Design criteria for studies examining individual fatty acid effects on cardiovascular disease risk factors: human and animal studies¹,²

Penny M Kris-Etherton and John Dietschy

ABSTRACT  Studies designed to examine individual fatty acid effects in humans and animals are critically dependent on subjects or animal species, experimental diets, and the experimental design used. For both human and animal studies, the numbers of subjects and animals must be adequate for achieving statistical significance and the subjects and animals must be grouped appropriately (eg, age, sex, and cholesterol responsiveness). In animal studies the appropriate species must be selected because some species are unacceptable models. In both human and animal studies, great attention must be paid to the design of the experimental diets. Test diets must be tightly controlled and nutrient specifications must be met and verified by chemical analysis. Ideally, only the fatty acid of interest should vary among the test diets. Two experimental designs are appropriate: crossover and parallel-arm designs. Feeding periods must be of adequate duration for stabilization of endpoints. Attention to these study design issues is imperative for meaningful conclusions to be reached about the effects of individual fatty acids. Am J Clin Nutr 1997;65(suppl):1590S–6S.

KEY WORDS  Experimental design, fatty acids, diet, human feeding studies, animal feeding studies

INTRODUCTION  

Central to our understanding of the effects of individual fatty acids on cardiovascular disease risk factors is the design and implementation of well-controlled clinical (and animal) studies. Historically, most of the research conducted to date has addressed the question of how fatty acid classes rather than individual fatty acids affect various cardiovascular disease risk factors. Only recently has the focus shifted to clarifying the effects of individual fatty acids. This shift in the emphasis of the clinical nutrition investigations has heightened our awareness of important design issues that must be implemented to accurately assess individual fatty acid effects.

The design of well-controlled fatty acid studies is complicated by many factors, including subject eligibility, the development of experimental diets, and the experimental design used. Animal studies play an important role in advancing our understanding of the underlying mechanisms of action by which individual fatty acids cause their effects.

Several experimental design and diet design issues are common to both human and animal studies. The purpose of this paper is to discuss experimental design issues that have an important bearing on the outcome of both human and animal studies of the effects of individual fatty acids on cardiovascular disease risk factors.

HUMAN STUDIES  

Well-controlled feeding studies are needed to clarify the biological effects of individual fatty acids. As shown in Table 1, many important issues must be considered when designing human studies that examine individual fatty acid effects. A key consideration is the design of the experimental diets. The diets must be designed to unambiguously establish an individual fatty acid effect. The other important design criteria (ie, subject eligibility and experimental design) are common to virtually all clinical studies, regardless of whether they focus on diet, drugs, or exercise. Important aspects of these criteria that are essential to well-controlled feeding studies designed to examine individual fatty acid effects will be discussed.

Subects  

A major goal of well-controlled feeding studies is to establish the generalizability of the results obtained to the population at large. Another common goal is to assess responses in distinct population groups (eg, persons with dyslipidemia, males compared with females, different ethnic groups, and younger compared with older individuals). Because some groups of individuals respond differently to diet modifications, it is important that the study subjects meet carefully defined eligibility criteria. Examples that illustrate this point have been described by Harris (1) and Denke and Frantz (2). For instance, the low-density-lipoprotein-cholesterol (LDL-C) response to n−3 fatty acids differs distinctly for the various dyslipidemias (1). In addition, the LDL-C response to a blood cholesterol-lowering diet is greater in individuals with hypercholesterolemia (excluding familial hypercholesterolemia) (2). Precisely defined eligibility criteria, thus, are important for subject selection for well-controlled feeding studies.

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TABLE 1
Important criteria for conducting well-controlled feeding studies designed to examine individual fatty acid effects

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<th>Human studies</th>
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<td><strong>Subjects</strong></td>
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<tr>
<td>1) The number of subjects should be adequate to achieve statistical significance.</td>
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<td>2) Subjects should be grouped appropriately by sex, age, race and ethnicity, health status, presence of lipid abnormality, and menopausal status.</td>
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<td>3) Subjects can be free-living or housed (eg, in a domiciliary or metabolic ward).</td>
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<td><strong>Experimental diets</strong></td>
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<td>1) All food should be provided.</td>
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<td>2) Food should be weighed and measured.</td>
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<td>3) Whole-food diets are ideal; liquid-formula diets are acceptable.</td>
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<td>4) Structured fats should be physiologically relevant.</td>
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<td>5) Experimental diets should be well controlled: essentially the only variable is the fatty acid of interest; diets should be high in the fatty acid of interest.</td>
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<td>6) Dietary composition should be chemically assayed for (at least) fatty acids, total fat, and cholesterol.</td>
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<td>7) Experimental diets should be validated chemically before the study begins.</td>
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<td><strong>Experimental designs</strong></td>
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<td>1) Crossover and parallel-arm designs are acceptable.</td>
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<td>2) Ideally, subjects should be randomly assigned.</td>
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<td>3) Study must last ≥2 wk or at least be sufficiently long enough for endpoint measures to stabilize.</td>
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<td>4) At least two well-controlled treatment periods are necessary; uncontrolled periods are not useful.</td>
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<td>5) Subjects should be fasted.</td>
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<td>6) More than one plasma lipid measurement should be made at each time.</td>
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<td>7) Plasma lipids and lipoproteins should be collected and assayed according to standardized procedures.</td>
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<th>Animal models</th>
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<td><strong>Species</strong></td>
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<tr>
<td>1) Acceptable: nonhuman primates (cynomolgus, cebus, rhesus, and African green monkeys and baboons), hamsters, guinea pigs, gerbils, dogs, and pigs.</td>
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<td>2) Unacceptable or cautionary models: mice, rabbits, squirrel monkeys, and rats.</td>
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<td>3) Animals should be grouped appropriately by age, sex, and strain.</td>
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<td><strong>Experimental diets</strong></td>
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<td>1) Laboratory dry-pellet diets and semipurified diets are acceptable and should be well controlled: only a single fatty acid should be substituted for another fatty acid, diets should be high in the fatty acid of interest, a finite amount of dietary cholesterol is necessary (not excessively high, eg, &lt;0.2%), and diets should be equienergetic.</td>
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<tr>
<td>2) Dietary composition should be chemically assayed, at least for fatty acid composition.</td>
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<td>3) Energy density is important and should be reported.</td>
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<td><strong>Experimental designs</strong></td>
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<tr>
<td>1) Study duration should be sufficient for equilibration of endpoints of interest.</td>
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<td>2) Fatty acid bioavailability should be documented, especially that of highly saturated fatty acids (eg, stearic acid or tristearin).</td>
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<td>3) Animals should not be deprived of food before blood collection for lipid assays.</td>
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<td>4) Studies that focus on plasma LDL-C and LDL-C metabolism should include hepatic cholesteryl ester, LDL turnover, and LDL receptor activity measurements.</td>
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The number of subjects included in a study is important for more than statistical power. The major consideration is whether there is a good rationale for grouping different individuals together. This underscores the need to first establish the response of different target groups to determine whether they can be appropriately grouped together. For example, can the results from men and women (and older and younger subjects) be analyzed together? If the response is similar in the groups studied, then subsequent studies can be conducted without specific sex or age eligibility criteria, thereby reducing sample size. In contrast, should men and women respond differently, then subsequent studies must either have a number of men and women sufficient for discerning a relevant sex-treatment effect or recruit only one sex. Questions about the effects of the menstrual cycle on diet response have led many investigators to exclude women from feeding studies. A recent study, however, showed that the plasma lipid-lipoprotein-apo lipoprotein response to an experimental diet is similar at all stages of the menstrual cycle (3). Because so little is known about the response to individual fatty acids in different target groups, much remains to be done to resolve this issue on a population-wide basis. To achieve this goal, great care must be taken in defining the eligibility criteria of the target populations studied.

**Experimental diets**

The design of experimental diets and control of nutrient intake are essential in the implementation of studies designed to elucidate individual fatty acid effects. Of primary importance, irrespective of the particular strategies and methods used, is that the experimental diets be well controlled, vary only in the individual fatty acid of interest, and provide known amounts of nutrients (and fatty acids), energy, and other dietary constituents (eg, cholesterol and fiber).

To determine individual fatty acid effects, experimental diets must be precisely formulated to ensure that the fatty acid of interest is the only dietary variable. In this regard, there are two acceptable options. One involves substituting just one fatty acid (ie, the target fatty acid) for another and ensuring that no other dietary changes are made. With this approach, the total fat content of the diet remains constant. The other approach
involves substituting just the target fatty acid for carbohydrate, which results in the total fat content of the experimental diets being different. Thus, despite both of these approaches being considered gold standards, there are, in fact, two dietary variables that are introduced in the experimental diets. As a result, questions arise about whether the response observed is due to the addition of the target fatty acid, whether it reflects the removal of the fatty acid for which it is substituted, or whether it is due to a change in the total fat content of the diet. As is apparent, neither approach is perfect. A fatty acid of interest cannot be substituted for protein, which, like dietary carbohydrate, elicits a neutral cholesterolemic response. Protein is present in the diet in smaller quantities than carbohydrate, which precludes feeding a large quantity of a fatty acid of interest.

Although it may seem straightforward to develop whole-food diets that meet the specified nutrient criteria, it is challenging to design experimental diets that vary only in one fatty acid and not in any other nutrient. Therefore, the nutrient specifications are usually defined not as absolute values but rather as ranges, albeit stringent ones.

Typically, experimental diets are composed of whole foods and are fed as meals and snacks. Whole-food experimental diets are designed with foods that are eaten (and chewed) as opposed to liquid-formula diets, which have the appearance and consistency of a shake. In general, liquid-formula diets are used less frequently than are whole-food diets. Although whole-food diets represent usual food-consumption practices, liquid-formula diets can meet the target nutrient criteria precisely. Whole-food diets clearly can be designed to meet specified nutrient criteria, although usually there is greater variation in the nutrient composition of these experimental diets than there is with liquid-formula diets. Cycle menus developed for short time periods (eg, a few days), however, minimize this concern. In addition, central procurement of fat sources and detailed food specifications for other foods will decrease nutrient variability.

An unresolved question of growing interest is whether liquid-formula diets elicit a plasma total and lipoprotein cholesterol response that is similar to that observed when whole-food diets are fed, assuming ideal adherence to both types of diets. Of greater importance is the question of whether the magnitude of the blood cholesterol response is similar when two liquid-formula experimental diets are compared as opposed to two whole-food diets that are compositionally similar to the liquid-formula diets. Some investigators have claimed that liquid-formula diets, as well as simply admitting study subjects to a metabolic ward, cause plasma total cholesterol concentrations to decrease $\approx 1.1$ mmol/L (40 mg/dL) (4, 5). Even if this does occur, however, it may be inconsequential if the size of the response difference noted between two liquid-formula experimental diets is the same as that reported for compositionally similar whole-food experimental diets. Because of the growing number of studies designed to examine individual fatty acid effects that are being conducted with liquid-formula diets, these important questions remain to be addressed. Despite the possible limitations of liquid-formula diets, however, studies with these diets have contributed significantly to our understanding of the relative effects of different fatty acids and continue to be used by investigators (6, 7).

Moreover, the results reported for liquid-formula diets agree with those from whole-food diets.

Whether to use a natural fat or a structured fat in an experimental diet is another important consideration. A structured fat is synthesized from glycerol and the fatty acids of interest. Structured fats are useful in designing diets that contain predominately one fatty acid or specified amounts of the fatty acids of interest. Key points to consider when using structured fats include their stereochemistry, their absorption, the randomization of the fatty acids of interest when structured fats comprising a mixture of fatty acids are used, and how the fats are incorporated into the diet. The stereochemistry of acylglycerols affects the absorption and metabolism of fatty acids as well as of the lipoprotein fraction in which they are transported (8). To ensure that the absorption and metabolism of the fatty acid mirrors a natural fat, the fatty acids in the structured fats must be randomized, because fatty acids in the sn-2 position may account for increased absorption (8, 9). Moreover, fatty acid absorption should be assessed. Last, there is some evidence to suggest that some structured fats must be heated (to 70–85 °C) to maximize absorption (10). Thus, the use of a structured fat necessitates conducting preliminary experiments to show that the structured fat is physiologically similar to a natural fat.

Adherence to the experimental diet is of paramount importance to the outcome of the study. In choosing a method for the delivery of the experimental diets to the study subjects, achieving maximal adherence to the diet must be considered along with rigorous control of nutrient intake (11). A study in which all foods are weighed and measured is considered the gold standard. For what is typically referred to as a metabolic-type diet, subjects are required to consume all foods provided and nothing that is not specified. For example, some studies permit the consumption of energy-free beverages and even alcoholic beverages within limits. Subjects usually eat some meals, if not all (as is the case if they are admitted to a metabolic ward), in a dining facility that is monitored by the research staff. Some meals are packed and eaten when and where the subjects choose. With this approach, subjects are free-living, although nutrient intake is well controlled. Many researchers use this approach to feed subjects and report excellent compliance (as measured both objectively and subjectively) (12–18). A run-in period of at least several days before the study begins is highly recommended to minimize the dropout rate during the study. A run-in gives potential participants a chance to experience the study protocol and assess their ability to adhere to the requirements of the study. An adequate run-in period is important to ensure that potential participants are well-aware of the commitment they are required to make. Studies in which some foods (eg, special fats and products made with them) but not all are given to free-living subjects together with dietary guidance and studies in which subjects are given instructions about food choices lack sufficient experimental control to allow accurate assessment of individual fatty acid effects.

Essential to the design and implementation of well-controlled feeding studies is ensuring that the studies meet the target nutrient specifications. Not only must experimental diets be chemically assayed and validated before they are fed to subjects but they should be monitored periodically throughout the study (19). To minimize variations in key nutrients such as fat and fatty acids, which are affected by season, geographic region, animal and plant genetics, processing, storage, and
shipping, among others, quality-control procedures such as purchasing fat sources at one time from the same lot and storing foods appropriately to prevent fatty acid and cholesterol oxidation should be implemented (20). Together, ongoing monitoring of diet composition (by chemical analysis) and rigorous oversight of quality control ensure that the defined experimental diet is being fed to study subjects (19).

In summary, the design and implementation of experimental diets requires careful planning, attention to quality control procedures and continuous monitoring. Considering all of these aspects makes it possible to carry out a well-controlled feeding study that can answer important questions about the effects of individual fatty acids on cardiovascular disease risk factors.

Experimental design

Well-designed feeding studies are essential for clarifying the effects of individual fatty acids on endpoints of interest. There are two widely used experimental design protocols that are appropriate for fatty acid feeding studies: crossover design and parallel-arm design. Important design criteria include length of the treatment periods, number of treatments evaluated, randomized treatment assignments, a double-blinded study design, and methods for collecting endpoint data.

In a crossover study, subjects are fed all experimental diets; they cross over to the different treatments. In a parallel-arm study, subjects are assigned usually to one treatment. Implicit to a crossover study is that fewer subjects are required but the feeding study lasts longer. In contrast, the parallel-arm feeding study requires more subjects, but although the feeding periods are the same length, the study is shorter because subjects are assigned to fewer treatments. Experimental design decisions are often made on the basis of the length of time required for the study. Few test diet treatments can be evaluated ideally in a crossover design, and a parallel-arm design facilitates inclusion of more treatments. If five test diets were compared with a parallel-arm design, six groups of 20 subjects (including control subjects) could follow a test diet for a 4-wk feeding period. If, however, three test diets were studied, 20 subjects could follow each diet for 4 wk. This crossover study design would require 100 fewer subjects but 8 additional weeks, and fewer treatments would be studied. Certainly a five-period crossover study could be done with 20 subjects for five 4-wk treatment periods, but this would be more demanding for the subjects and thus possibly increase the risk of a high dropout rate.

A control group (that is randomly assigned) is essential and is inherently part of a crossover study but must be included in a parallel-arm study. A control diet can be given to a separate group studied concurrently with the treatment group in a parallel design or subjects can serve as their own controls by receiving both the experimental and control diet in a crossover protocol (21). In a crossover study, experimental diets are randomly assigned to ensure a balanced order of diet assignment and that an equal number of subjects is on each treatment at one time. An inadequately controlled study design frequently used is a sequential treatment design in which there is a baseline control diet phase followed by an experimental period and then sometimes a control diet period. A comparison of the treatment with the control periods is inappropriate because of the lack of a control group during the experimental period to control for confounding variables present during this time. An appropriate (concurrent and essentially identical to the treatment group) control group is necessary to protect against numerous confounding variables, including seasonal variation in biological measurements, regression to the mean, and other identifiable influences (22). An advantage of a crossover study is that having all subjects follow each treatment controls for any confounding variable that may be present in one of the treatment groups. For example, if more hyperresponsive subjects were allocated to one group in a parallel-arm study, the difference in the response observed between two experimental diets would erroneously increase. A crossover design avoids this problem by studying all subjects on all treatments; the hyperresponsive subjects' results would be observed for all treatments, not just for one.

Subjects usually make the transition from their habitual diet to a controlled feeding regimen during the baseline period. It is inappropriate to make any treatment comparisons with any baseline measurements because of the appreciable variability in subjects' habitual diets unless the controlled diet is fed for an adequate time (often referred to as the run-in period). Thus, the appropriate comparisons are between treatment groups, and a comparison of an experimental diet with a typical American diet requires rigorous control of all test diets that are studied.

The duration of the study depends on the length of the feeding periods, the number of treatments, and whether wash-out periods are included. These are important considerations because they affect the scope and cost of the study, as well as the morale of subjects and staff, which has a marked effect on subject adherence to the study protocol and dropout rate. In practice, it is difficult to conduct a single feeding study for >9–12 mo because of the significant commitment required from the subjects.

The actual feeding periods should be sufficiently long to ensure a stable endpoint measurement. Obviously, this will vary depending on the endpoint measurements selected. For example, for plasma lipids and lipoproteins, a 3–4-wk feeding period is adequate. Another important consideration is whether to include a washout period (or break). A washout period is not necessary if the endpoint measurements are stable. However, although a washout period adds to the study length, it is beneficial to both subjects and staff in promoting long-term adherence to the study protocol. Scheduled breaks provide a means by which feeding studies can be conducted and yet consider holidays that are important to subjects and that would otherwise preclude the subjects' participation. Whenever possible, a blinded study design should be used because it adds further control.

More than one endpoint measurement should be taken. At least two and preferably three plasma lipid and lipoprotein measurements are recommended and should be taken on sequential days at the end of the treatment period. This procedure minimizes regression to the mean, which is commonly observed, and also reduces variance in the measurement taken (2). Implicit to this is that samples are collected by using standardized procedures and assayed by using methods that are widely accepted among experts in the field.

Thus, experimental design is a key component of well-controlled feeding studies. Historically, this is one of the problematic areas that has contributed to inconsistent observations (23). It cannot be emphasized strongly enough how important
a well-conceived and implemented experimental design is to a successful study outcome.

Future studies

In recent years we have advanced our understanding of the experimental methodologies appropriate for conducting feeding studies designed to examine individual fatty acid effects on plasma lipids and lipoproteins. The major objective now is to use this knowledge to design studies that address key questions. Of greatest relevance is understanding specific fatty acid effects at physiologic doses. Determining the dose-response effect at amounts that are likely to be consumed as well as at supraphysiologic amounts will provide a sound basis for future dietary recommendations. Also, knowing the relative potency of individual fatty acids at maximal doses will be essential for conducting subsequent studies designed to examine the mechanisms by which individual fatty acids elicit diverse cholesterolemic effects.

Studies should include a Step 1 diet (< 30% of energy from fat, < 10% of energy from saturated fat, and < 300 mg cholesterol/d) as recommended by the American Heart Association as a point of reference to enable comparisons among different studies. In addition, it will be beneficial to manipulate the concentration of individual fatty acids within the context of a Step 1 diet. Collectively, this information will help us identify the ideal fatty acid profile to achieve the most desirable plasma total and lipoprotein cholesterol effects.

Summary

Gaining a better understanding of the effects of individual fatty acids on cardiovascular disease risk factors is a major area of inquiry. Essential to this quest is the need to conduct well-controlled feeding studies. Of critical importance to the success of these studies are well-defined subject eligibility criteria, precisely defined experimental diets in which the only variable is the fatty acid of interest, excellent quality-control procedures that ensure delivery of the experimental diets, and the development and implementation of an appropriate experimental design including the collection and analysis of end-point measurements. Within the context of each of these categories, other important considerations have been discussed that collectively have an important effect on the success or failure of the feeding study. Careful attention to all of these factors is crucial for ensuring that the outcome of the study provides useful information that expands our knowledge base about how individual fatty acids affect cardiovascular disease risk factors.

ANIMAL STUDIES

Experiments designed to evaluate the ability of specific fatty acids to alter plasma cholesterol concentration require the use of an appropriate animal model in which cholesterol balance across liver can be carefully controlled and in which there is an appropriate neutral, control fatty acid available. Ideally, such experiments would involve the measurement of hepatic LDL receptor activity, the LDL-C production rate, the steady state hepatic free and esterified cholesterol concentrations, and plasma LDL-C and high-density-lipoprotein (HDL)-C concentrations.

Innumerable studies have been carried out in which various diets were fed to many different types of experimental animals. Unfortunately, many of these studies were undertaken in animal species that behave very differently from primates and used inappropriate dietary cholesterol and triacylglycerol concentrations. Many of these studies also did not incorporate an absolute neutral control, so that it is difficult to interpret the results of a particular experiment. Thus, in reviewing and interpreting these older experiments, it is important to consider the type of experimental animal that was used, the absolute quantity of cholesterol in the diet, the types of triacylglycerols in the diet, and the specific measurements that were made.

Appropriateness of different animal models

The absolute rate of cholesterol synthesis varies inversely with animal size (24). Small animals such as mice can synthesize as much as 150 mg cholesterol·d⁻¹·kg body wt⁻¹ whereas large primates, including humans, synthesize only 9–10 mg cholesterol·d⁻¹·kg body wt⁻¹. There are two critical issues in choosing a particular animal model. First, the rate of cholesterol synthesis in liver should be relatively low compared with that of the whole animal. Second, animals should not be able to activate bile acid synthesis (ie, cholesterol 7α-hydroxylase) in response to a dietary cholesterol challenge. When fed cholesterol, animals do not begin to expand their intrahepatic pool of sterol and down-regulate LDL receptor activity until the rate of hepatic cholesterol synthesis approaches zero (25). Thus, if a particular species has a high rate of hepatic cholesterol synthesis to begin with or if that species can markedly increase its rate of bile acid synthesis, then such an animal will not elevate its plasma lipoprotein cholesterol concentration when challenged with dietary cholesterol and fatty acids. Rats, and to a lesser degree mice, are prime examples of this type of animal. Such species primarily respond to dietary cholesterol challenge by down-regulating hepatic synthesis and up-regulating hepatic bile acid production so that the plasma lipoprotein concentrations remain relatively unchanged. This type of response is distinctly different from that of other species, particularly primates.

Thus, in considering earlier experiments, those undertaken in rats and mice have been largely excluded as not being relevant for humans. Other animal models, however, do behave similarly to humans and are included in this discussion. These animal models include hamsters, guinea pigs, cynomolgus monkeys, cebus monkeys, African green monkeys, rhesus monkeys, baboons, and pigs. There are other acceptable animal models, although few have been characterized in detail.

Experimental diets

In general, commercial animal diets contain various complex carbohydrates, proteins, and soluble and insoluble fiber. Highly purified diets contain primarily sugars and protein hydrolysates; these diets characteristically alter several aspects of hepatic biochemistry and lipoprotein metabolism and the response of the plasma cholesterol concentration to lipid feeding.

Many studies have been undertaken with high dietary cholesterol concentrations of 1–3 g/100 g diet. This range far exceeds the dietary cholesterol load experienced by most primates. On average, humans take in ~3–5 mg cholesterol·d⁻¹·kg body wt⁻¹. This is an amount of sterol...
equal to \( \approx 50\% \) of the cholesterol synthesized each day in this species. A comparable dose of dietary cholesterol in hamsters would be \( \approx 20 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg body wt}^{-1} \) and in the cynomolgus monkey would be \( \approx 5-6 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg body wt}^{-1} \). A rigorous cholesterol challenge in any of these species would come from a diet formulated to give them a cholesterol intake of 5–10 times their daily synthesis rate. In small animals such as hamsters, for example, such a load is achieved with dietary cholesterol concentrations of only 0.1–0.3%. In many of the older studies in which animals were challenged with cholesterol loads that were 10 times greater than this amount, it is unlikely that the changes induced in liver biochemistry and plasma lipoprotein concentrations have any relevance to the regulation of lipoprotein concentrations in primates in general and in humans in particular. Thus, in this discussion, earlier studies using very high dietary cholesterol loads have been excluded.

**Dietary triacylglycerol additions**

One of the major problems encountered in many older studies is how triacylglycerols were added to the diet. First, in many investigations the effect on cholesterol absorption of adding triacylglycerol to the diet was not quantitated. Obviously if an added oil increased the absolute amount of cholesterol absorbed from the diet, then the effect observed on the plasma cholesterol concentration might result from this increased cholesterol load reaching the liver and not from an effect of the triacylglycerol. Second, many studies used commercial triacylglycerols that contain a mixture of fatty acids. It is not always clear whether a particular effect on the plasma cholesterol concentration resulted from the deletion or the addition of a specific fatty acid to the diet. Third, in almost none of the early studies was a truly neutral control fatty acid present in the diet of the control animals. Thus, it is difficult to determine whether a change in the plasma cholesterol concentration represents a return to the neutral level or whether a particular fatty acid in the experimental triacylglycerol played an active role in regulating LDL receptor activity or the LDL-C production rate.

**Duration of feeding**

How long the various experimental diets were fed varied markedly among studies. From the theoretical considerations outlined earlier, it is important to feed the experimental diets long enough for all variables of the regulatory process to reach new steady states. This includes the relative concentrations of different fatty acids in the hepatic lipid pools, the distribution of unesterified and esterified cholesterol in liver, the level of hepatic LDL receptor activity, the size of LDL-C production, and the concentration of LDL-C in the plasma. In several species it has been shown that new steady states are reached in all of these variables when diets are fed for periods long enough for \( \approx 100 \) pools of LDL-C to be turned over. This period equals 3 wk in mice, 4–5 wk in hamsters, 3 mo in monkeys, and probably >3 mo in humans.

**Experimental measurements**

Unfortunately, in many early studies, only total cholesterol concentration was measured at the end of the dietary feeding period. Such data are essentially uninterpretable. Ideally, data should be available on the concentration of cholesterol and cholesteryl esters in liver; the profile of fatty acids in the total lipids, phospholipids, and cholesteryl esters of liver; hepatic LDL receptor activity; LDL-C production rates; and affinity constants (\( K_a \)) for the lipoprotein particles after feeding various diets. In addition, in studies designed to look at the regulation of HDL-C concentrations, it would be important to have measurements of apolipoprotein A-I turnover, cholesteryl ester transfer protein concentrations, and absolute flux of cholesterol through the HDL pathway. Unfortunately, few published studies include such measurements. Most studies discussed in this review, therefore, are those in which there was at least determination of LDL-C concentrations and, in many instances, some measure of LDL receptor activity or another variable of lipoprotein turnover.

**REFERENCES**


