Regression Studies with Safflower Oil and Sitosterol in Rabbit Atherosclerosis

By JONATHAN P. MILLER, PH.D., G. FREDERICK LAMBERT, PH.D., AND DOUGLAS V. FROST, PH.D.

Hypercholesteremia with atherosclerosis was induced in male rabbits by feeding hydrogenated coconut oil as the only lipid in a purified diet. Replacing the hydrogenated coconut oil by safflower oil produced a profound fall in plasma cholesterol in rabbits. Supplementing the safflower oil with β-sitosterol at 3 per cent of the oil weight caused an even greater decrease in plasma cholesterol. Although in both cases the further development of aortic atheroma was arrested, there was no detectable regression of existing lesions over 24 weeks. Hydrogenated coconut oil feeding produced high plasma and liver cholesterol levels in rabbits. When small amounts of safflower oil were fed in addition to the hydrogenated coconut oil, the elevation of plasma or liver cholesterol was much less.

We have induced marked hypercholesteremia and aortic atheromas in rabbits fed a purified diet containing 20 per cent hydrogenated coconut oil* (HCO), but no cholesterol.† A similar diet, but with 20 per cent safflower oil† (SO) caused only a small rise in plasma cholesterol and no atheroma. This paper describes experiments using these cholesterol-free diets with HCO to induce atheroma and to test their regression when the HCO was replaced by SO. In human studies, Beveridge et al.1 reported that the unusual hypocholesteremic effect of corn oil is due to its relatively high sitosterol content. We were interested, therefore, to test the value of both SO and SO plus β-sitosterol (S:S) to regress atheroma in rabbits. The lowering of cholesterol levels by replacing HCO with SO might merely be due to removal of HCO rather than to any effect of SO. Experiments are also reported showing that the dietary addition of SO, without a concomitant decrease in HCO, lowers both plasma and liver cholesterol levels.

METHODS

Our procedure for rabbit experiments has been described.1 Per cent composition of the basic diet is as follows: cellulose 10, casein 25, dextrin 39.9, hydrogenated coconut oil 20, macrominerals 5, minor minerals 0.1. Macro mineral composition in grams: Ca3(C8H5O7)•4H2O 308.2, Ca(H2PO4)2 104.7, K2HPO4 218.7, KCl 124.7, NaCl 77.0, CaCO3 68.5, 3MgCO3 • Mg(OH)2 • 3H2O 35.1, MgSO4 38.3. Minor mineral composition in gram: FeC6H2O7 • 5H2O 463.85, CuSO4 • 5H2O 28.15, MnSO4 • H2O 16.5, KI 1.5. Vitamins added as follows: (milligrams or I.U./100 Gm. diet) thiamin • HCl 0.07, riboflavin 0.6, calcium-DL-pantothenate 1.5, pyridoxine • HCl 0.7, niacin 20, choline chloride 100, betaine chloride 100, inositol 10, p-aminobenzoic 0.2, folie acid 0.1, d-riboflavin 0.05, vitamin E 665 I.U., vitamin B12 .005, vitamin D2 1 I.U., vitamin E 7.5 I.U., menadione 0.075. I experiments 3 and 4, one diet contained 17.5 per cent hydrogenated coconut oil and 2.5 per cent safflower oil while the other contained 17.5 per cent hydrogenated coconut oil with the dextrin increased to 42.4 per cent. When β-sitosterol was used it was dissolved in safflower oil and added to the diet at the expense of cellulose. All rabbits were pair-fed, daily food ingestion being limited to an amount which all rabbits would eat (50 Gm. daily except in experiment 2 after 6 weeks regression when food offered was increased to 60 Gm.). The daily allotment of diet was completely consumed by each rabbit except in very few instances.

Cholesterol determinations in plasma were done as before.1 Livers were removed at death and kept frozen in sealed plastic bags until analysis. Total lipid and cholesterol content was

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*Hydrol (Durkee Famous Foods), approximate composition: linoleic acid 0.70 per cent, sitosterol 0.13 per cent.
†Pacific Vegetable Oil Co., approximate composition: linoleic acid 70.5 per cent, sitosterol 0.40 per cent.
determined from aliquots of a chloroform extract as prepared by the method described by Cohn. In this method 30 per cent w/v aqueous KOH was added to the entire liver on a 1:1 basis (ml: Gm. wet weight) and the mixture autoclaved in sealed bottles at 10 p.s.i. for 2 hours. The resulting mixture was diluted to 500 ml. with 95 per cent 3A alcohol and filtered. Fifty milliliter aliquots were pipetted into centrifuge bottles which were then placed in boiling water until all alcohol evaporated. The residue was acidified with HCl, diluted with 25 ml. water, and then extracted once with 100 ml. redistilled chloroform. Centrifugation produced a clean separation of layers. After filtration of the chloroform layer, suitable aliquots were used for the determination of cholesterol. Following evaporation of the chloroform from such aliquots, cholesterol was estimated by the method previously described. Total lipid content of the livers was determined gravimetrically from aliquots of the filtered chloroform extract.

Aortas were stained with Sudan IV and graded for gross atherosclerotic lesions by 2 independent observers using a scale of 0 to 4. A grade of 4 indicates that lesions cover 100 per cent of the area while a grade of 0 indicates the absence of any lesions.

The first experiment is outlined in figure 1. To facilitate the identification of groups in experiments 1 and 2, we use a letter code derived from abbreviations for each type of fat studied. Thus in the preregression period the group fed hydrogenated coconut oil is identified as group H and the group fed the mixed safflower oil-hydrogenated coconut oil MR, that on safflower oil group SR, that on mixed safflower oil-hydrogenated coconut oil MR, and that on safflower oil (3 rabbits of this group were sacrificed at 22 weeks). Survivors in all groups were killed after 24 weeks of regression.

In both experiments 3 and 4 one group of rabbits was fed a 17.5 per cent HCO diet and the other group received a diet containing 17.5 per cent HCO plus 2.5 per cent SO. Survivors in experiment 3 were sacrificed at 14 weeks, and those in experiment 4 at 16 weeks. The data of experiments 1 and 2 were treated by analysis of variance. Whenever the F test was significant at the 5 per cent level the data were further analyzed by Hartley's sequential method of testing using Q tables as described by Snedecor. In experiments 3 and 4 statistical significance was determined by the t test.

**Results**

Experiment 1 (figs. 1 and 3, table 1). During the preregression part of the experiment (0 to 14 weeks), the rabbits on the mixed fat diet (group M) gained more weight than those on HCO (group H), i.e., 1.1 vs. 0.52 Kg. Feed conversion (food eaten/weight gained) was more efficient for group M (4.31 vs. 7.54). The 5 rabbits on (SO group SR) that were sacrificed after only 4 weeks of regression showed the same general trend with respect to plasma cholesterol and liver composition as those that were continued for the full regression period of 12 weeks. During the regression portion of the experiment the rabbits that were changed to SO or HCO:SO mixture gained more weight than did those continued on HCO.
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(0.48 and 0.55 vs. 0.15 Kg. respectively). The group on SO and SO:HCO also converted more feed to body weight than did group HR, maintained constantly on HCO (7.04 and 6.44 vs. 20.83).

Experiment 2 (figs. 2 and 4, table 1). During regression the rabbits on SO (group SR) or sitosterol-supplemented SO (group SSR) gained more weight (0.96 and 0.80 vs. 0.58 Kg.) and had more favorable feed efficiency (9.34 and 10.85 vs. 14.71) than did those on HCO (group HR). The 3 rabbits on S:S (group SSR) that were sacrificed after only 12 weeks of regression showed the same general trend with respect to plasma cholesterol and liver composition as those that were continued for the full regression period of 24 weeks.

Experiments 3 and 4 (tables 2 and 3). In experiment 3 the rabbits on HCO-SO gained more weight (0.81 vs. 0.18 Kg.) and used feed more efficiently than those on HCO (6.26 vs. 24.87). Also in experiment 4 the rabbits on HCO-SO gained more weight (0.7 vs. 0.5 Kg.), with better feed conversion than those on HCO (6.90 vs. 8.82). This was to be expected since the HCO-SO diets contained more total fat and had higher caloric density.

Although experiment 4 was simply a repeat of experiment 3, there are considerable discrepancies between feed efficiency and plasma cholesterol of rabbits on HCO in the earlier experiment and those on the same diet in the latter test. The reason for this is not clear and may represent difference in HCO composition, differences in susceptibility between groups of rabbits, or something as subtle as differences in temperature or time of the year. The cause of this variability between experiments should be investigated more thoroughly since its counterpart in human investigation is also frequently seen.

DISCUSSION

During the preregression phase of experiment 1, replacement of part of the HCO by
Table 1.—Probability Values for Liver Composition in Experiments 1 and 2

<table>
<thead>
<tr>
<th>Group comparison</th>
<th>Wet weight (Gm.)</th>
<th>Lipid (%)</th>
<th>Total lipid (mg.)</th>
<th>Cholesterol (mg. %)</th>
<th>Total cholesterol (mg.)</th>
<th>Cholesterol in lipid (%)</th>
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<td><strong>Experiment 1</strong></td>
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<td>H vs. MR</td>
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<td>HR vs. SR</td>
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<td><strong>Experiment 2</strong></td>
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<td>C vs. SR</td>
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<td>C vs. SSR</td>
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<tr>
<td>HR vs. SR</td>
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<tr>
<td>SR vs. SSR</td>
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</table>

For explanation of groups see figures 1 and 2. Under column labeled group comparison, the group listed first represents that of highest magnitude. In cases where this order is reversed, the change is noted in the table. For instance H vs. M indicates that group H had higher per cent lipid, mg. per cent cholesterol, total cholesterol, and per cent cholesterol in lipid than did group M (p < .01). All probability differences of p > .05 were omitted from the table.

SO produced significantly lower plasma cholesterol levels and atheroma scores (p < .05). During the regression phase of the experiment, although replacement of HCO by SO led to a prompt and profound drop in plasma cholesterol, no regression of preformed aortic atheroma could be observed by gross visual examination. This was also supported histologically.* Perhaps some changes occurred that we did not detect by our methods. In experiment 2, even though this drop in plasma cholesterol did not result in regression of lesions, it appeared to arrest further development of atheroma. At the end of the experiment, lesion scores of groups changed to safflower oil or β-sitosterol-supplemented safflower oil were lower (p < .05) than those continued on coconut oil. Furthermore, animals remaining on HCO developed progressively higher atheroma scores (group HR higher than group C; p < .05).

It is noteworthy that the addition of β-sitosterol to SO brought the plasma cholesterol below normal without regressing the atheromas. Anitschkow5,6 stated that, following the removal of dietary cholesterol, a definite regression of lesions occurred with a disappearance of much lipid from the aortic atherosclerotic plaques of rabbits. The rabbits were allowed to regress for about 1½ to 3 years. However, McMillan et al.7 found no regression of lesions over a period of 6 months, either in terms of visual grading or in the sense of decreasing the cholesterol content of the aortas. These latter observations were also made following removal of cholesterol from rabbit diets. Beher et al.,8 in rabbit studies, found that although the concurrent addition of β-sitosterol and removal of cholesterol caused greater drops in plasma cholesterol than the removal of cholesterol alone, no regression in aorta lipid, cholesterol or plaques was seen after 4 months.

In experiment 2 the plasma cholesterol level of the rabbits fed unsupplemented safflower oil, fell to slightly subnormal levels near the end of the experiment. The magnitude of this difference from normal levels

*We are indebted to Dr. R. J. Stein for histologic examination of the aorta.
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Table 2.—Average Plasma Cholesterol Levels in Experiments 3 and 4

<table>
<thead>
<tr>
<th>Dietary fat</th>
<th>0%</th>
<th>4%</th>
<th>8%</th>
<th>12%</th>
<th>16%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCO</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>HCO-SO</td>
<td>88</td>
<td>91</td>
<td>88</td>
<td>94</td>
<td>97</td>
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<tr>
<td>p</td>
<td>&lt;.01</td>
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Experiment 3

Experiment 4

HCO, 17.5 per cent hydrogenated coconut oil; HCO-SO, 17.5 per cent hydrogenated coconut oil plus 2.5 per cent safflower oil.

Each group started with 10 rabbits. The number of survivors at termination of the experiment were as follows: experiment 3 (HCO, 8; HCO + SO, 10), experiment 4 (HCO, 7; HCO + SO, 9).

As noted in the experimental data, all calculations of liver composition were made per 100 Gm. body weight. This was done to relate effect of diet to liver composition independently of growth rate. For instance, whenever rabbits were changed from HCO to any diet containing SO the growth rate was stimulated, increasing absolute liver size, but not liver weight relative to body weight.

At the end of the preregression phase of experiment 1 the livers of group H on HCO were gorged with lipid and cholesterol as compared to group M in which part of the HCO was replaced by safflower oil. In order to survive when continued on HCO, the animal seemingly must compensate by decreasing this lipid or cholesterol concentration to a more normal range by increasing liver size without changing total cholesterol or lipid content, decreasing total cholesterol or lipid content without changing liver size, or both. The data from both regression experiments indicate that the liver increases in size, lipid and cholesterol concentration decreases, and total cholesterol decreases. Further evidence for this mechanism is shown by the fact that, at the end of regression, there was no significant difference.
in liver cholesterol content between animals continued on HCO and those transferred to SO. On the other hand, total liver lipids were higher in the HCO group. When dietary fat is changed from HCO to SO, HCO-SO mixture or S:S, both total lipid and cholesterol decrease and the liver weight remains approximately constant.

As compared to safflower oil alone, the addition of β-sitosterol does not significantly affect either liver composition or atheroma scores, i.e., no difference between groups SR and SSR.

The liver data of experiments 3 and 4 again indicate that the effects observed when SO replaces all or part of HCO in diets is not due solely to removal of HCO. In both experiments the livers of the rabbits receiving 17.5 per cent HCO plus 2.5 per cent SO contained much less lipid and cholesterol than those of rabbits fed 17.5 per cent HCO with no SO.

In human studies the decrease in serum cholesterol caused by feeding unsaturated fats has been verified by Hellman et al., Gordon et al., Lewis and Haust et al. These authors reported the decrease in serum cholesterol was accompanied by increased fecal excretion of cholesterol and its transformation products. Although we have not studied excretion per se, our results suggest that, in this respect, rabbits may behave similarly to man. Transferring rabbits from HCO to SO causes significant decreases in both plasma and liver cholesterol. Presumably cholesterol or its transformation products is eliminated via fecal excretion.

Coconut oil was used in these experiments simply as a means to produce aortic lesions. We do not intend to imply that it is necessarily typical of saturated fats or representative of the saturated fats in human diets. It differs from many food fats in its high content of short chain saturated fatty acids.

Whether these data are indicative of processes occurring in the human is a moot question. Everyone is aware that there are species differences with respect to a great many facets of metabolism. Regression studies

![Regression charts](http://circres.ahajournals.org/)

FIG. 3 Top. Average grade of aortas and liver composition in experiment 1. HCO, 20 per cent hydrogenated coconut oil; SO, 20 per cent safflower oil; HCO-SO, 15 per cent hydrogenated coconut oil-5 per cent safflower oil. H, M, HR, SR, and MB, groups (R, regression phase). Liver data expressed per 100 Gm. weight except per cent composition as shown.

FIG. 4 Bottom. Average grade of aortas and liver composition in experiment 2. HCO, 20 per cent hydrogenated coconut oil; SO, 20 per cent safflower oil; S:S, 20 per cent safflower oil plus β-sitosterol. C, HR, SR, and SSR, groups (R, regression phase). Liver data as in figure 3.
Atherosclerosis regression studies are obviously very difficult, if not impossible, to do and we are anxiously awaiting definitive evidence as to the long range effects of unsaturated fat administration. While this information is accumulating, animal studies may offer some insight into the general problem and provide clues for further studies in the human.

Summary

Substituting safflower oil in purified diets for hydrogenated coconut oil produced a rapid and profound fall in plasma cholesterol in rabbits. Supplementing the safflower oil with β-sitosterol at 3 per cent of the oil weight caused an even greater decrease in plasma cholesterol. Although in both cases the further development of aortic atheroma was arrested, there was no detectable regression of existing lesions over 24 weeks. Rabbits fed hydrogenated coconut oil alone at first developed unusually high concentrations of liver lipids, including cholesterol. Rabbits surviving on such a diet develop very large livers with decreasing liver cholesterol and lipid concentration. This compensation appears necessary for survival. Substituting safflower oil or sitosterol-supplemented safflower oil for hydrogenated coconut oil increased growth rate and decreased concentration and amount of liver lipids, including cholesterol. Rabbits surviving on such a diet develop very large livers with decreasing liver cholesterol and lipid concentration. This compensation appears necessary for survival. Substituting safflower oil or sitosterol-supplemented safflower oil for hydrogenated coconut oil increased growth rate and decreased concentration and amount of liver lipids, including cholesterol. Rabbits surviving on such a diet develop very large livers with decreasing liver cholesterol and lipid concentration. This compensation appears necessary for survival. Substituting safflower oil or sitosterol-supplemented safflower oil for hydrogenated coconut oil increased growth rate and decreased concentration and amount of liver lipids, including cholesterol. Rabbits surviving on such a diet develop very large livers with decreasing liver cholesterol and lipid concentration. This compensation appears necessary for survival. Substituting safflower oil or sitosterol-supplemented safflower oil for hydrogenated coconut oil increased growth rate and decreased concentration and amount of liver lipids, including cholesterol. Rabbits surviving on such a diet develop very large livers with decreasing liver cholesterol and lipid concentration. This compensation appears necessary for survival.

SUMMARY

Le substitution de oleo de carthamo pro hydrogenate oleo de coco in dietas purificate de coñillos produciva un marcato reduction del contento de cholesterol in le plasma del animales. Le supplementation del oleo carthamo con sitosterol beta amontante a 3 pro cento del peso del oleo resultava in un ancora plus grande reduction del cholesterol del plasma. Ben que in ambe casos de disveloppamento de atheroma aortic eseva ar-restate, nulle regression del existente lesions eseva detegite in le curso de 24 septimanas. Conilios nutritie con hydrogenate oleo de coco sol disveloppava inicialmente unusualmente alte concentrationes de lipidos hepatic, include cholesterol. Le animales que superviveva con un tal dieta disveloppava largissime hepates con descrecente concentrationes hepatic de cholesterol e lipid. Il pare que iste compensation es necessari pro le supervivientia. Le substitution de oleo de carthamo o de oleo de carthamo con un supplemento de sitosterol pro le hydrogenate oleo de coco acelerava le crescentia del delipidios hepatic, include cholesterol. Le addition de un micre quantitate de oleo de carthamo a un dieta de hydrogenate oleo de coco protegeva in parte contra le elevation de cholesterol in plasma e hepate ben que le contenoto total de grassia in le dieta eseva augmentate.

REFERENCES


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