

A green tea extract lowers plasma cholesterol by inhibiting cholesterol synthesis and upregulating the LDL receptor in the cholesterol-fed rabbit

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Abstract

Green tea extracts enriched in catechins decrease plasma cholesterol in hamsters, mice and rats. The aims of this study were to determine whether a catechin-enriched extract of green tea could lower plasma cholesterol in the cholesterol-fed rabbit and to determine the mechanism of action. Four groups of six New Zealand White rabbits were initially made hypercholesterolaemic by feeding a 0.25% (w/w) cholesterol diet for 2 weeks before the diet was supplemented with a catechin extract from green tea at 0, 0.5, 1 or 2% (w/w) for 4 weeks. Administration of the crude catechin extract from green tea significantly ($p < 0.05$) lowered cholesterol in plasma (–60%), VLDL + IDL (–70%), LDL (–80%), liver (total by –25% and unesterified by –15%) and aorta (–25%) compared to control. There was a significant reduction in the cholesterol synthesis index (–60%) and a significant increase in hepatic LDL receptor activity (+80%) and protein (+70%) but there was no change in the intrinsic capacity to absorb cholesterol from the intestines. These results suggest that green tea catechins lowered plasma, liver and aortic cholesterol in the cholesterol-fed rabbit by lowering cholesterol synthesis and upregulating the hepatic LDL receptor.

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1. Introduction

Green tea is a widely consumed beverage brewed from the plant species ‘*Camellia sinensis* (L.) O. Kuntze’. It contains an abundance of naturally occurring polyphenols called catechins of which epigallocatechin gallate (EGCG) is the most prevalent. Epidemiological studies [1–3] have found that drinking between 5 and 10 cups of green tea per day is associated with lower plasma cholesterol concentrations. Intervention studies in rats, mice and hamsters have also found that green tea or green tea extracts enriched in catechins exhibit hypocholesterolaemic effects [4–8]. In contrast, Tijburg et al. [9] found that a green tea extract, included in

the drinking water, did not significantly decrease cholesterol concentrations in the cholesterol-fed hypercholesterolaemic rabbit.

Inhibition of cholesterol absorption has been proposed as a mechanism to explain the cholesterol lowering effects of green tea. This is because the faecal excretion of total lipids and cholesterol were found to be higher in animals consuming green tea extracts [4,5,8]. The EGCG has also been observed to inhibit the uptake of ¹⁴C-cholesterol from the intestine [10]. This apparent reduction in intestinal cholesterol absorption has been ascribed to EGCG reducing the solubility of cholesterol into mixed bile salt micelles [11]. It has also been found that hamsters and rats fed green tea extracts had increased faecal excretion of bile acids [7,8].

This apparent decrease in cholesterol absorption and bile acid reabsorption by green tea should lead to a reduction

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in liver cholesterol concentrations. In order to compensate for this it would be expected that LDL receptor activity and cholesterol synthesis in the liver would increase [12]. These effects have been noted in studies using inhibitors of cholesterol absorption such as tiqueside [13] and inhibitors of intestinal reabsorption of bile acids such as cholestyramine [14]. The increase in the LDL receptor can mediate the lowering of plasma cholesterol by enhancing the uptake of LDL cholesterol from the circulation. However, the cholesterol lowering potential of these agents can be offset by an increase in cholesterol synthesis [12].

Studies *in vitro* [15–17] have provided evidence that green tea extracts and its catechin constituents can upregulate the LDL receptor and modulate cholesterol metabolism in HepG2 cells. Indirect evidence has also been found *in vivo*. When rats were fed EGCG, the removal of intravenously injected ^{14}C -cholesterol from the plasma was enhanced [10]. This increase in the plasma clearance of cholesterol may have been due to upregulation of the LDL receptor as it is the main mechanism by which the sterol is removed from the circulation [12]. There is, however, no study to date that has investigated the effects of green tea or green tea extracts on the LDL receptor *in vivo*.

Little is known about the effects of green tea on cholesterol synthesis. Studies have found no effect of green tea on the “*in vitro*” activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase [7,8]. The HMG-CoA reductase enzyme catalyses the rate-limiting step in cholesterol biosynthesis, but measurement of its activity “*in vitro*” may not always reflect the level of cholesterol synthesis. For example, treatment with inhibitors of cholesterol synthesis (e.g. the statins) has been found to elevate, not decrease, “*in vitro*” HMG-CoA reductase activity [18,19]. More direct measures of cholesterol synthesis such as the incorporation of tritium in cholesterol using tritiated water [20] and the plasma ratio of lathosterol to cholesterol have confirmed that whole body cholesterol synthesis is, in fact, lowered with statin treatment. This is despite often marked increases in “*in vitro*” HMG-CoA reductase activity [19].

The aims of this study were to determine if a crude catechin extract from green tea could lower plasma cholesterol concentrations in the cholesterol-fed hypercholesterolaemic rabbit and to determine if a green tea extract could upregulate the LDL receptor and increase cholesterol synthesis.

2. Materials and methods

2.1. Catechin extract

The crude catechin extract was prepared from commercially available “Special Gunpowder” green tea, packaged by the China National Native Products and Animal By-products Import and Export Corporation, Zhejiang Tea Branch, China. The method used was based on the method of Huang et al.

[21]. Briefly, 15 kg of green tea was extracted with 3 volumes (v/w) of methanol at 50 °C for 3 h. Solvent was removed from the extract using a reduced pressure rotary evaporator. The residue was dissolved in 2 volumes of water (v/w) at 50 °C and extracted twice with equal volumes of hexane (v/v) and once with an equal volume of chloroform (v/v). The remaining aqueous phase was then extracted once with an equal volume of ethyl acetate (v/v) which extracts the polyphenolic compounds including the catechins. The ethyl acetate was then evaporated, the residue redissolved in the minimum amount of warm water (50 °C) and freeze dried. The extract contained at least 58% (w/w) catechins and the composition of the measured constituents were: 30% EGCG, 21% ECG, 10% caffeine, 6% moisture, 4% EGC, 2% GCG and 0.5% theanine.

2.2. Animal study

Twenty-four (4 month old) male New Zealand White rabbits (IMVS, Gillies Plains, SA, Australia) were housed in individual cages at the CSIRO Health Sciences and Nutrition animal facility (Kintore Avenue, Adelaide, SA, Australia). Ethics approval for the study was obtained from the University of Adelaide and CSIRO Health Sciences and Nutrition Animal Ethics Committees. The rabbits were housed individually in surroundings of controlled temperature (20 ± 1 °C) and a 12 h light cycle (06:00 h to 18:00 h).

All rabbits were initially fed a diet containing 0.25% (w/w) cholesterol that was mixed with their basic rabbit chow (IMVS, Gillies Plains, SA). This diet was fed to the rabbits for a period of 2 weeks to increase their plasma cholesterol concentrations prior to the administration of the crude catechin extract.

The rabbits were then randomised into four different treatment groups and the crude catechin extract was fed at concentrations of 0, 0.5, 1 or 2% (w/w). The extract was mixed in with their normal rabbit chow along with 0.25% (w/w) cholesterol and fed to the rabbits for a period of 28 days. Daily consumption of the diets was determined. Rabbits were fasted overnight and blood samples for lipid analysis were taken from the ear artery prior to and after the two-week cholesterol-only feeding period. Following the 4 weeks of dietary intervention with the crude catechin extract, the rabbits were fasted overnight and the following morning were injected intramuscularly (i.m.) with 1.2 ml (2.5 mg) of Acepromazine. Once sedated, rabbits were injected i.m. with a muscle relaxant (0.75 ml Rompun, 15 mg) and a general anaesthetic (1.5 ml Ketamine, 150 mg). Under deep anaesthesia the rabbits were bled by cardiac puncture until euthanasia. Blood was collected into EDTA tubes (1 mM) and plasma was isolated by centrifugation at $3000 \times g$ for 10 min at 4 °C. The entire aorta, from the ascending arch to the ileac bifurcation, was carefully removed and divided into three segments: the aortic arch, the descending aorta and the abdominal aorta. The aortic arch and the abdominal aorta were fixed and stained for atheroma assessment then quantified using

TM/TC Image Analysis Systems (Digithurst, Herts, England) and MicroScale software. The remaining descending thoracic aorta was frozen in liquid nitrogen and stored at -80° for determination of artery cholesterol. Rabbit livers were also removed, frozen in liquid nitrogen and stored at -80° C.

2.3. Plasma lipids

Plasma cholesterol and triglyceride concentrations were measured on a Cobas Bio automated centrifugal analyser (F. Hoffmann-LaRoche, Basel, Switzerland) using enzymatic test kits (Roche Diagnostica, Basel, Switzerland). Lipoprotein fractions containing VLDL + IDL ($1.006 < d < 1.019$), LDL ($1.019 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml) were isolated by sequential ultracentrifugation from 1 ml of fasting rabbit plasma obtained after treatment with the green tea extract. The cholesterol, triglyceride and protein content of the different lipoprotein fractions were then determined using the Cobas Bio.

2.4. Cholesterol synthesis and intrinsic capacity to absorb dietary cholesterol

Plasma lathosterol and phytosterols (campesterol and β -sitosterol) were measured by gas chromatography (GC) as described by Wolthers et al. [22]. The ratios of serum lathosterol and phytosterol concentrations in the plasma to plasma cholesterol concentration have been found to correlate with whole body cholesterol synthesis [23] and the intrinsic capacity to absorb dietary cholesterol [24] respectively.

2.5. Hepatic LDL receptor binding assay

To prepare LDL-gold conjugates, normolipidemic human blood (Australian Red Cross, Adelaide, Australia) was used to isolate LDL ($1.025 < d < 1.050$) by sequential ultracentrifugation. Colloidal gold was prepared and the isolated LDL was then conjugated to the colloidal gold as previously described [25]. A 2–3 g piece of liver was homogenised and microsomal membranes ($800\text{--}100,000 \times g$ centrifuge fraction) were prepared and solubilised with 1% (w/v) Triton X-100, 5 mM phenylmethylsulfonyl fluoride and 5 mM *N*-ethylmaleimide, to prevent degradation and dimerisation of the rabbit LDL receptor protein. Once solubilised, Triton X-100 was removed using Amberlite XAD-2 [26] and the protein content of the microsomal membranes was determined.

To measure LDL receptor binding activity, 8 μ g of the solubilised liver membranes were applied to nitrocellulose paper (Schleicher and Schuell, Westborough, MA) which was then blocked with 4% (w/v) bovine serum albumin (BSA) solution [25,27]. The nitrocellulose membranes were then incubated in buffer containing either 20 μ g/ml LDL-gold in the absence and presence of 20 mM EDTA to determine total and non-specific binding, respectively. The nitrocellulose paper was soaked in water for 30 min and then incubated with IntenSE

BL silver enhancement kit (Amersham, UK) for a further 30 min. This was washed with water, dried and scanned using an LKB Ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, North Ryde, NSW, Australia). The specific binding (total minus the non-specific binding) was taken to be the LDL receptor binding activity which is expressed as peak height, determined from the laser densitometer scan.

2.6. Quantification of LDL receptor protein

To determine relative amounts of LDL receptor protein, solubilised rabbit liver membranes (100 μ g) were subjected to electrophoresis on 3–15% SDS polyacrylamide gradient gels and electrotransferred onto nitrocellulose paper. The membranes were then overlaid with a polyclonal antibody against the LDL receptor followed by an anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO USA). The LDL receptor band was then detected on X-ray film (Hyperfilm-ECL, Amersham, North Ryde, NSW, Australia) using enhanced chemiluminescence (Amersham). Quantification of LDL receptor protein was performed by laser densitometry. Results are expressed as peak area, determined from the densitometer scan.

2.7. Liver lipid determinations

Total cholesterol, unesterified cholesterol and triglycerides were measured on the liver homogenate and the solubilised liver membranes. Both liver preparations were initially sonicated, then diluted 1:1 with a 2% (w/w) Triton X-100 and 2 mM CaCl_2 solution. This was agitated on a rotating wheel for 30 min at 4° C and protein content determined. Lipid measurements were performed using enzymatic methods on the Cobas Bio and expressed relative to the protein concentrations.

2.8. Artery cholesterol measurements

The total cholesterol in the descending aorta was determined on approximately 15–20 mm segments of aorta, weighing 0.3–0.5 g which were homogenised and then sonicated on ice for 30 s. Cholesterol was extracted by the Folch method [28] and then subjected to saponification. The cholesterol was then extracted from the aqueous phase using 2 ml of hexane. This was dried under a stream of nitrogen and resuspended in 50 μ l of hexane for GC injection [22].

2.9. Statistical analysis

All values are expressed as the mean \pm standard error of the mean (S.E.M.). Data was analysed using a linear regression or a one way analysis of variance (ANOVA) with the Scheffe or Fishers least significant difference (L.S.D.) tests

of significance where appropriate. A value of $p < 0.05$ was the criterion of significance.

3. Results

3.1. Crude catechin extract from green tea lowers plasma cholesterol

Rabbits were initially fed a cholesterol only diet (0.25%, w/w) for two weeks. During these two weeks the average cholesterol concentrations significantly increased from 0.91 ± 0.06 to 4.26 ± 0.43 mmol/L ($p < 0.001$). There were no differences in daily food consumption between treatment groups (data not shown).

Administration of the crude catechin extract along with 0.25% (w/w) cholesterol for 4 weeks was found to significantly reduce plasma cholesterol concentration by 60% in the 2% (w/w) treatment group ($p < 0.05$) compared to the control group (Fig. 1A). There was a significant inverse trend between the dose of crude catechin extract and plasma cholesterol concentration ($p < 0.05$).

3.2. Crude catechin extract from green tea lowers plasma lipoprotein cholesterol

Administration of crude catechin extract at the highest dose (2%) significantly reduced cholesterol in the LDL fraction (80%, $p < 0.05$) and in the VLDL + IDL fraction (65%, $p < 0.05$) compared to control rabbits (Fig. 1B and C). There was a significant inverse trend between the dose of the crude catechin extract and both LDL cholesterol and VLDL + IDL cholesterol ($p < 0.05$). There were no significant differences in cholesterol in the HDL fraction between treatment groups (Fig. 1D). The recovery of cholesterol in the lipoprotein fractions isolated from rabbit plasma after administration of the crude catechin extract was approximately 70%.

3.3. Crude catechin extract from green tea lowers cholesterol in the arteries

The concentration of cholesterol in the descending thoracic segment of the aorta from rabbits is shown in Table 1. There was a significant 30% reduction in the rabbits fed 2% (w/w) crude catechin extract compared to the control group

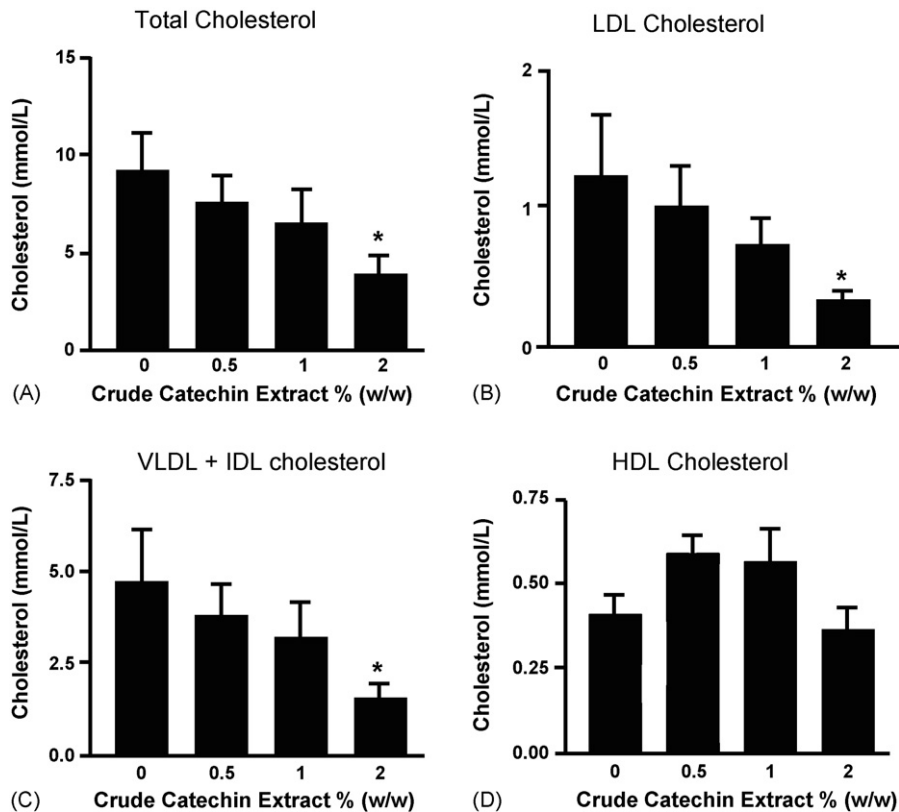


Fig. 1. (A–D) Crude catechin extract from green tea lowers plasma total cholesterol concentration and cholesterol in the lipoprotein fractions. Twenty-four hypercholesterolaemic rabbits, randomised into four treatment groups of six rabbits each, were fed a crude catechin extract at concentrations of 0, 0.5, 1 or 2% (w/w) mixed in with normal rabbit chow and 0.25% (w/w) cholesterol for 28 days. Lipoproteins, VLDL + IDL ($1.006 < d < 1.019$ g/ml), LDL ($1.019 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml) were isolated from plasma using sequential ultracentrifugation. Cholesterol was measured using enzymatic techniques. Values are expressed as mean \pm S.E.M. *Significant difference compared to control group using a one-way ANOVA and Fishers L.S.D. ($p < 0.05$).

Table 1

Cholesterol content and fatty streak assessment in aorta dissected from rabbits following dietary intervention with a crude catechin extract for 28 days

	Crude catechin extract (% w/w)			
	0	0.5	1	2
Thoracic aortic cholesterol ($\mu\text{mol cholesterol/g}$)	0.98 ± 0.13	1.22 ± 0.12	0.87 ± 0.05	$0.73^* \pm 0.06$
Aortic arch fatty streak (% total surface area with Lipophilic stain)	2.35 ± 0.73	2.28 ± 1.05	1.57 ± 0.53	1.93 ± 0.67

* Significant difference compared to control group using a one-way ANOVA and Fishers L.S.D. ($p < 0.05$).

Table 2

Total and unesterified cholesterol concentrations in rabbit liver homogenate and membranes after dietary intervention with a crude catechin extract for 28 days

Liver fraction	Crude catechin extract (% w/w)			
	0	0.5	1	2
Homogenate				
Total cholesterol ($\mu\text{M/g}$)	316.5 ± 15.1	309.8 ± 10.3	301 ± 25.1	$244.5^* \pm 21.1$
Unesterified cholesterol ($\mu\text{M/g}$)	146.6 ± 8.9	151.5 ± 5.8	145.9 ± 12.7	$123.5^* \pm 7.7$
Liver membranes				
Total cholesterol ($\mu\text{M/g}$)	483 ± 43.2	512.2 ± 48.3	440.3 ± 61	$377.7^* \pm 29.8$
Unesterified cholesterol ($\mu\text{M/g}$)	252.9 ± 19	268.8 ± 12	248.2 ± 19	$199.4^* \pm 16$

* Significant difference compared to control group using a one-way ANOVA and Fishers L.S.D. ($p < 0.05$).

($p < 0.05$). There were, however, no significant differences in the percent surface area of the aortic arch stained with lipophilic oil red O between treatment groups (Table 1).

3.4. Crude catechin extract from green tea lowers liver cholesterol

Administration of the crude catechin extract significantly reduced ($p < 0.05$) total and unesterified cholesterol in liver homogenates (25 and 15% respectively) in the 2% (w/w) treatment group compared to controls (Table 2). There was a significant inverse trend between the dose of the crude catechin extract and both total and unesterified cholesterol in liver homogenates ($p < 0.05$). Unesterified cholesterol constituted approximately 50–55% of the total cholesterol concentration and consumption of the crude catechin extract did not alter this percentage significantly. In the liver membrane fraction there were also significant reductions ($p < 0.05$) in both total and unesterified cholesterol (22 and 21% respectively) in the 2% (w/w) treatment group. There was a significant inverse trend between the dose of the crude catechin extract and both total and unesterified cholesterol in the liver membranes ($p < 0.05$). Unesterified cholesterol was approximately 47% of the total cholesterol concentration and remained unchanged with crude catechin consumption.

3.5. Crude catechin extract from green tea inhibits cholesterol synthesis

The ratio of plasma lathosterol to cholesterol concentration was determined as an index of cholesterol synthesis. Rabbits administered the 1 and 2% (w/w) green tea extract had significant reductions ($p < 0.05$) in cholesterol synthesis

(50 and 60%, respectively) (Fig. 2). No significant differences were found in the intrinsic capacity to absorb dietary cholesterol between treatment groups as measured using the ratio of plasma phytosterol to cholesterol.

3.6. Crude catechin extract from green tea upregulates LDL receptor activity and protein

The calcium-dependant LDL-gold binding capacity of solubilised liver membranes was used to determine hepatic LDL receptor binding activity [25]. Administration of the highest dose of crude catechin extract (2%, w/w) signifi-

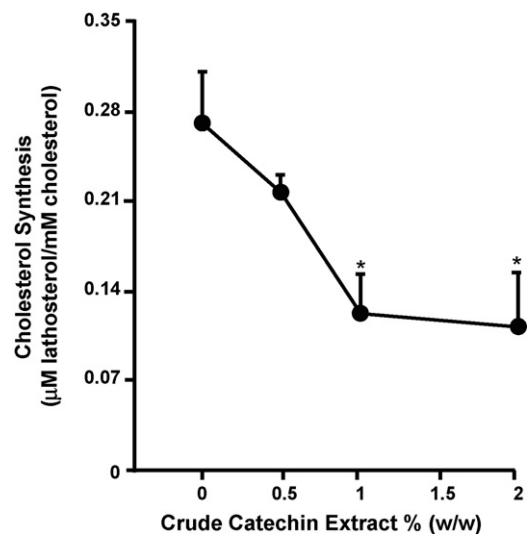


Fig. 2. Crude catechin extract from green tea inhibits cholesterol synthesis. The plasma ratios of lathosterol to cholesterol were determined using gas chromatography. Values are expressed as mean \pm S.E.M. *Significant difference compared to control group using a one way ANOVA and Scheffe post hoc test ($p < 0.05$).

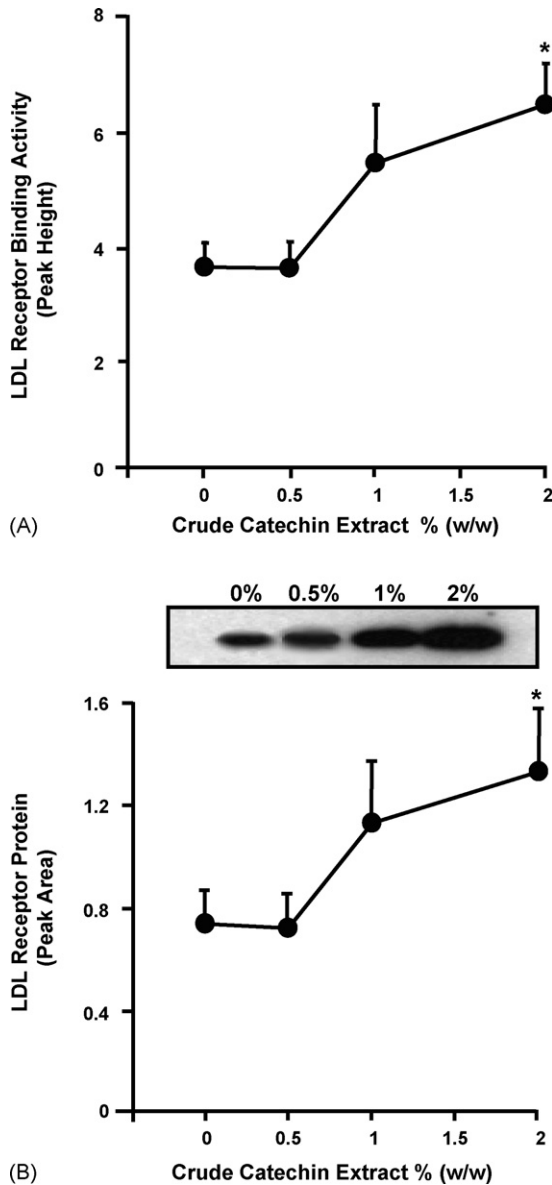


Fig. 3. Crude catechin extract from green tea increases (A) hepatic LDL receptor binding activity and (B) the relative amounts of LDL receptor protein. Hepatic LDL receptor binding activity was determined as the calcium-dependant-binding of LDL-gold to solubilised membrane proteins dot blotted onto nitrocellulose. The relative amounts of hepatic LDL receptor protein were determined using a polyclonal antibody against the hepatic LDL receptor and Western Blotting. Values are expressed as mean \pm S.E.M. *Significant difference compared to control group using a one-way ANOVA and Fishers L.S.D. ($p < 0.05$). Image in 3B depicts representative western blot of LDL receptor protein levels from the different treatment groups.

cantly increased hepatic LDL receptor binding activity by 80% ($p < 0.05$). Using a polyclonal antibody against the LDL receptor, the relative amounts of LDL receptor protein in the 2% (w/w) treatment group was found to be significantly higher (70%) than in the control group. (Fig. 3). There was a significant positive trend between the dose of the crude catechin extract and both LDL receptor binding activity and LDL receptor protein ($p < 0.05$).

4. Discussion

The present study set out to investigate if a green tea extract, enriched in catechins, could lower the plasma cholesterol concentration in the cholesterol-fed hypercholesterolaemic rabbit. We also wanted to determine a possible mechanism of action for this effect. This study reports the following important findings: administration of a crude catechin extract from green tea significantly lowered total plasma cholesterol concentrations by 60%. There were also significant reductions in VLDL + IDL (65%) and LDL (80%) cholesterol fractions, thereby improving the atherogenic profile. This was reflected by a significant decrease in the cholesterol measured in the descending thoracic aorta. Administration of the crude catechin extract was able to dramatically decrease cholesterol synthesis (60%) and increase the hepatic LDL receptor binding activity (80%) and protein (70%), providing mechanisms to explain the hypocholesterolaemic effect of the green tea extract used in this study.

The finding that the crude catechin extract from green tea lowered plasma cholesterol in the cholesterol-fed rabbit is consistent with other animal intervention studies in hamsters, rats and mice [4–8]. This is the first study, however, to demonstrate this effect in the cholesterol-fed hypercholesterolaemic rabbit model. In contrast, Tijburg et al. [9] found no changes in plasma cholesterol when they fed a green tea extract to their cholesterol-fed hypercholesterolaemic rabbits. One difference between the two studies was the way the extract was presented to the rabbits. In the present study, the extract was mixed in with the solid diet along with cholesterol while Tijburg et al. [9] dissolved their green tea extract in water and gave it to the rabbits as their sole drinking source. It may be that the active components in green tea are better absorbed when in the solid diet than in the drinking water. It is also possible that catechins are better able to complex with the cholesterol when they are both in the same medium, thereby preventing their incorporation into mixed bile salt micelles [11]. Another difference between the two studies is the hypercholesterolaemia achieved. The rabbits had an average plasma cholesterol concentration four times higher in Tijburg's study compared to the present study. This is possibly because we fed our rabbits the control diet for a shorter period (2 weeks compared with 9 weeks) and it contained a lower fat concentration. This may have made it easier for plasma cholesterol to be lowered by our green tea extract.

The hypocholesterolaemic effects of green tea catechins have been attributed to their ability to inhibit cholesterol absorption from the intestine [10,11]. In this study, the intrinsic capacity of the intestine to absorb dietary cholesterol, as measured by the plasma ratio of phytosterols to cholesterol [24], did not change after the consumption of the crude catechin extract. These results are supported by the observations of Chan et al. [7] who found that green tea catechins did not alter the activity of intestinal acyl Co A:cholesterol acyl-transferase (ACAT), an enzyme which is rate limiting for the intestinal esterification and absorption of cholesterol [12].

The crude catechin extract therefore does not appear to inhibit cholesterol absorption by decreasing the intrinsic capacity of the intestine to absorb the sterol. As proposed by Ikeda et al. [11], the catechins are more likely to inhibit cholesterol absorption by interfering with the biliary micelle system in the lumen of the intestine.

The upregulation of the LDL receptor observed in this study is consistent with the increased faecal excretion of cholesterol and bile acids found in other animal studies [8,11]. It is one mechanism by which the crude catechin extract could lower plasma cholesterol concentrations.

The decrease in cholesterol synthesis, however, is not consistent with an increase in the faecal excretion of cholesterol and bile acids. It appears to be a separate, systemic effect of the crude catechin extract where it is acting directly to inhibit cholesterol synthesis. This is possible because catechins are known to be absorbed into the circulation and can be measured in the plasma and the tissues [29].

Furthermore, this decrease in cholesterol synthesis may be the initiating mechanism by which the crude catechin extract produces its cholesterol lowering effects rather than the increase in the LDL receptor. This may suggest a possible mechanism for the increase in the LDL receptor whereby the decreased level of cholesterol synthesis may reduce the size of the “active pool” of cholesterol which is thought to regulate the LDL receptor [30]. These effects of the crude catechin extract are similar to hypocholesterolaemic drugs such as lovastatin, simvastatin and pravastatin, which reduce cholesterol synthesis and increase the LDL receptor [27,31].

In conclusion this study found that the administration of a crude catechin extract from green tea could reduce plasma, liver and thoracic aorta cholesterol in the cholesterol-fed hypercholesterolaemic rabbit. The crude catechin extract also reduced cholesterol synthesis and increased the LDL receptor which can both contribute to lowering plasma cholesterol concentrations.

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