cholesterol intake with increased presence of dietary saturated fat. The higher concentration of cholesterol and detection of cholesterol oxides in diseased aortas from quail that we report in the present study confirm the similarities in aortic plaque composition between this animal model and human atherosclerosis. Although previous workers have reported the recovery of cholesterol oxides from LDL extracted from human aortic plaque at autopsy (Steinbrecher & Lougheed, 1992), our results show for the first time the important interaction between a high cholesterol intake combined with increased dietary energy derived from saturated fat on aortic plaque development and sterol composition in this animal model of human atherosclerosis.

Conclusion

In summary, atherosclerosis-susceptible Japanese quail fed on commercial Turkey Starter diets supplemented with 5·0 g cholesterol/kg diet and a low or high level of saturated fat exhibited both hyperlipoproteinaemia and aortic plaque development. The hypertriacylglycerolaemia associated with cholesterol feeding was further enhanced in quail fed on beef tallow-supplemented diets, which supports the 'lipid hypothesis' that an increased intake of energy from saturated fat can increase plasma lipids and potentiate atherosclerosis. Moreover, the findings of a significant interaction between dietary cholesterol intake and saturated fat content observed for both plasma TG as well as aortic plaque score in Japanese quail strengthens the importance of plasma TG levels in reducing risk of coronary artery disease.

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