Influence of dietary cholesterol and fat source on atherosclerosis in the Japanese quail (*Coturnix japonica*)

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The Japanese quail has been used as a model of human atherosclerosis to investigate the mechanisms underlying the development of vascular lesions, i.e. hyperlipoproteinaemia and impaired endogenous antioxidant status. In the present study, Japanese quail were fed on semi-purified diets containing butter, beef tallow or soyabean-oil blends, with either 0·5 or 5 g cholesterol/kg for 9 weeks to examine the effects of dietary fat blends varying in fatty acid composition and cholesterol intake on plasma lipids and aortic atherosclerotic plaque and sterol composition. These findings were related to possible diet-induced changes in antioxidant status of selected tissues. Hypercholesterolaemia was confirmed ($P < 0·001$) in birds fed on high-cholesterol (HC) diets. Plasma total cholesterol concentration and cholesterol content of lipoprotein fractions in hypercholesterolaemic birds were lower ($P < 0·05$) in quail fed on the soyabean-oil blend. Plasma triacylglycerol content was increased ($P < 0·001$) in HC-fed birds. Dietary fat blends did not influence plasma triacylglycerol levels. Tissue antioxidant status (catalase (*EC* 1.11.1.6), glutathione peroxidase (*EC* 1.11.1.9), glutathione reductase (*EC* 1.6.4.1) and superoxide dismutase (*EC* 1.15.1.1) activities and glutathione content) was generally not greatly affected by dietary fat blend or cholesterol treatment. Birds fed on HC diets exhibited severe ($P < 0·001$) atherosclerotic plaque in aortas which was not influenced by the source of dietary fat blend. Scanning electron microscopy confirmed results of visual aortic plaque scoring using dissecting light microscopy. Several cholesterol oxides were identified and quantified in aortic plaque from HC-fed birds (5,6α-epoxy-5α-cholesterol, 7β-hydroxycholesterol and 7-ketocholesterol) regardless of dietary fat blend. The results indicate that dietary fat blends varying in polyunsaturated : saturated fatty acid ratios only marginally influence the degree of hypercholesterolaemia in atherosclerosis-susceptible quail fed on atherogenic diets only, and are not a factor, compared with sterol feeding, in modulating the degree of atherosclerosis or the aortic oxysterol content in these same birds. Moreover, diet-induced hyperlipoproteinaemia had only a small effect on antioxidant status of selected tissues examined.

Atherosclerosis: Lipoproteins: Dietary fats: Japanese quail

Studies of patients with CHD suggest an association between increased susceptibility to lipid peroxidation and reduced levels of specific antioxidant enzymes in the plasma and erythrocytes (Jayakumari *et al.* 1992; Bonithon-Kopp *et al.* 1997), platelets (Buczynski *et al.* 1983) and diseased aortic tissue (Hunter *et al.* 1991). The role of oxidized lipoproteins in the formation of atherosclerotic lesions (Jurgens *et al.* 1987) and the presence of

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cholesterol oxides in aortic plaque material collected from both human subjects (Stringer *et al.* 1989) and animal models (Rosenfeld *et al.* 1990) further supports the hypothesis that lipid peroxidation and impaired antioxidant status are implicated in the initiation of atherosclerosis.

Histological studies of aortic plaque obtained from Japanese quail (*Coturnix japonica*) fed on atherogenic diets indicate that endothelial cell populations involved in the initiation and propagation phases of plaque development in this animal model are very similar to human atherosclerosis (McCormick *et al.* 1982; Shih, 1983; Shih *et al.* 1983). The characterization of atherosclerosis-susceptible and -resistant strains of Japanese quail has validated the use of this animal as a model for human atherosclerotic disease (Shih *et al.* 1983; Godin *et al.* 1994). In studies examining the endogenous antioxidant status of atherosclerosis-susceptible and -resistant Japanese quail, Godin *et al.* (1994) reported differences between strains in aortic tissue, erythrocyte and plasma antioxidant enzyme activities which were tissue- and enzyme-specific.

While the Japanese quail has been characterized as a model of human atherosclerosis using dietary cholesterol manipulation alone (Smith & Hilker, 1973; Radcliffe *et al.* 1982), no attempt has been made to examine the role of dietary fat composition in modulating hyperlipoproteinaemia and atherosclerosis in this animal model. Previous studies using the atherosclerosis-susceptible White Carneau pigeon (*Columba livia*) reported that marked elevations in serum cholesterol resulted in aortic cholesterol deposition and corresponded to the severity of atherosclerosis in cholesterol-fed birds (Lofland *et al.* 1961). More recently, a number of studies using rodents have shown the important interaction between fatty acid profile and cholesterol intake in regulating lipoprotein metabolism and plasma cholesterol levels (Nishina *et al.* 1993; Hayes *et al.* 1995). In the avian model, hypercholesterolaemia has been observed to lead to the down-regulation of cholesterol synthesis, but with little change in the percentage LDL receptor-mediated clearance, thus indicating that the response to cholesterol feeding is species specific (Reagan *et al.* 1990). There is little information however, on how diet-induced changes in plasma lipids correspond to changes in antioxidant status and the specific development of atherosclerotic plaque. The purpose of the present study, therefore, was to examine the influence of dietary fat blends and level of cholesterol intake on plasma lipid composition and subsequent changes in aortic plaque deposition, as well as aortic cholesterol and cholesterol oxide levels in atherosclerosis-susceptible Japanese quail. In addition to comparing fat blends which predominate in saturated *v.* polyunsaturated fatty acids, the potential atherogenic character of butter and tallow fats was also examined for the purpose of comparing two saturated fat sources that are different in short- and medium-chain fatty acid content. Furthermore, the effect of dietary fat blends on endogenous antioxidant status was studied to explore the possible relationship between lipid-induced changes in antioxidant status of selected tissues and the severity of aortic plaque deposition in these birds.

MATERIALS AND METHODS

Animals

Seventy-two, 6-week-old male atherosclerosis-susceptible Japanese quail (University of British Columbia Quail Genetic Resource Centre, Vancouver, BC, Canada) were randomly allocated to one of six dietary treatments (*n* 12 in each group) varying in dietary lipid

composition. All animal housing and experimentation procedures were conducted as set forth by guidelines of the Canadian Council of Animal Care.

Formulation of semi-purified diets

The semi-purified diets (Table 1) differed only in composition of dietary fat sources and cholesterol–cholic acid content. All experimental diets contained 30 g rapeseed oil/kg diet (Neptune Food Services, Richmond, BC, Canada) as a component of the fat

Table 1. *Composition of diets fed to atherosclerosis-susceptible Japanese quail*

Diets...	Butter blend LC	Butter blend HC	Beef-tallow blend LC	Beef-tallow blend HC	Soyabean-oil blend LC	Soyabean-oil blend HC
Dietary component (g/kg)						
Soyabean protein meal*	340	340	340	340	340	340
Ca-free mineral mix†	20	20	20	20	20	20
$CaCO_3$‡	50	50	50	50	50	50
Poultry vitamin premix*§	3	3	3	3	3	3
DL-Methionine ‖	4	4	4	4	4	4
Choline chloride*	3	3	3	3	3	3
Maize starch¶	395	395	395	395	395	395
Sucrose¶	25	25	25	25	25	25
Alphacel†	50	50	50	50	50	50
Monofos*,**	30	30	30	30	30	30
Rapeseed oil¶	30	30	30	30	30	30
Butter††	60	60	–	–	–	–
Beef tallow‡‡	–	–	50	50	–	–
Soyabean oil§§	–	–	–	–	50	50
Cholesterol:						
Naturally occurring	0·12	0·12	0·06	0·06	–	–
Added†	0·38	4·88	0·44	4·94	0·5	5·0
Total	0·50	5·0	0·50	5·0	0·5	5·0
Cholic acid†	0·25	2·5	0·25	2·5	0·25	2·5
Gross energy (kJ/g)	15·94	16·44	16·50	16·68	16·56	16·55

LC, low cholesterol; HC, high cholesterol.

* Van Waters & Rogers, Abbotsford, BC, Canada.

† ICN Biochemicals Inc., Cleveland, OH, USA. Mineral mix supplied the following (mg/kg diet): K_2HPO_4 10600, NaH_2PO_4 2100, $MgSO_4.7H_2O$ 1600, NaCl 4600, ferric citrate 900, KI 30, $MnSO_4.H_2O$ 150, $ZnCl_2$ 20, $CuSO_4.5H_2O$ 10.

‡ BDH Chemicals, Toronto, ON, Canada.

§ Vitamin premix supplied the following (mg/kg diet): thiamine hydrochloride 1·0, riboflavin 5·0, niacin 75·0, pantothenic acid 10·0, pyridoxine hydrochloride 3·0, choline 402, pteroylmonoglutamic acid 1·0, D-biotin 0·1, cyanocobalamin 0·012, menadione sodium bisulfite 1·6, D-calcium pantothenate 30, retinyl palmitate 40, cholecalciferol 8 μg, dl-α-tocopherol 120.

‖ United States Biochemical Co., Cleveland, OH, USA.

¶ Neptune Food Services, Richmond, BC, Canada.

** Monofos supplied the following (mg/kg diet): nitrogen 3000, phosphorus 6300, fluorine 72, arsenic 0·6, iron 450, lead 1·1.

†† Dairyworld Foods, Burnaby, BC, Canada. Fatty acid composition of butter-blend diets was (g/100 g total fatty acids): 12:0 2·4, 14:0 6·5, 16:0 22·3, 16:1*n*-7 1·0, 18:0 7·4, 18:1*n*-9 36·4, 18:1 (isomers) 1·8, 18:2*n*-6 13·7, 18:3*n*-3 3·7, 20:1 0·8.

‡‡ Cargill Foods, High River, AB, BC, Canada. Fatty acid composition of beef-tallow-blend diets was (g/100 g total fatty acids): 14:0 2·2, 16:0 18·5, 16:1*n*-7 2·0, 18:0 10·8, 18:1*n*-9 46·7, 18:1 (isomers) 2·4, 18:2*n*-6 13·5, 18:3*n*-3 4·0.

§§ Bioforce Canada, Burnaby, BC, Canada. Fatty acid composition of soyabean-oil blend diets was (g/100 g total fatty acids): 14:0 0·3, 16:0 9·0, 16:1*n*-7 0·2, 18:0 4·3, 18:1*n*-9 47·6, 18:1 (isomers) 3·4, 18:2*n*-6 25·3, 18:3*n*-3 6·7, 20:0 0·4, 20:1 0·8.

blend, and were formulated with mixing of dietary ingredients before the addition of experimental dietary fat sources and sterols. Following thorough mixing of the basal diet ingredients in an industrial-sized stainless steel mixing vat, the powdered diet was pelleted and crumbled in a feed mill (Agriculture Canada Research Station, Agassiz, BC, Canada). No additional antioxidants were added to the diets, with the exception of the vitamin E that was present as a component of the poultry vitamin-premix (see legend of Table 1). The crumbled basal diet was stored in doubled, dark plastic bags at $-15°$ until the experimental dietary fat sources were added to complete the composition of the different experimental fat blends. Dietary fat sources consisted of unsalted butter (Dairyworld Foods, Burnaby, BC, Canada), beef tallow (Cargill Foods, High River, AB, Canada) and soyabean oil (Bioforce Canada, Burnaby, BC, Canada). The water content of the butter (18 g/kg by manufacturer's specifications) was accounted for in the formulation of the butter-blend diets (e.g. 60 g butter/kg diet was incorporated into diets, compared with 50 g/kg diet of beef tallow or soyabean oil in other diets). Dietary fats with sterols at levels reported in Table 1 were slowly added to the crumbled basal diet during reblending and mixed in uniformly using a Hobart mixer with an aluminum bowl. Individual diets were stored frozen ($-15°$) in doubled, dark plastic bags throughout the experimental period. A sample of each experimental diet was taken for analysis of fatty acid, gross energy and DM content.

The experimental fat sources were added to the basal diet at a level of 50 g/kg for a final calculated fat blend content of 80 g dietary fat/kg. This level of fat in the experimental diets was equivalent to the level of dietary lipid in commercial quail feed (Otter Co-op, Aldergrove, BC, Canada). The differences between the fatty acid composition of the butter-blend and beef-tallow-blend diets were minimal, e.g. total saturates (g/100 g total fatty acids) were 38·6 for butter-blend diets and 31·5 for beef-tallow-blend diets; polyunsaturated fatty acids (PUFA; g/100 g total fatty acids) were 17·4 for butter-blend diets and 17·5 for beef-tallow-blend diets; *n*-6 : *n*-3 ratios were 3·7 for butter-blend diets and 3·4 for beef-tallow-blend diets, but both varied considerably in comparison with the soyabean-oil-blend diets, e.g. total saturates (g/100 g total fatty acids) were 14·0, PUFA (g/100 g total fatty acids) were 32·0 and the *n*-6 : *n*-3 ratio was 3·8 (Table 1) for soyabean-oil-blend diets. The mono-unsaturated fatty acid content of the beef-tallow-blend diets (e.g. 51·1 g/100 g total fatty acids) resembled that of the soyabean-oil-blend diets (e.g. 52·0 g/100 g total fatty acids) rather than the butter-blend diets (e.g. 40·0 g/100 g total fatty acids). The polyunsaturated : saturated fatty acid ratio (P : S ratio) values for the butter-blend and beef-tallow-blend diets (e.g. 0·45 and 0·56 respectively) were lower than those for the soyabean-oil-blend diets (e.g. 2·28). The levels of cholesterol incorporated into diets were adjusted to 0·5 g/kg diet for the low-cholesterol (LC) diets (on the basis of energy density, 0·0306 mg/kJ) and 5·0 g/kg diet for the high cholesterol (HC) diets (0·302 mg/kJ). Crystalline cholesterol and cholic acid (ratio 2 : 1) were both incorporated into the lipid sources before addition to the diets. All diets were isonitrogenous and contained a comparable level of energy (i.e. 15·94–17·00 MJ/kg; Table 1).

Diet gross energy determination

The energy content of experimental diets was determined using a bomb calorimeter and was corrected for the dry weight of the diet (Miller & Payne, 1959).

Dietary fatty acid analysis

Samples were extracted with Folch's reagent (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. column coated with 0·25 μ Supelcowax 10 (Supelco; Bellefont, PA, USA). The internal standard included in these fatty acid analyses was 17 : 0 (Supelco). The results of the fatty acid analyses of diets are presented in the legend of Table 1.

Animal feeding

Quail were housed in heated brooder cages with one treatment group per brooder cage. Feed and distilled deionized water were provided *ad libitum* to birds in separate feeding troughs. Feed was replaced daily to minimize lipid oxidation.

Experimental procedures

After 9 weeks on their respective diets, quail were killed at 09.00 hours. Quail were decapitated, trunk blood was collected into chilled, heparinized tubes and plasma was separated by low-speed centrifugation (1000 ***g***, 5 min, 4°). Heart tissues were excised and placed into chilled 50 mM-Tris, 0·1 mM-EDTA, pH 7·6 homogenizing buffer. In addition, the aortic tree (the brachiocephalic arteries to their bifurcations and the aorta to the iliac branching) was dissected out, opened longitudinally and examined under a 10–30× dissecting microscope for lesions on the inner wall. Briefly, aortic lesion scores from 0 to 4 were assigned according to Shih *et al.* (1983) and Godin *et al.* (1994). Scoring was performed in a blinded fashion by two independent investigators as follows: 0 = clean surface; 1 = $\leq$ 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 present. Immediately following completion of plaque scoring, the aortic tissue was placed into chilled 50 mM-Tris, 0·1 mM-EDTA, pH 7·6 homogenizing buffer before homogenization for analysis of antioxidant enzyme activities. Additional birds were added to each treatment group to provide aortic specimens for GC with mass spectrometry (GC–MS) analysis of cholesterol oxides, as well as specimens for scanning electron microscopy of aortic lumen cell morphology once they had been examined microscopically for aortic plaque score as detailed earlier.

Plasma lipid and lipoprotein analyses

Lipoprotein fractions were separated from pooled plasma samples (i.e. two birds/sample) from quail by density gradient ultracentrifugation (Lasser *et al.* 1973; Terpstra *et al.* 1981). Plasma samples from each treatment group were prepared in duplicate, which consisted of an unstained sample for lipoprotein analysis and a stained reference sample prepared for visual identification of the various lipoprotein fractions. Individual lipoprotein fractions in the gradients were visualized in the reference sample by staining with Sudan Black in ethylene glycol. This enabled distinction and confirmation of different lipoprotein fractions at measured densities for normo- and hypercholesterolaemic birds. In hypercholesterolaemic quail, a distinct lipid plug corresponding to the portomicron fraction surfaced (i. e. $\rho_{20} < 1{\cdot}006$) at the top of the centrifuge tube after ultracentrifugation. This fraction was carefully removed and attention given to preventing contamination of other fractions

(Terpstra *et al.* 1981). Remaining quail lipoprotein fractions were separated based upon gradient densities as follows: fraction I: $1{\cdot}006 < \rho_{20} < 1{\cdot}020$; fraction II: $1{\cdot}030 < \rho_{20} < 1{\cdot}046$; fraction III: $1{\cdot}056 < \rho_{20} < 1{\cdot}180$; and fraction IV: $\rho_{20} > 1{\cdot}21$ using an sw40Ti rotor at 272 000 ***g***, 22 h, 20° in a Beckman L2-65 ultracentrifuge (Beckman, Montreal, Quebec, Canada). Careful removal of the different fractions according to the procedure of Terpstra *et al.* (1981) was performed to prevent contamination of individual lipoprotein fractions.

Portions of whole plasma and lipoprotein fractions were analysed for total cholesterol (Siedel *et al.* 1983), triacylglycerol (Ziegenhorn, 1975), phospholipid (Takayama *et al.* 1977) and protein (Bradford, 1976).

Tissue preparation for enzymic analyses

Erythrocytes (RBC) were washed twice with isotonic 0·15 M saline for use in biochemical assays. Haemolysates were prepared by diluting RBC 1 : 10 with double-distilled water and freeze-thawing three times in solid CO_2–acetone to ensure complete cell disruption. Heart tissue was blotted dry, weighed and prepared as a homogenate (10 g/l) in fresh, chilled 50 mM-Tris, 0·1 mM-EDTA, pH 7·6 homogenizing buffer using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY, USA) at 25 % maximum speed, for 30 s (two bursts of 15 s). Tissue cytosolic fractions used in enzymic assays were prepared by ultracentrifugation at 105 000 ***g***, 15 min, 4° using a Beckman L2-65 ultracentrifuge with an sw40Ti rotor. Aortic tissues were blotted dry and any adhering tissue removed before recording aortic tissue weights. Chilled 50 mM-Tris, 0·1 mM-EDTA, pH 7·6 homogenizing buffer (1·0 ml) was added to the aortic tissue in a test-tube and a homogenate was prepared using a micro-probe attachment for a Polytron homogenizer at 50 % maximum speed, for 30 s (two bursts of 15 s). Aortic cytosolic fractions were prepared from the homogenates by centrifugation (12 000 ***g***, 4°, 15 min). Enzyme activity determinations were carried out using a Perkin-Elmer model Lambda 6B spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) with temperature control set for 25°.

Tissue antioxidant analyses

Tissue antioxidant analyses were performed according to methods described by Yuan *et al.* (1996). A brief description of methods used follows.

Tissue GSH (sulfhydryl group) content. Heart and RBC acid-soluble sulfhydryl group contents (taken as a measure of GSH) were determined according to the method of Moron *et al.* (1979) with minor modifications as described by Yuan *et al.* (1996). Briefly, heart homogenates were combined with ice-cold 250 g/l TCA (Sigma, St Louis, MO, USA) and supernatant fractions obtained by centrifugation (12 000 ***g***, 4°, 15 min) were assayed for acid-soluble sulfhydryl groups at 412 nm using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma) in 0·1 M phosphate buffer, pH 8·0. Packed RBC were lysed with cold double distilled water, and treated with cold 50 g/l TCA–1 mM-Na_2-EDTA followed by centrifugation (12 000 ***g***, 4°, 15 min). A portion of the cold supernatant fraction was assayed for acid-soluble sulfhydryl groups as described earlier.

Tissue susceptibility to in vitro *oxidative challenge.* Susceptibility of heart tissue to *in vitro* oxidative challenge was determined by two methods (Yuan *et al.* 1996). Briefly, individual samples of tissue homogenates were incubated with an equal volume of various concentrations of H_2O_2 (prepared in 0·15 M NaCl–2 mM-NaN_3) for 30 min at 37°. The first set of homogenates was used in the determination of acid-soluble sulfhydryl group

depletion, and the second group for measurement of 2-thiobarbituric acid reactive substances (TBARS; an indirect measurement of lipid peroxidation). For determination of heart acid-soluble sulfhydryl group depletion, the incubation was terminated by the addition of cold 250 g/l TCA, followed by centrifugation (12 000 ***g***, 4°, 15 min) to obtain supernatant fractions for determination of acid-soluble sulfhydryl groups as described earlier.

Production of TBARS in heart homogenates following incubation with H_2O_2 was determined according to the method of Buege & Aust (1978) with modifications (Yuan *et al.* 1996). The incubation reactions were terminated by the addition of cold 280 g/l TCA–0·1 M-Na-arsenite, followed by centrifugation (12 000 ***g***, 4°, 15 min). A portion of the supernatant fraction was reacted with 5·0 g/l 2-thiobarbituric acid (TBA; Sigma) in 0·025 M-NaOH and heated in a boiling water bath for 15 min. When the tubes were cooled, the absorbance was read at 532 nm.

Determination of antioxidant enzyme activities

(1) Catalase (CAT) activity. Heart and RBC cytosolic CAT (*EC* 1.11.1.6) activities were determined according to the method of Aebi (1974) which measures absorbance of H_2O_2 at 240 nm. Enzyme activity in heart tissue was expressed as *k*/g tissue wet weight, where *k* is the first-order rate constant. RBC CAT activity was determined using RBC haemolysates prepared as described earlier and expressed as *k*/g haemoglobin (Hb).

(2) Glutathione peroxidase (GSH-Px) activity. Heart GSH-Px (*EC* 1.11.1.9) activity was determined according the method of Paglia & Valentine (1967) and expressed on the basis of nmol NADPH oxidized to NADP per min per mg tissue wet weight using the extinction coefficient of NADPH at 340 nm of $6{\cdot}22 \times 10^{-6}$/M per cm. RBC GSH-Px activity was determined using RBC haemolysate diluted 1 : 10 with double-distilled water. Enzyme activity was expressed as nmol NADPH oxidized to NADP per min per g Hb.

(3) Glutathione reductase (GSSG-Red) activity. Heart GSSG-Red (*EC* 1.6.4.2) activity was determined using the method of Long & Carson (1961) and expressed as nmol of NADPH oxidized to NADP per min per mg tissue wet weight. RBC GSSG-Red activity was determined using RBC haemolysates, with enzyme activity expressed as nmol NADPH oxidized to NADP per g Hb.

(4) Superoxide dismutase (SOD) activity. Heart SOD (*EC* 1.15.1.1) activity was determined according to the method of Winterbourn *et al.* (1975) and expressed as units of SOD per mg tissue wet weight. One unit of SOD activity is defined as the amount of enzyme activity that causes 50 % inhibition of nitroblue tetrazolium (NBT) reduction. The rate of inhibition of NBT reduction by superoxide generated by photoreduction of riboflavin was determined by measuring the absorbance at 560 nm. RBC SOD activity was determined using RBC haemolysate, and enzyme activity expressed as units of SOD enzyme activity per g Hb.

(5) Correction of enzyme activities for blood contamination. Since RBC contain substantial activities of the antioxidant enzymes assayed herein, it was necessary to correct tissue cytosolic enzyme activity values for any contribution due to the presence of contaminating RBCs. For the determination of Hb content, RBC haemolysate and tissue cytosolic fractions were analysed using the method of Drabkin & Austin (1935).

Aortic cholesterol oxides

Aortic cholesterol oxidation products (COP) were analysed by GC, with confirmation by mass spectrometry (GC–MS) on preselected tissues with similar known plaque scores

assessed as described earlier. Aortas were cleansed of adhering tissue, weighed and lipids extracted according to the method of Folch *et al.* (1957). An internal standard (5α-cholestane, 100 μg) was added to samples before lipid extraction. Extracted lipids were evaporated to dryness under a stream of N_2 and subjected to a cold saponification with 1 M-KOH (in methanol) overnight at room temperature (25°). The saponified samples were extracted with diethyl ether (three times), washed with 0·5 M-KOH (once) followed by distilled deionized water (twice). The non-saponifiable fraction was dried (anhydrous Na_2SO_4) before reducing the sample volume with a N_2 stream for transfer to Reacti-Vials (Pierce Chemical Co., Rockford, IL, USA). Samples were placed under vacuum to remove further traces of moisture, before solubilization in dry pyridine. Samples were derivatized with Sylon BTZ (Supelco, Inc., Oakville, ON, Canada) with the reaction allowed to proceed to completion (30 min) at room temperature. Derivatized standards (cholesta-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-5-ene-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 (5α-cholestane) and samples were analysed using a Carlo Erba GC (Carlo Erba Strumentazione, Italy) with a flame ionization detector (GC–FID) equipped with a DB-1 column (15 m × 0·25 mm i.d., 0·1 μ 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 3° per min, with the final temperature held for 15 min. The identity of COP was confirmed using a Kratos MS80 mass spectrometer (Ramsey, NJ, USA) coupled to a Carlo Erba GC as described earlier. Quantification of COP was performed after determining the response linearity of each derivatized sterol.

Scanning electron microscopy

Aortic specimens previously selected based on visual plaque score performed as described earlier, were prepared for scanning electron microscopy according to methods of Peng *et al.* (1985). Briefly, samples were immersed in 30 g/l glutaraldehyde buffer, pH 7·6, followed by immersion in a 5·0 g/l OsO_4 solution, rinsed and dehydrated with ethanol. Samples were then dried by the critical point drying method using CO_2. Finally, specimens were mounted onto stubs and coated with gold before viewing using scanning electron microscope facilities at the Electron Microscopy Laboratory, Department of Zoology, University of British Columbia.

Statistics

All data are expressed as means with their standard errors (SEM). 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 for Windows). Two-way multiple ANOVA (SPSS) was used to identify potential interactions between dietary fat blend and dietary cholesterol level. Linear regression analysis (SPSS) was performed to investigate associations between plasma lipids and aortic plaque variables.

RESULTS

Animal growth and plasma lipid composition

Final body weights of birds were not different between dietary treatment groups (mean 126 (SEM 1) g). The total cholesterol concentration of whole plasma was elevated ($P < 0.001$) in birds fed on the HC diets (Table 2). Birds fed on soyabean-oil-blend diets had reduced ($P = 0.041$) plasma total cholesterol levels compared with butter- and beef-tallow-blend-fed counterparts. Plasma triacylglycerol levels were also elevated ($P < 0.001$) in birds fed on HC diets. However, there was no effect of dietary fat blend on plasma triacylglycerol concentrations (Table 2). Similarly, plasma phospholipid concentrations were also elevated ($P = 0.031$) in HC-fed birds, although there was no effect of dietary fat blend on plasma phospholipid levels. Plasma protein levels were not influenced by either dietary fat blend or cholesterol intake level. Birds fed on a commercial quail diet without cholesterol normally have a plasma cholesterol concentration level in the range of 5–6 mmol/l (Y. V. Yuan and D. D. Kitts, unpublished results).

Plasma samples from birds fed on HC diets exhibited a thick layer of lipid at $\rho_{20} < 1.006$ following ultracentrifugation, which represented the chylo- or portomicron fraction. This fraction was negligible in plasma samples from birds fed on LC diets (Table 3). The cholesterol content of the chylo- or portomicron fraction collected from birds fed on HC diets was reduced ($P = 0.044$) in those fed on the soyabean-oil-blend diet. Birds fed on soyabean-oil-blend HC diets also exhibited lower levels of cholesterol in lipoprotein fractions I ($P = 0.031$), II ($P = 0.031$) and III ($P < 0.001$), but not fraction IV, compared with butter-blend and beef-tallow-blend fed counterparts. No effect of dietary fat blend on lipoprotein fraction cholesterol content was observed in birds fed on LC diets.

There was no effect of dietary fat blend on the triacylglycerol content of the portomicron fraction from HC-fed birds or on lipoprotein fractions I–IV (Table 4).

Table 2. *Plasma lipid and protein concentrations in Japanese quail fed on diets containing different types of fat and either 0·5 or 5·0 g cholesterol/kg*

(Mean values with their standard errors for twelve birds per group)

	Total cholesterol (mmol/l)		Triacylglycerol (mmol/l)		Phospholipid (mmol/l)		Protein (g/l)	
Dietary cholesterol (g/kg diet)...	0·5	5·0	0·5	5·0	0·5	5·0	0·5	5·0
Butter blend								
Mean	6·89	61·2	2·30	5·02	6·58	6·07	31·4	35·5
SEM	0·46	6·0	0·57	0·65	0·39	0·36	1·6	2·5
Beef-tallow blend								
Mean	7·49	64·8	2·17	5·76	6·73	7·70	35·0	32·1
SEM	0·87	5·6	0·31	0·56	0·42	0·42	2·5	6·5
Soyabean-oil blend								
Mean	5·86	51·4	1·90	4·32	6·57	7·52	30·0	34·3
SEM	0·36	4·0	0·31	0·52	0·29	0·35	2·1	3·0
ANOVA *P* value*								
C	< 0·001		< 0·001		0·031		NS	
F	0·041		NS		NS		NS	
C × F	NS		NS		NS		NS	

* C, cholesterol intake level effect; F, dietary fat blend effect; C × F, cholesterol intake and dietary fat blend effect interaction by two-way multiple ANOVA.

Table 3. *Cholesterol content (mmol/l) of lipoprotein fractions from plasma of Japanese quail fed on diets containing different types of fat and either 0·5 or 5·0 g cholesterol/kg*

(Mean values with their standard errors for twelve birds per group)

Fraction*...	Portomicron		Fraction I		Fraction II		Fraction III		Fraction IV	
Dietary cholesterol (g/kg diet)...	0·5	5·0	0·5	5·0	0·5	5·0	0·5	5·0	0·5	5·0
Butter blend										
Mean	ND	37·9[a]	1·68	7·44[a]	0·41	3·17[a]	4·88	9·22[a]	0·16	0·18
SEM		3·6	0·36	0·29	0·04	0·10	0·21	0·52	0·01	0·01
Beef-tallow blend										
Mean	ND	43·2[a]	0·928	6·90[a]	0·47	3·14[a]	5·35	8·08[a]	0·19	0·18
SEM		3·0	0·109	0·75	0·08	0·35	0·16	0·51	0·01	0·01
Soyabean-oil blend										
Mean	ND	34·6[b]	0·881	5·87[b]	0·43	2·31[b]	4·52	5·35[b]	0·18	0·21
SEM		4·1	0·090	0·52	0·02	0·20	0·26	0·15	0·01	0·01

ND, not detected.

[a,b] Mean values within a column with unlike superscript letters were significantly different, $P < 0·05$.

* Fraction number refers to position in density gradient: portomicron $\rho_{20} < 1·006$; fraction I $1·006 < \rho_{20} < 1·020$; fraction II $1·030 < \rho_{20} < 1·046$; fraction III $1·056 < \rho_{20} < 1·180$; fraction IV $\rho_{20} > 1·21$.

Similarly, the portomicron and lipoprotein fraction phospholipid concentrations also were not influenced by dietary fat blend (Table 5).

Erythrocyte and tissue antioxidant status

(1) Catalase activity. CAT activity associated with quail RBC and heart tissue was negligible, as determined by the methods used in the present study.

(2) Superoxide dismutase activity. SOD activity of RBC (mean 5·51 (SEM 0·49) U/mg Hb), heart (mean 1·41 (SEM 0·12) U/mg wet weight) and aortic tissues (0·98 (SEM 0·17) U/mg wet weight) from quail were not influenced by either dietary fat blend or cholesterol intake level.

(3) Glutathione peroxidase activity. Both RBC and heart GSH-Px activities were significantly affected by dietary fat blend, but were not influenced by cholesterol intake level. Birds fed on soyabean-oil-blend diets exhibited greater ($P = 0·004$) RBC GSH-Px activity than counterparts fed on butter-blend or beef-tallow-blend diets (Fig. 1(a)). Heart GSH-Px activity was lower ($P = 0·024$) in birds fed on soyabean-oil-blend diets, both with and without cholesterol, compared with those fed on butter-blend or beef-tallow-blend diets (Fig. 1(b)). Dietary fat blend and cholesterol intake did not significantly influence aortic tissue GSH-Px activities (Fig. 1(c)).

(4) Glutathione reductase activity. GSSG-Red activity of RBC (mean 10·2 (SEM 0·4) nmol NADPH/min per mg Hb), heart (0·444 (SEM 0·02) nmol NADPH/mg tissue wet weight) and aortic (0·342 (SEM 0·09) nmol NADPH/mg tissue wet weight) tissues collected from Japanese quail fed on experimental diets was not significantly influenced by either dietary fat blend or cholesterol intake level.

(5) Tissue GSH content. Levels of acid-soluble sulfhydryl groups (taken as a measure of GSH content) in quail RBC (mean 4·64 (SEM 0·22) nmol GSH/mg RBC) and heart tissue (mean 1·88 (SEM 0·07) nmol GSH/mg tissue wet weight) were not affected by dietary fat blend or level of cholesterol intake.

Table 4. *Triacylglycerol content (mmol/l) of lipoprotein fractions from plasma of Japanese quail fed on diets containing different types of fat and either 0·5 or 5·0 g cholesterol/kg*

(Mean values with their standard errors for twelve birds per group)

Fraction*...	Portomicron		Fraction I		Fraction II		Fraction III		Fraction IV	
Dietary cholesterol (g/kg diet)...	0·5	5·0	0·5	5·0	0·5	5·0	0·5	5·0	0·5	5·0
Butter blend										
Mean	ND	3·35	0·55	0·44	0·11	0·23	1·50	0·78	0·51	0·50
SEM		0·70	0·08	0·03	0·01	0·02	0·25	0·06	0·08	0·07
Beef-tallow blend										
Mean	ND	3·80	0·59	0·51	0·09	0·20	1·18	0·97	0·44	0·47
SEM		0·40	0·09	0·06	0·01	0·04	0·08	0·06	0·03	0·07
Soyabean-oil blend										
Mean	ND	2·78	0·47	0·37	0·12	0·13	1·09	0·76	0·47	0·55
SEM		0·61	0·11	0·03	0·03	0·01	0·07	0·04	0·02	0·06

ND, not detected.

* Fraction number refers to position in density gradient: portomicron $\rho_{20} < 1{\cdot}006$; fraction I $1{\cdot}006 < \rho_{20} < 1{\cdot}020$; fraction II $1{\cdot}030 < \rho_{20} < 1{\cdot}046$; fraction III $1{\cdot}056 < \rho_{20} < 1{\cdot}180$; fraction IV $\rho_{20} > 1{\cdot}21$.

Tissue susceptibility to in vitro *oxidative challenge*

Heart GSH depletion and thiobarbituric acid reactive substances production. The *in vitro* oxidative challenge of heart tissue at a single concentration of H_2O_2, with treatment differences identified, is presented in Table 6. Depletion of GSH from heart tissue was greater ($P = 0{\cdot}049$) in birds fed on HC diets compared with counterparts fed on LC diets. Dietary fat blend did not influence GSH depletion from quail heart tissue. Susceptibility of heart tissue to *in vitro* TBARS production was not affected by dietary fat blend or cholesterol intake level (Table 6).

Table 5. *Phospholipid content (mmol/l) of lipoprotein fractions from plasma of Japanese quail fed on diets containing different types of fat and either 0·5 or 5·0 g cholesterol/kg*

(Mean values with their standard errors for twelve birds per group)

Fraction*...	Portomicron		Fraction I		Fraction II		Fraction III		Fraction IV	
Dietary cholesterol (g/kg diet)...	0·5	5·0	0·5	5·0	0·5	5·0	0·5	5·0	0·5	5·0
Butter blend										
Mean	ND	1·69	0·54	1·06	0·23	0·80	5·91	3·09	0·51	0·33
SEM		0·21	0·12	0·07	0·04	0·04	0·42	0·13	0·06	0·03
Beef-tallow blend										
Mean	ND	1·96	0·35	1·01	0·23	0·83	5·76	3·04	0·57	0·39
SEM		0·42	0·03	0·08	0·04	0·06	0·31	0·22	0·04	0·02
Soyabean-oil blend										
Mean	ND	1·56	0·49	1·07	0·23	0·86	5·78	3·63	0·59	0·45
SEM		0·36	0·02	0·08	0·01	0·02	0·21	0·35	0·04	0·03

ND, not detected.

* Fraction number refers to position in density gradient: portomicron $\rho_{20} < 1{\cdot}006$; fraction I $1{\cdot}006 < \rho_{20} < 1{\cdot}020$; fraction II $1{\cdot}030 < \rho_{20} < 1{\cdot}046$; fraction III $1{\cdot}056 < \rho_{20} < 1{\cdot}180$; fraction IV $\rho_{20} > 1{\cdot}21$.

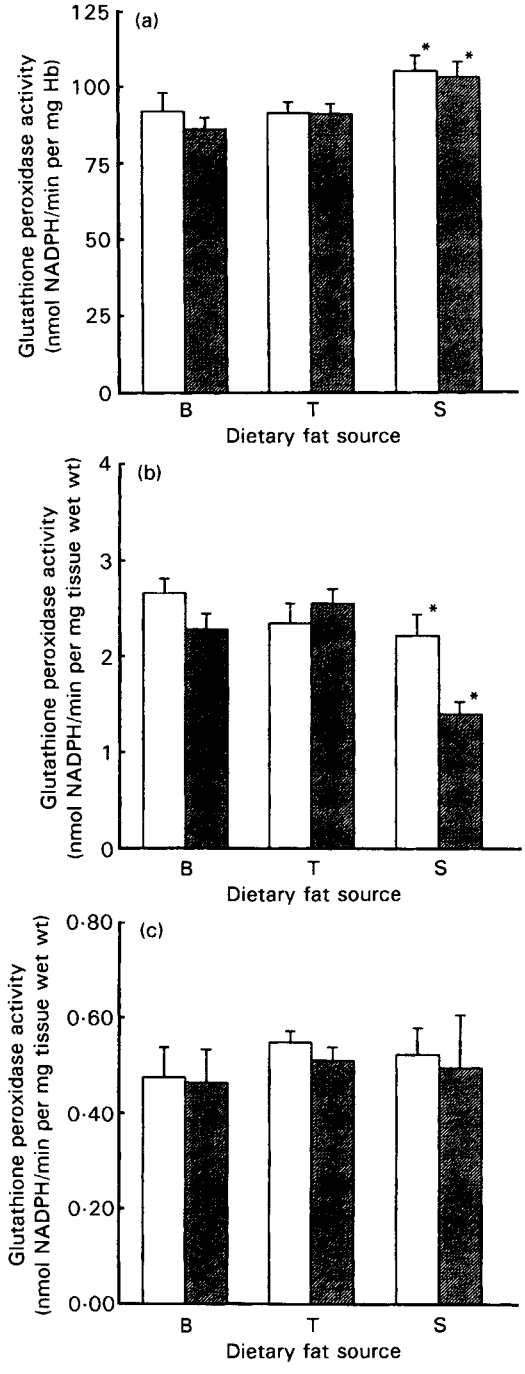


Fig. 1. Glutathione peroxidase activity in (a) erythrocytes, (b) heart, and (c) aortic tissues of atherosclerosis-susceptible Japanese quail fed semi-purified diets containing different fat blends (B, butter; T, beef tallow; S, soyabean-oil) with either 0·5 (□) or 5·0 (▨) g cholesterol/kg diet. * Difference between the two fat blend treatments was statistically significant, $P < 0{\cdot}05$. Hb, haemoglobin.

Aortic tissue atherosclerotic plaque deposition

All birds fed on HC diets exhibited significant ($P < 0{\cdot}001$) atherosclerotic plaque deposition in the aortic tree compared with birds fed on LC diets (Table 7). Dietary fat blend did not influence the aortic plaque scores of experimental quail. Similar to the results obtained with the numerical plaque scores, aortas from birds fed on HC diets also exhibited greater ($P < 0{\cdot}001$) coverage of lumen surface area by aortic plaque than aortas collected from birds fed on the LC diets. Significant positive correlations were recorded between aortic plaque score and plasma total cholesterol (r 0·872, $P = 0{\cdot}001$) as well as plasma triacylglycerol (r 0·667, $P < 0{\cdot}001$) concentrations. Similarly, the percentage aortic area covered by plaque was also positively related to plasma total cholesterol (r 0·870, $P < 0{\cdot}001$) and plasma triacylglycerol (r 0·657, $P < 0{\cdot}001$) levels. Aortic plaque score was also determined to be positively correlated with the cholesterol content of plasma lipoprotein fractions I (r 0·836, $P < 0{\cdot}001$), II (r 0·883, $P < 0{\cdot}001$) and III (r 0·618, $P < 0{\cdot}001$).

Aortic tissue which was free from plaque involvement (e.g. score of zero) had an undulating, intact surface when examined by scanning electron microscopy (Fig. 2(a)). Ovoid protrusions from the lumen surface probably represented nuclei and overlying cytoplasm (Peng *et al.* 1985). Aortas from HC-fed quail which had extensive plaque material visible when viewed by dissecting microscope (e.g. score of 4) exhibited distinct areas of raised tissue with disruption of the epithelial cells when viewed by scanning electron microscopy (Fig. 2(b)).

Aortic plaque cholesterol oxides

The cholesterol and cholesterol oxide contents of the non-saponifiable extracts of aortic tissue samples collected from a smaller number of individual quail for GC–FID analysis are presented in Table 8. Aortic tissue from HC-fed birds consistently exhibited a greater amount of native, unoxidized cholesterol than aortic tissue from birds fed on LC diets. This was particularly true in aortic tissues chosen from birds fed on the soyabean-oil-blend diets. The presence of cholesterol oxides was detected only in the non-saponifiable extracts of aortic tissue from birds fed on HC diets. Considerable individual variability in the profile of cholesterol oxides detected in aortic tissue was observed between birds fed on the different dietary fat blends, as well as within specific dietary fat blend groups with the same aortic plaque score (Table 8). Three cholesterol oxides: 5,6α-epoxy-5α-cholesterol, 7β-hydroxycholesterol and 7-ketocholesterol were present in aortic tissue from hypercholesterolaemic birds fed on butter-blend, beef-tallow-blend or soyabean-oil-blend diets. Additional cholesterol oxides present in the aortas from hypercholesterolaemic birds fed on the soyabean-oil-blend diet were: 7α-hydroxycholesterol, 4β-hydroxycholesterol, cholestanetriol, and 25-hydroxycholesterol (Table 8). The detection of these four additional oxysterols was associated with the relatively higher aortic cholesterol content in these particular birds.

DISCUSSION

In the present study, cholesterol-supplemented diets were designed to alter lipoprotein metabolism and induce hypercholesterolaemia for the purpose of evaluating the atherogenicity of different dietary fat blends. Alteration in target tissue antioxidant enzymes and susceptibility to lipid oxidation were also examined, in view of the

Table 6. *Heart homogenate susceptibility to hydrogen peroxide-induced GSH depletion and production of 2-thiobarbituric acid reactive substances (TBARS) in atherosclerosis-susceptible Japanese quail fed on diets containing different types of fat and either 0·5 or 5·0 g cholesterol/kg*

(Mean values with their standard errors for twelve birds per group)

Dietary cholesterol level (g/kg diet)...	GSH depletion (%) 0·6 mM-H_2O_2				TBARS (A_{532}) 1·0 mM-H_2O_2			
	0·5		5·0		0·5		5·0	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Dietary treatment								
Butter blend	24·8	3·7	29·3	4·9	0·124	0·013	0·156	0·021
Beef-tallow blend	28·2	5·4	30·4	4·5	0·142	0·029	0·121	0·010
Soyabean-oil blend	21·6	3·5	38·8	7·7	0·107	0·014	0·142	0·020
ANOVA *P* value*								
C	0·049				NS			
F	NS				NS			
C × F	NS				NS			

* C, cholesterol intake level effect; F, dietary fat blend effect; C × F, cholesterol intake and dietary fat blend interaction by two-way multiple ANOVA.

hypothesized role of lipid peroxidation in the development of atherosclerosis (Jayakumari *et al.* 1992; Buczynski *et al.* 1993; Bonithon-Kopp *et al.* 1997).

Table 7. *Aortic plaque score and area covered by plaque in atherosclerosis-susceptible Japanese quail fed on diets containing different types of fat and either 0·5 or 5·0 g cholesterol/kg*

(Mean values with their standard errors for twelve birds per group)

Dietary cholesterol level (g/kg diet)...	Plaque score*			Area covered (%)†		
	0·5	5·0		0·5	5·0	
		Mean	SEM		Mean	SEM
Dietary treatment:						
Butter blend	ND	3·4	0·4	ND	80	10
Beef-tallow blend	ND	3·8	0·1	ND	88	4
Soyabean-oil blend	ND	3·5	0·2	ND	79	7
ANOVA *P* value‡						
C		< 0·001			< 0·001	
F		NS			NS	
C × F		NS			NS	

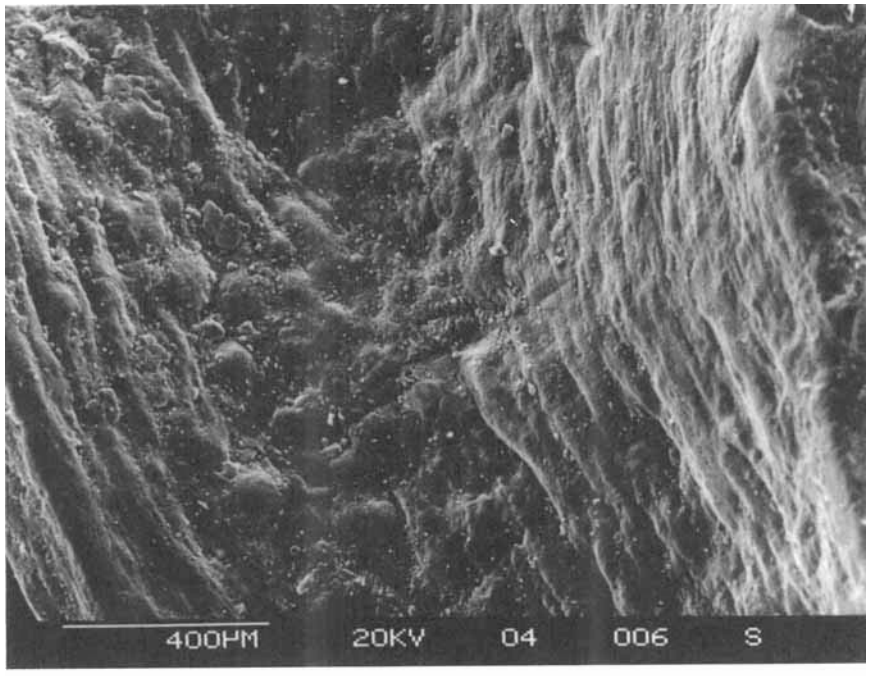
ND, not detected.

* Plaque score based on scale of 0 (ND), clean surface; 1, ≤ 5 plaques; 2, 6–20 plaques; 3, > 20 plaques; 4, massive atheromas seen. Values represent mean values made by two judges evaluating in a double-blind protocol.

† Percentage of aortic epithelium covered by plaque.

† C, cholesterol intake level effect; F, dietary fat blend effect; C × F, cholesterol intake and dietary fat blend interaction by two-way multiple ANOVA.

(a)



(b)

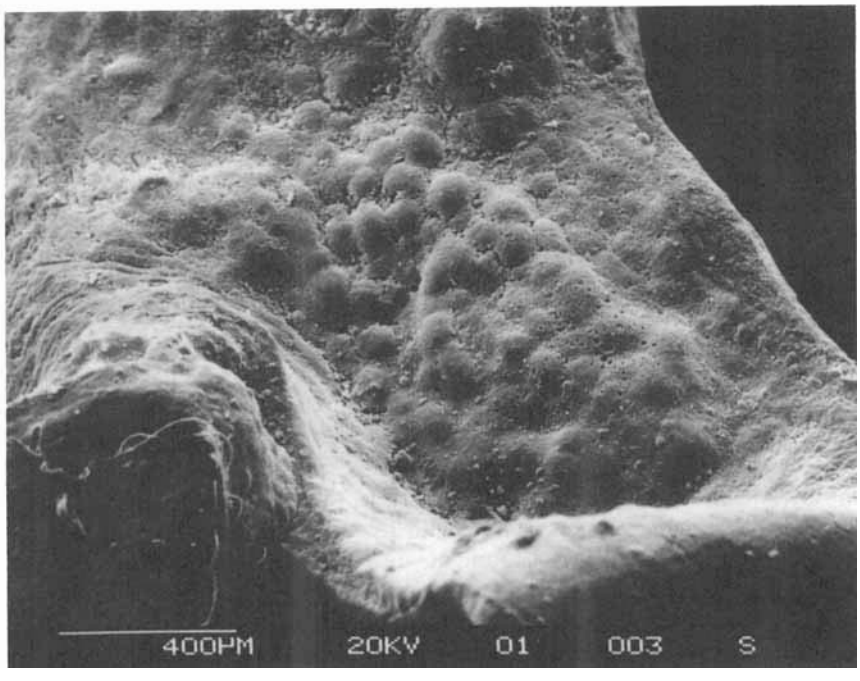


Fig. 2. (a) A representative scanning electron micrograph of aortic tissue from atherosclerosis-susceptible Japanese quail fed on a low-cholesterol diet (0·5 g cholesterol/kg). This micrograph depicts the intact, luminal surface of aortic tissue with a plaque score of zero, which was determined using a visual scoring scale. (b) A corresponding micrograph from a Japanese quail fed on a high cholesterol diet (5·0 g cholesterol/kg). This micrograph depicts the atherosclerotic luminal surface of aortic tissue with a plaque score of 4. The coverage of the aortic lumen by plaque is characterized by a focal area of raised tissue.

Table 8. *GC quantification of cholesterol and cholesterol oxide content of aortic tissue (mg/g tissue) from atherosclerosis-susceptible Japanese quail fed on diets containing different types of fat and either 0·5 or 5·0 g cholesterol/kg*

(Values are for individual birds)

Dietary treatment	Plaque score*	Cholesterol and oxides							
		Cholesterol	7α-OH	5,6α-epoxide	7β-OH	4β-OH	triol	7-keto	25-OH
Butter blend									
0·5 g chol./kg diet	0/0/0	1·61	ND	ND	ND	ND	ND	ND	ND
	0/0/0	2·38	ND	ND	ND	ND	ND	ND	ND
5·0 g chol./kg diet	4/4/4	16·52	ND	0·51	0·41	ND	ND	0·57	ND
	3/4/4	19·17	ND	0·53	0·65	ND	ND	0·38	ND
Beef-tallow blend									
0·5 g chol./kg diet	0/0/0	2·68	ND	ND	ND	ND	ND	ND	ND
	0/0/0	2·38	ND	ND	ND	ND	ND	ND	ND
5·0 g chol./kg diet	4/4/4	19·67	ND	ND	0·44	ND	ND	0·53	ND
	4/4/4	19·03	ND	0·26	0·36	ND	ND	0·37	ND
Soyabean-oil blend									
0·5 g chol./kg diet	0/0/0	2·20	ND	ND	ND	ND	ND	ND	ND
	0/0/0	2·71	ND	ND	ND	ND	ND	ND	ND
5·0 g chol./kg diet	4/4/4	28·30	ND	0·16	0·61	0·16	ND	0·42	ND
	4/4/4	24·82	0·12	1·06	0·60	0·05	0·25	0·80	0·54
	4/4/4	23·99	0·18	0·14	0·63	0·08	0·07	0·36	0·74

Chol., cholesterol; ND, not detected; 7α-OH, 7α-hydroxycholesterol; 5,6α-epoxide, 5,6α-epoxy-5α-cholesterol; 7β-OH, 7β-hydroxycholesterol; 4β-OH, 4β-hydroxycholesterol; triol, cholestane-triol; 7-keto, 7-ketocholesterol; 25-OH, 25-hydroxycholesterol.

* Plaque score is based on scale of 0 (ND), clean surface; 1, ≤ 5 plaques; 2, 6–20 plaques; 3, > 20 plaques; 4, massive atheromas seen. Values represent two judges evaluating in a blinded protocol. x/y/z, individual score for each of three vessels in aortic tree (aorta and brachiocephalic arteries).

Validation of quail as a model for human atherosclerosis

The hypercholesterolaemia in quail observed in the present study was due to feeding cholesterol, as an approximately ten-fold elevation in plasma total cholesterol was observed in birds fed on diets containing 5 g cholesterol/kg, regardless of the source of dietary fat blend. Previous studies have induced atherosclerosis in quail by feeding higher levels of cholesterol (e.g. 10 or 20 g/kg diet) with 5 g cholic acid/kg (Day *et al.* 1977; McCormick *et al.* 1982). Similar extreme hypercholesterolaemia has been observed in atherosclerosis-susceptible pigeons fed on cholesterol-supplemented diets (Lofland *et al.* 1961; Reagan *et al.* 1990). In addition to marked elevations in plasma total cholesterol level, quail fed on HC diets also exhibited elevated plasma triacylglycerol levels. This hyperlipoproteinaemia could be traced primarily to the absorptive portomicron fraction. The association of hypercholesterolaemia with hypertriacylglycerolaemia in this experimental model of atherosclerosis is significant in light of the proposed role of elevated plasma triacylglycerol levels as a risk factor for CHD (Carlson & Böttiger, 1972; Castelli, 1986). Our present study also confirms the importance of the relative distribution of cholesterol within plasma lipoprotein fractions as an important predictor of susceptibility to CHD (Grundy & Vega, 1988). Other studies using a different animal model (e.g. apolipoprotein E-null mouse) have shown a positive correlation between atherosclerotic lesion area and the VLDL + LDL : HDL cholesterol ratio (Nishina *et al.* 1993). Although it can be argued that avian species possess a contrasting mode of lipid transport (e.g. portal *v.* lymphatic system in mammals), and unlike man are a predominate HDL animal species,

notable similarities to man with respect to lipid lipoprotein metabolism have been reported (Chapman, 1980).

Effect of dietary fat blends on plasma lipid profiles

Previous studies have shown that short- and medium-chain fatty acids with chain lengths ranging from six to ten C atoms are more thermogenic than long-chain fatty acids due to preferential oxidation for energy (Leyton *et al.* 1987). Since butter contains these particular fatty acids in a relatively greater proportion than other fats and oils, the fact that previous clinical studies have reported elevated total serum cholesterol in subjects fed on diets containing 50 % energy from butterfat (Wood *et al.* 1993), suggests that other fatty acids in butter are important in potentially elevating plasma cholesterol. Hegsted *et al.* (1965) reported a markedly greater hypercholesterolaemic effect with the fatty acid 14 : 0, while Keys *et al.* (1957) reported an equivalent hypercholesterolaemic effect of 12 : 0 compared with 16 : 0. In the present study, the relative lack of an effect of butter-blend diets on plasma total cholesterol, or the cholesterol content of individual lipoprotein fractions in normocholesterolaemic birds can be partially attributed to the relatively small contribution of 12 : 0 and 14 : 0 to the total energy (e.g. < 2 %) of these diets. Other studies conducted with normocholesterolaemic human subjects fed on diets supplemented with 12 : 0 and 14 : 0 at a higher proportion of the total energy content have reported greater elevations in plasma cholesterol compared with individuals fed on diets supplemented with 16 : 0 (Sundram *et al.* 1994) or 18 : 1*n*-9 (Grundy & Vega, 1988). In our present study with normocholesterolaemic birds fed on either the butter-blend or beef-tallow-blend diets, dietary 16 : 0 content did not influence plasma lipids relative to soyabean-oil-blend-fed counterparts. It is generally believed that 16 : 0 can contribute to elevated total and LDL-cholesterol relative to 18 : 0 (Hegsted *et al.* 1965; Grundy & Vega, 1988). Our results obtained with normocholesterolaemic quail are similar to those of previous studies, which showed that a lack of a distinctive effect of different dietary fat blends on plasma cholesterol was due to no change in LDL-receptor activity in both normocholesterolaemic rodents and monkeys (Woollett *et al.* 1992; Khosla & Hayes, 1993; Sundram *et al.* 1994). Dietary fat source has also been shown to have no effect on plasma cholesterol levels in atherosclerosis-susceptible pigeons in the absence of dietary cholesterol (Lofland *et al.* 1961). Furthermore, human subjects consuming self-selected diets containing butter or margarine have been reported to exhibit no specific effect of consuming a butter diet on plasma cholesterol levels (Flynn *et al.* 1991). Other studies conducted with individuals with normal lipoprotein profiles have also reported no effect of 16 : 0 on either total or LDL-cholesterol when consumed in the absence or presence of very-low-cholesterol diets (Sundram *et al.* 1995).

Effect of dietary cholesterol on plasma lipid profiles

The greater degree of hypercholesterolaemia observed in birds fed on butter-blend or beef-tallow-blend HC diets reflects the hypercholesterolaemic potential of 16 : 0 in these quail. Furthermore, the 18 : 2*n*-6/14 : 0 ratio of butter-blend and beef-tallow-blend diets (e.g. range 2·1–6·1) *v.* that of soyabean-oil-blend diets (e.g. 84·3) should be noted as an indicator of the greater hypercholesterolaemic potential of the butter-blend and beef tallow diets in the presence of high amounts of cholesterol (Hayes *et al.* 1995). The greater hypercholesterolaemic potential of saturated fatty acid sources used in fat blends fed to these birds, but not in normocholesterolaemic quail, suggests that diet-induced alterations

involving LDL-receptor activity, reported by others with the feeding of saturated fat with cholesterol (Woollett *et al.* 1992; Khosla and Hayes, 1993) may have occurred. It is noteworthy however, that in other avian species such as the pigeon, cholesterol feeding has been found to have little effect on LDL-receptor activity, although hepatic sterol synthesis was reduced markedly (Reagan *et al.* 1990). Thus, the combined effects of a primarily saturated fat diet and high cholesterol intake in amplifying the degree of hypercholesterolaemia in the quail model may not be explained by diet-induced changes in avian LDL-receptor activity.

Evaluating differences in quail lipoprotein cholesterol content due to dietary fatty acid composition was somewhat obscured by the presence of the lipid-rich portomicron fraction in plasma collected from quail fed on the HC diets. Regardless of this characteristic of avian dietary lipid absorption, the relative hypercholesterolaemic effects of dietary 16:0 content and 18:2*n*-6/14:0 ratio previously observed in animal (Woollett *et al.* 1992; Pronczuk *et al.* 1994) and clinical studies (Hayes *et al.* 1995; Sundram *et al.* 1995) was extended to quail fed on butter-, beef-tallow- and soyabean-oil-blend diets in the present study. Other workers have attributed the relative reduction in plasma cholesterol associated with PUFA diets to the ability of PUFA to increase catabolism of LDL- and HDL-cholesterol (Fernandez & McNamara, 1991; Stucchi *et al.* 1991). The relatively greater increase in cholesterol content of the portomicron fraction and lipoprotein fraction I (analogous to VLDL) in birds fed on butter-blend and beef-tallow-blend HC diets may be similar to the prominent effect of 16:0 to increase VLDL secretion previously observed in rodents (Khosla & Hayes, 1991).

Aortic plaque deposition in quail

The severity of diet-induced aortic plaque development in Japanese quail has been reported to vary with dietary cholesterol level as well as duration of feeding (Shih *et al.* 1983; Godin *et al.* 1994). The fact that comparable atherogenic effects were obtained with HC diets containing butter, beef-tallow or soyabean-oil blends, indicates that the subtle differences in plasma cholesterol induced by the different dietary fat blends were not sufficient to influence the degree of aortic plaque formation in this study. An explanation for this conclusion can be drawn from the *in vitro* findings in atherosclerosis-susceptible pigeons which have shown a failure of aortic cells to internalize LDL via the LDL-receptor pathway (Randolph & St Clair, 1984). Unlike mammalian cells, the absence of a functional LDL-receptor pathway in smooth muscle cells from the atherosclerosis-susceptible pigeon does not allow these cells to maintain cholesterol homeostasis by regulating the uptake of LDL-cholesterol (Randolph *et al.* 1984). Thus, it can be speculated that the severe hyperlipidaemia observed in quail in our study may have masked more subtle effects associated with the differences in dietary fatty acid composition.

The role of oxidized cholesterol species and oxidatively modified lipids in the initiation and progression of atherosclerosis has been substantiated by reports of the presence of oxidized LDL in the plasma and diseased aortas in animal models (Rosenfeld *et al.* 1990) as well as in diseased human aortic tissue (Stringer *et al.* 1989). Our results indicate that atherosclerotic aortas from birds fed on butter- or beef-tallow-blend diets contained cholesterol-5,6α-epoxide, 7β-hydroxycholesterol and 7-ketocholesterol, while those fed on soyabean-oil-blend diets contained in addition, 7α-hydroxycholesterol, 4β-hydroxycholesterol, cholestanetriol, and 25-hydroxycholesterol. The atherogenic potential of cholesterol oxides has been demonstrated with *in vitro* cell culture studies (Peng *et al.* 1978; Hennig & Boissonneault, 1987; Caboni *et al.* 1994), intravenous administration to

experimental animals (Peng *et al.* 1985) and animal feeding studies (Donaldson, 1982). Previous studies in which cholesterol oxides were examined in human plasma and aortic tissue have also identified cholest-3,5-diene-7-one, cholestanetriol, 7-hydroxycholesterol, cholesterol-5,6α- and β-epoxides, 7-ketocholesterol, 24-hydroxy, 25-hydroxy- and 26-hydroxycholesterol (Morin & Peng, 1992; Dzeletovic *et al.* 1995). In our study, not only were many of these same cholesterol oxides present in aortic tissue of atherosclerotic birds but also, substantial individual variability occurred in the profile of oxides from birds within the same dietary treatment group. Despite the fact that plasma cholesterol concentrations were relatively lower in soyabean-oil-blend-fed hypercholesterolaemic birds compared with counterparts fed on butter- or beef-tallow-blend diets, the absolute amount of cholesterol and the percentage of cholesterol oxides in aortic tissue were similar between dietary fat groups. The observation that only aortic specimens from birds fed on the soyabean-oil-blend HC diet exhibited detectable amounts of the potentially cytotoxic 25-hydroxycholesterol and atherogenic cholestanetriol (Peng *et al.* 1985) may be noteworthy, suggesting a relative difference in tissue sterol oxidative metabolism between birds fed on dietary fat blends predominantly containing PUFA *v.* saturated fatty acids.

Antioxidant status of quail

In the present study, we also report that a high level of dietary cholesterol had a minimal effect on the antioxidant enzyme activities of quail RBC, heart and aortic tissues. In other studies in which quail were fed on a cholesterol-supplemented commercial diet, plasma GSH-Px and SOD activities were positively related to plasma total cholesterol and triacylglycerol levels, while aortic SOD activity was negatively correlated with plasma total cholesterol and aortic plaque score (Godin *et al.* 1994). However, other antioxidant enzymes of aortas (i.e. GSH-Px and GSSG-Red) and RBC (i.e. GSH-Px, GSSG-Red and SOD) were not affected by cholesterol feeding in these same animals. Taken together, these findings in the hyperlipidaemic quail suggest that compensatory changes in antioxidant enzymes were limited in determining the severity of aortic plaque when initiated by feeding cholesterol-containing diets. One possible exception to this was the indirect manner in which hyperlipoproteinaemia may have affected the antioxidant status of tissues by enhancing tissue sensitivity to oxidative stress. For example, the observation that the *in vitro* GSH depletion from heart tissue was increased in quail fed on HC diets, suggests a potential detrimental effect of hypercholesterolaemia on the resistance of the myocardium to oxidative stress.

The present study is the first to investigate the effect of dietary fat blends on the antioxidant status of specific tissues in the Japanese quail. Several studies have demonstrated an increased requirement for antioxidants when diets high in PUFA are consumed (L'Abbé *et al.* 1991; Skúladóttir *et al.* 1994). It is of interest, therefore, that while GSH-Px activity was increased in RBC of soyabean-oil-blend-fed quail, activity of this enzyme in heart tissue was reduced in these same birds. Other workers have reported reduced activities of SOD and GSH-Px in heart tissue from rodents fed on high fat, marine oil, *n*-3 PUFA diets (L'Abbé *et al.* 1991). The mechanism(s) for the association between altered tissue antioxidant enzyme activity and dietary PUFA is probably complex and remains to be elucidated. Our results indicate that these relationships are probably tissue-specific and dependent on the specific target tissue examined.

CONCLUSION

In summary, diets containing 5 g cholesterol/kg combined with a moderate, nutritionally adequate level of dietary fat derived from blends containing butter, beef tallow or soyabean oil as the dominant lipid source resulted in severe hyperlipoproteinaemia and associated atherosclerosis in Japanese quail. Counterparts fed on similar diets, but with a very much lower cholesterol content, did not develop atherosclerosis or hyperlipoproteinaemia. The extreme hypercholesterolaemia characteristic of quail fed on atherogenic diets was associated with altered lipoprotein lipid composition and aortic sterol–oxysterol content. Tissue antioxidant enzymes were minimally affected by dietary treatment and did not appear to be a major factor in determining the severity of aortic plaque deposition.

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