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Promotion of Colonic Microadenoma Growth in Mice and Rats Fed Cooked Sugar or Cooked Casein and Fat[1]

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ABSTRACT

We studied the effect of cooked food components on the promotion of microadenoma growth in the colons of mice and rats. CF1 mice and Fisher 344 rats were initiated with azoxymethane, with 152 mice receiving four weekly i.p. injections of 5 mg/kg, 59 rats receiving a single injection of 20 mg/kg, and 24 rats receiving 30 mg/kg. A week after the last injection, the animals were randomly assigned to one of eight diets with identical ingredients, but with the three components, sucrose, casein, and beef tallow, either uncooked or cooked. Control animals were given diets with uncooked ingredients. Experimental animals were fed diets in which one, two, or three of the components were cooked in an oven at 180°C until golden brown before they were added to the diet. After 100 days on the diets, the colons were fixed, stained with methylene blue, and scored for microadenomas. The mice and the rats fed cooked sucrose, or casein and beef tallow cooked together, had three to five times more large microadenomas than did the controls (P ranging from 0.02 to 0.0001). No significant increase was observed with the five other cooked diets. Two rats fed the casein and beef tallow cooked together had adenocarcinomas. Thus, a diet containing 20% of cooked sucrose, or 40% of casein and beef tallow cooked together, promotes the growth of colonic microadenomas in initiated mice and rats, and would appear to contain promoters for colon cancer.

INTRODUCTION

Epidemiological studies suggest that factors associated with the diet are responsible for colon cancer. Although dietary components such as fat, protein, or calories may be involved, it is also possible that the factors are not the nutrients themselves, but are rather products of the cooking process (1, 2). Thermal processing of foods is known to lead to the formation of many compounds not normally present in foods. Some of these, such as the polycyclic aromatic hydrocarbons and heterocyclic amines, are known to be carcinogenic. However, they affect primarily organs other than the colon, and concentrations found in foods are not carcinogenic in rodents (3–5). Most of the others have never been tested for their carcinogenic effects.

A recently developed biological assay for colon carcinogens has allowed us to test for the initiating and promoting properties of cooked foods. Bird (6) has shown that the colons of rodents treated with colon carcinogens develop aberrant crypts and aberrant crypt foci within a period of 2 weeks. These crypts, which we will collectively refer to as MA,[4] can be easily visualized with light microscopy and counted in unsectioned, methylene blue-stained preparations. MA are distinguished from normal crypts by their intense blue staining, increased size, elongated shape, and more prominent epithelial cells (6). They are induced in a dose-dependent manner specifically by colon carcinogens (7–9). They often display typical dysplasia in histological section (8). The number of crypts per MA increases with time, and animals harboring MA eventually show typical adenocarcinomas (10). MA can thus be used to provide a relatively rapid measure for the carcinogenic effect of dietary components (11).

The objective of the present study was to evaluate the promoting activity of cooked components, by assessing their effect on the growth of MA in rodents initiated by AOM. After initiation, the animals were assigned to one of eight diets with identical ingredients, in which sucrose, casein, or fat, alone or in combinations, were either cooked or uncooked. The study end point was the number of large MA per colon, as a measure of MA growth. Preliminary experiments showed that a feeding period of 100 days was adequate to quantify promotion of MA growth. The study was replicated in both mice and rats.

MATERIALS AND METHODS

Animals. Five-week-old female CF1 mice and Fischer 344 rats (Harlan Sprague Dawley, Indianapolis, IN) were housed in plastic cages with wire tops and sawdust bedding, with a 12-h light-dark cycle and a temperature of 21–24°C. They had free access to water and diet.

Diets. All the animals were fed a modified AIN-76 diet (12), in which 20% fat was added to the diet at the expense of carbohydrate on a caloric basis (Table 1). The control and experimental diets all contained the same uncooked core component constituting 40% by weight of the total diet. To this were added the variable components, either uncooked or cooked: sucrose (20%), casein (20%), and beef tallow (20%). Control diet (Diet A) consisted of the core and the uncooked variable components. The seven experimental diets (Diets B to H) had the same composition, but one, two, or three of the components of the variable part were cooked before mixing into the remainder of the diet (Table 2). Cooking was carried out as described in Tables 1 and 2 at a temperature of 180°C until the general color of the component(s) was golden brown to simulate the normal cooking of foods. Temperature profiles, measured inside the foods with a mercury thermometer, are given in Figure 1.

Experimental Design. Three separate experiments were carried out to test the promoting effect of the diets, one for mice (Experiment 1), and two for rats (Experiments 2 and 3). Starting one week after their arrival, the mice were given four weekly i.p. injections of 5 mg of AOM (Ash Stevens, Detroit, MI) per kg body weight (Experiment 1), and the rats were given a single i.p. injection of 20 mg/kg (Experiment 2), or 30 mg/kg (Experiment 3) AOM. One week after the last injection, the animals were randomly assigned to groups of approximately 15 mice or six rats. The control groups were larger than experimental ones to obtain more precise comparisons of the seven experimental diets against the single control. Each group was then fed one of the eight diets (Diets A to G). Only two cooked diets, C and G, were tested in Experiment 3. Experiments 1 and 2 were repeated, to test for the initiating effects of the diets, in 62 rats and 100 mice that were not treated with AOM but were randomly allocated to one of the eight diets (Experiments 4 and 5). After 100 ± 1 days on the diets, the animals were sacrificed and weighed, and the colons were scored for MA. Data were analyzed by one-way analysis of variance, followed by a Student's t test comparison between the control and each experimental group.

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[4] The abbreviations used are: MA, microadenomas; AOM, azoxymethane.

Table 1 *Composition of the diets: ingredients*

Ingredients	% weight of total diet	
Sucrose	3.5	(sugar from local supermarket)
Casein	3.5	(ICN Biomedicals, Cosa Maesa, CA)
Corn oil	3.5	(Mazola, from local supermarket)
Corn starch	17.7	(ICN)
Cellulose	5.9	(ICN)
AIN-76 mineral mix	4.1	(ICN)
AIN-76 vitamin mix	1.2	(ICN)
DL methionine	0.36	(ICN)
Choline chloride	0.24	(ICN)
Sucrose (raw or cooked)	20.0	
Casein (raw or cooked)	20.0	
Fat (raw or cooked)	20.0	(beef tallow, Hubbert's, Toronto, Ontario, Canada)
Total	100.0	

Table 2 *Composition of the diets: cooked components*

Diet	Cooked components	Cooking conditions: Cooked mixture[a]	Cooking conditions: Cooking time (h)
A	None		
B	Sucrose	Sucrose-water (1:1)	2.5
C	Casein	Casein-water (1:2)	2.5
D	Fat	Beef tallow[b] (3 kg)	72.0
E	Sucrose + casein	Sucrose-casein-water (1:1:1)	1.0
F	Sucrose + fat	Sucrose-beef tallow (1:1)	2.5
G	Casein + fat	Casein-beef tallow (1:1)	2.5
H	Sucrose + casein + fat	Sucrose-casein-water-beef tallow (1:1:1:1)	1.3

[a] Cooking was done by spreading the mixture as a 2-cm-thick layer in four Pyrex dishes, which were then placed into a kitchen electric oven preheated at 180°C. The dishes were rotated in the oven when the first dish started to brown.
[b] Beef tallow was heated at 180°C under continuous stirring in a stainless-steel beaker.

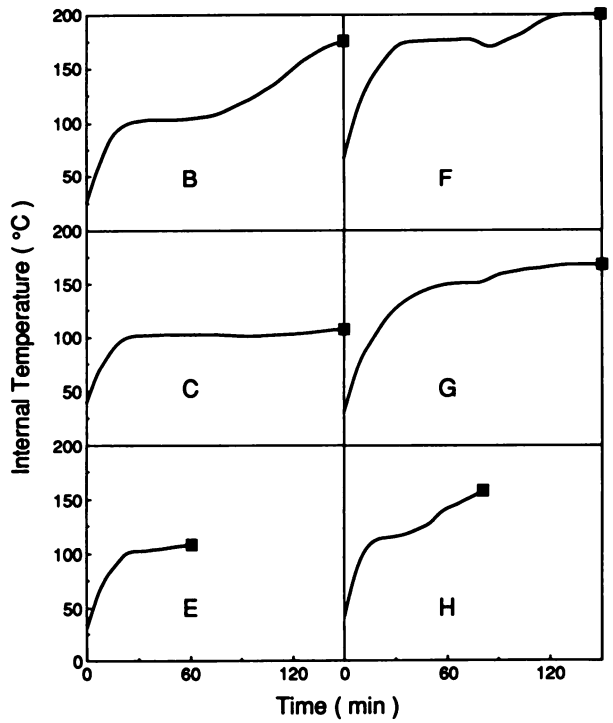

Fig. 1. Internal temperature profiles during the cooking of foods in a kitchen electric oven set at 180°C, until they were golden brown (■). Cooking mixtures *B* to *H* are described in Tables 1 and 2. The profile for fat (D) is not given since it reached a plateau in 2 h, and remained at 180 ± 5°C for 70 h.

Assay. Immediately following the termination of the animals by CO_2 asphyxiation (rats) or by cervical dislocation (mice), the colons were removed, flushed with Krebs-Ringer bicarbonate buffer, slit open from cecum to anus, and fixed flat in 10% phosphate-buffered formalin, between filter papers. The coded colons were scored blindly for MA by a single observer, after they were stained with methylene blue (0.2% in Krebs-Ringer, 15–30 min), according to the method of Bird (6).

RESULTS

The weight gains of the animals after 100 days on the diets (Table 3) suggest that three of the diets may be toxic. Both mice and rats fed cooked sucrose (Diet B), sucrose and beef tallow cooked together (Diet F), and casein and beef tallow cooked together (Diet G) were lower in weight than the animals fed the control diet (Diet A). Similar results were obtained in the animals not treated with AOM (data not shown). On gross examination, both the mice and rats fed cooked sucrose and those fed sucrose and beef tallow cooked together showed other signs of distress: watery feces, dilated mesenteric blood vessels, and enlarged cecums. For instance, the full cecums of the rats fed these diets weighed 7.9 ± 0.8 and 10.8 ± 2.4 g, respectively, whereas those of the rats fed uncooked components weighed 2.2 ± 0.5 g ($P < 0.001$). The animals fed casein and beef tallow cooked together showed no such signs of distress, and no consistent evidence of toxicity was detected in other groups. Some colons could not be scored because they were too heavily stained ($n = 8$) or damaged ($n = 6$).

The total number of MA per colon for each of the diet groups, after 100 d on the diets, is shown in Table 4, except for mice that had no MA and were not treated with AOM. Less than 1 MA was found per rat not treated with AOM, and the cooked diets had no significant effect in those rats. In AOM-treated animals, the numbers of MA are larger for rats than for mice, and larger for rats given injections of 20 mg/kg AOM than those given injections of 30 mg/kg. This paradoxical dose-effect relationship on the total number of MA has already been observed and could be due to the cytotoxicity of the higher dose of AOM (10). Mice fed cooked sucrose (Diet B), casein and beef tallow cooked together (Diet G), and sucrose, casein, and beef tallow cooked together (Diet H) had about twice as many MA as did controls (Diet A), but this effect was not observed in rats.

The distribution of MA sizes, based on the number of aberrant crypts in each MA, is illustrated in Figure 2. The MA were larger in rats than in mice, and in both species the size distribution was skewed. The size distributions of MA in animals fed some of the cooked diets were shifted further towards large values when compared with controls. For example, Figure 2 illustrates the difference of this distribution for mice and rats

Table 3 *Mean body weights (g) of mice and rats fed cooked diets for 100 days*

Diets	Experiment 1: mice, *n*	Experiment 1: mice, Mean ± SD	Experiment 2: rats, *n*	Experiment 2: rats, Mean ± SD	Experiment 3: rats, *n*	Experiment 3: rats, Mean ± SD
A[a]	45	40[b] ± 9	17	230 ± 19	10	214 ± 14
B	15	35[c] ± 6	6	208[c] ± 11		
C	16	39 ± 7	6	211 ± 14	7	212 ± 13
D	16	33[d] ± 4	6	214 ± 18		
E	15	39 ± 6	6	204[c] ± 17		
F	15	31[d] ± 3	6	193[d] ± 11		
G	15	35[c] ± 5	6	185[d] ± 11	7	177[d] ± 9
H	15	37 ± 7	6	222 ± 16		

[a] Diets A to H contained identical ingredients. Sucrose, casein, and fat were uncooked in Diet A, whereas one, two, or three of those components were cooked before mixing in Diets B to H. Detailed compositions are given in Tables 1 and 2.
[b] The initial weight of mice (Experiment 1) was 23 ± 2 g, rats (Experiment 2) 95 ± 9 g, and rats (Experiment 3) 61 ± 11 g.
[c] $P \leq 0.01$.
[d] $P < 0.001$.

Table 4 *Total number of MA per colon after AOM injections, in rats and mice fed cooked diets for 100 days*

	Experiment 1: mice[a] [4 × 5 mg][b]		Experiment 2: rats [20 mg]		Experiment 3: rats [30 mg]		Experiment 4: rats [0 mg]	
Diets	*n*	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD
A[c]	44	28 ± 13	17	131 ± 49	10	53 ± 18	15	0.3 ± 0.6
B	13	48[d] ± 16	6	144 ± 37			6	0.0 ± 0.0
C	16	34 ± 11	6	124 ± 36	7	46 ± 13	6	0.7 ± 1.0
D	16	39 ± 21	6	120 ± 46			6	0.7 ± 0.5
E	15	31 ± 14	6	96 ± 39			6	1.2 ± 1.2
F	8	14[d] ± 6	5	122 ± 22			6	0.5 ± 0.5
G	15	43[e] ± 16	6	166 ± 50	6	42 ± 13	11	0.7 ± 0.8
H	14	48[e] ± 17	6	127 ± 44			6	0.8 ± 0.7
P[f]		<0.01		0.26				0.18

[a] No MA were detected in any of the 100 mice, which had not received AOM, in Experiment 5.
[b] Numbers in brackets, AOM dose.
[c] Diets A to H contained identical ingredients. Sucrose, casein, and fat were uncooked in Diet A, whereas one, two, or three of those components were cooked before mixing in Diets B to H. Detailed compositions are given in Tables 1 and 2.
[d] $P < 0.01$.
[e] $P < 0.001$.
[f] P is the significance of the F value in the one-way analysis of variance. The pairwise Student's t test comparisons are done only when the whole experiment shows some inter-group difference, that is, when this P is less than 0.05.

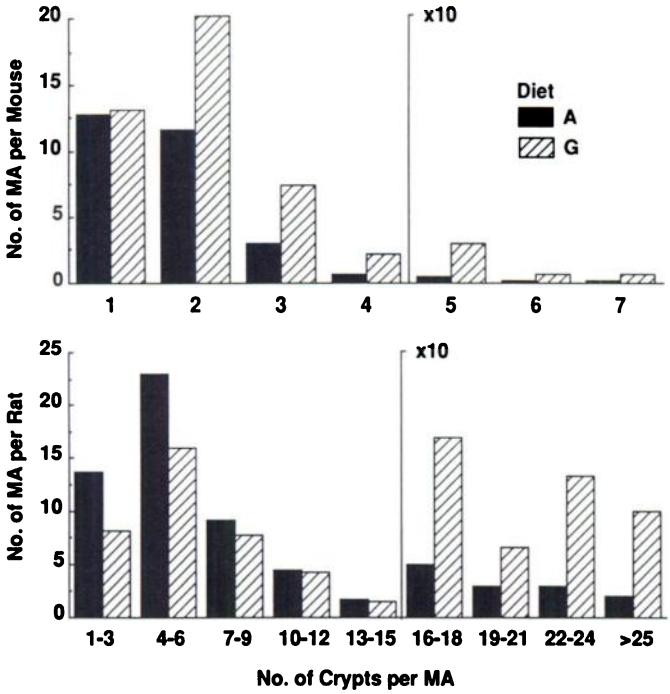

Fig. 2. Size distribution of microadenomas in the colons of mice (*top*) or rats (*bottom*) given AOM and then fed either an uncooked control diet (*A*) or a diet containing beef tallow and casein cooked together (*G*). *Ordinate* scale is expanded 10 times for mouse MA larger than four crypts and for rat MA larger than 15 crypts.

fed casein and beef tallow cooked together (Diet G) compared with those fed the control, uncooked diet (Diet A). With the cooked diet, the number of small MA are lower or equal to control values, while the numbers of large MA in these groups were two to five times higher than controls. Some of the cooked diets thus stimulated the growth of MA.

In order to measure more specifically the effect of cooked diets on the larger MA, we defined for each experiment an arbitrary cutoff size for "large MA." This size was chosen so that there was the same total number of large MA in the control group, as the number of animals in the group. In other words, there was an average of approximately one large MA per control animal. For instance, a total number of 10 MA containing 18 crypts or more was scored in the 10 control rats in Experiment 3. We thus chose 18 crypts as a cutoff for large MA in Experiment 3. Similarly, we chose a cutoff of four crypts per MA for mice in Experiment 1, and 11 crypts per MA for rats in Experiment 2.

The number of large MA per colon is shown for all the groups in Figure 3. There was an increase of more than threefold in all groups of mice and rats fed the cooked sucrose (Diet B) and casein and beef tallow cooked together (Diet G) when compared with controls (Diet A). No significant difference was found for the other experimental groups. This conclusion was insensitive to the definition of large MA. For instance, including the largest 5% of the MA in control animals as large MA led to P values for differences between control groups (Diet A) and the groups fed cooked sucrose (Diet B) and casein and beef tallow cooked together (Diet G) that were below 0.02. The conclusions were also insensitive to the statistical method used in their assessment. Dunnett's (13) method, which allows for multiple comparisons with a single control group, also showed these two groups to be significantly different ($P < 0.05$).

Some rats given AOM 30 mg/kg had macroscopic lesions that were examined histologically. No gross lesions were found in rats fed the control diet, but a polyp was observed in one rat

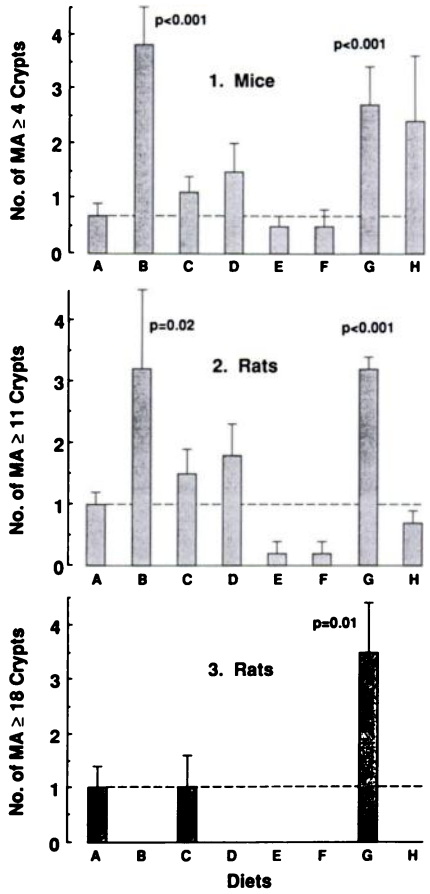

Fig. 3. Number of large microadenomas in the colons of mice or rats given AOM and then fed either control or cooked diets for 100 days. The mice in Experiment 1 (*1*) were given four weekly injections of AOM 5 mg/kg, the rats in Experiment 2 (*2*) received a single injection of 20 mg/kg, and the rats in Experiment 3 (*3*) received 30 mg/kg. Diet *A* is the uncooked control diet, Diet *B* contains 20% cooked sucrose, and Diet *G* contains 40% casein and beef tallow cooked together. Detailed composition of the diets is given in Tables 1 and 2. The rats in Experiment 3 were fed only Diets A, C, and G. *Bars*, SE; *P*, Student's t test comparison with Diet A.

fed cooked casein (Diet C), and a polyp was found in each of three rats fed the casein and beef tallow cooked together (Diet G). One of the polyps in the latter group was an adenoma, whereas the other two were polypoid adenocarcinomas.

DISCUSSION

The results suggest that a diet containing sucrose cooked to the consistency of hard caramel (Diet B) and a diet containing casein and beef tallow cooked together (Diet G) can act as promoters of colon carcinogenesis.

Several dietary variables are known to affect the promotion of colon cancer. Perhaps the best known is caloric restriction and excess, though the diets we have used do not appear to act in this way. They lead to an increase in large MA even in the absence of a weight gain compared with controls. A second dietary variable that has been extensively studied is the level of dietary fat. Some dietary fats have been found to increase the yield of colonic tumors (14). However, a diet containing 20% fat increases the yield of MA by a factor of only 1.5 times in a period of about 100 days, compared with a 5% fat control (8). The increase in large MA we have observed is thus not likely to be a consequence of differences in available fat in the diet. These considerations indicate that the effect of cooked foods is not a consequence of some previously described phenomena.

Cooked sucrose contains anhydrosugars and furans, among the hundreds of compounds that are generated during the cooking of carbohydrates (15). Many of these compounds are known to be genotoxic under *in vitro* conditions (16, 17) and could possibly act as promoters. Cooked sugar appears to act as a promoter, whereas cooked sugar and beef tallow do not (Fig. 3, Diets B and F). One explanation is that the two cooking conditions used were different (see Table 2). The promoting compounds would be produced in the presence of water and/or air, but would not under a fat layer. A second explanation is that the difference is a result of the greater toxicity of sugar when cooked with fat. Both products showed evidence of toxicity in terms of reduced weight gain and gross intestinal change. The toxicity could be associated with an effect on the growth of colonic mucosal cells and, perhaps, of MA as well.

The cooking of protein and fat produces amides (18), whose toxicity is not documented. At the temperatures of cooking, heterocyclic aromatic amines can be formed, though in low yield (3, 4). Among the many compounds that are generated during the cooking of protein and fat are the atypical di-amino acids such as lysinoalanine. This compound can be found in a variety of home-cooked and commercial foods and is known to be toxic to the kidney of the rat (19). Protein cooked with fat (Diet G) appears to have a much greater promoting activity than either cooked fat (Diet D) or the cooked protein (Diet C) alone. One possible explanation is that the fat acts as an efficient medium for the transfer of heat (20) and so the protein is more efficiently heated in the presence of the fat (see Fig. 1) (supported by preliminary *in vivo* results). A second possible explanation is that the active product is due to the interaction of fat and protein.

If the findings of this study can be extrapolated to longer periods of time and to the conditions of human food preparation, they may have important implications for the origin of human colon cancer. Foods typical of a Western diet, such as baked, toasted, and fried starchy foods, and fried, roasted, and broiled meats, may act as promoters, whereas fruits, vegetables, olive oil, and milk products that are eaten raw or boiled may not. Conceivably, significant reductions in the development of colon cancer may be possible, with the use of generally lower cooking temperatures for all the foods, without changing the major dietary components.

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