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Alimentary production of gallstones in hamsters

20. Influence of dietary cholesterol on gallstone formation

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With 3 tables

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Diets containing 20% casein, 12% rice starch, 52.3% glucose and 10% fat, plus salts, choline chloride and vitamins have previously been found to produce a rather high incidence of cholesterol gallstones in young hamsters when the fat is butterfat, but a low incidence of cholesterol gallstones when the fat is that of a dietetic margarine rich in linoleic acid. Under the same circumstances, the incidence of amorphous pigmented gallstones was found to be low or moderate with both fats (1).

We are now reporting an examination of the influence on gallstone production resulting from addition of cholesterol in the amount of one per cent to diets of the above mentioned types.

Experimental

The hamsters were young animals born in our stock colony [which has been renewed with animals from a dealer since our last report (2)]. They were about one month old at the beginning of the experiment and were housed in individual cages with wire screen bottom. Diet and water were available ad libitum. The duration of the feeding was 42 to 46 days. At the end of the feeding period the animals were killed with chloroform and autopsied. The gallbladder was taken out, placed on a glass slide, opened and inspected for gallstones. The type of the stones was determined by the aid of dissecting and polarizing microscopes. The results are based only on animals living through the entire experimental period and not having had diarrhea.

The composition of the diets is shown in table 1.

Table 1. Diets.

	Experiment series 129		Experiment series 130	
Casein[1])	20.0	20.0	20.0	20.0
Glucose	52.3	51.3	52.3	51.3
Rice starch	12.0	12.0	12.0	12.0
Salt mixture[2])	5.0	5.0	5.0	5.0
Vitamin mixture[3])	0.5	0.5	0.5	0.5
Choline chloride	0.2	0.2	0.2	0.2
Butterfat			10.0	10.0
Margarine fat	10.0	10.0		
Cholesterol		1.0		1.0
	100.0	100.0	100.0	100.0

[1]), [2]), [3]), see the correpondingly numbered footnotes to table 1 in Reference (1).

Preparation of the diets, separation of butterfat from the butter and of margarine fat from the margarine were as previously described (1). In the cholesterol supplemented diets, cholesterol replaced an equal weight of glucose. The composition of the high linoleic acid margarine used in the present experiment was somewhat different from that used previously (1, 3). The content of octadecadienoic acid was 50% compared with 40% of the fat component of the previously used margarine. According to information obtained from the manufacturer, the fat component of the new margarine consisted of 79.7% sunflower seed oil, 0.3% sesame oil, 20% hydrogenated soybean oil 42/44 °C, plus the prescribed addition of vitamins A and D_3.

The fatty acid patterns of the butterfat and margarine fat were examined by gas-liquid chromatography of the methyl esters as previously described (4). Samples of the sunflower seed oil and hydrogenated soybean oil from which the margarine had been prepared were obtained from the manufacturer and analyzed similarly. The results are shown in table 2. Since the hydrogenated soybean oil did not contain octadecadienoic acid, the content of this acid (18 : 2) in the new margarine must have originated from the unhydrogenated vegetable oils and, therefore, be identical with cis,cis-linoleic acid. This also applied to the octadecadienoic acid contained in the margarine used in our previous studies (1,3).

Table 2. Fatty acid composition of butterfat and margarine fat and of the two main components of the margarine fat.

Fatty acid[1])	Butterfat	Margarine fat	Sunflower seed oil	Hydrogenated soybean oil 42/44°
12 : 0	5.1	0.5		
14 : 0	10.8	0.2		0.3
14 : 1	2.9			
16 : 0	31.8	6.8	6.3	7.4
16 : 1	5.1			
18 : 0	10.0	6.5	3.9	15.8
18 : 1	30.6	34.8	23.8	76.5
18 : 2	2.5	50.0	66.0	
18 : 3	1.2	1.2	tr.	

[1]) Number of carbon atoms and double bonds.

As a further check on the absence of stereoisomers of linoleic acid, and in order to determine the amount of trans-octadecenoic acid (elaidic acid) in the 18 : 1 fraction, the following procedure was carried out. The methyl esters of the fatty acids of the margarine fat and of the hydrogenated soybean oil were subjected to thin-layer chromatography on silicagel G impregnated with silver nitrate as described by CUBERO and MANGOLD (5) using petroleum ether (b. p. 50–70 °C): benzene, 1 : 9 (v : v) as mobile phase. The spots in the TLC were visualized in ultraviolet light after spraying with a 0.2% solution of 2′,7′-dichloro-fluorescein in ethanol. The spots were scraped off, eluted with petroleum ether and examined individually by gas-liquid chromatography. The thin-layer chromatograms of the methyl esters from margarine fat and from the hydrogenated soybean oil each contained two spots which in the GLC behaved like 18 : 1. A second TLC of the methyl esters from the margarine fat was sprayed with 42% phosphoric acid (aqueous) and charred at 200 °C. Measurement of intensity and extinction of the spots, using a Densicord instrument (Photovolt Corporation, New York) showed that 29% of the total 18 : 1 in the margarine fat were elaidic acid (corresponding to 66% of the total 18 : 1 in the hydrogenated soybean oil). No stereoisomers of linoleic acid (18 : 2) were found.

The content of cholesterol in the butterfat was determined in a 6 g sample by hot saponification with KOH in aqueous methanol, extraction with ether, washing of the

Table 3. Frequencies of cases with cholesterol gallstones (C), cases with amorphous pigmented gallstones (A) and cases without gallstones (0).

Exp. series no.	Age of animals at start of feeding, days	Duration of feeding period, days	Diet characteristics	Sex and number of animals in group	Number of animals having			Percentage[2]) of animals having			Mean weight at start of experiment ± sd	Mean weight gain during first 42 days ± sd [3])	Range of individual weight gains during first 42 days,
					C	A	O	C	A	O	g	g	g
129	30–37	42	10% margarine fat	m 27	0	6	21	0	22.2	77.8	45.3 ± 1.5	31.1 ± 1.9	23–45
	30–37	43		f 38	0	12	26	0	31.6	68.4	45.7 ± 1.2	30.4 ± 1.7	10–51
	30–34	46	10% margarine fat	m 28	1	3	24	3.6	10.7	85.7	45.0 ± 1.3	35.9 ± 1.7	26–50
	30–34	46–47	+1% cholesterol	f 39	0	33	6	0	84.6	15.4	44.8 ± 1.1	30.9 ± 2.0	17–61
130	28–31	42	10% butter fat	m 29	17	4	8	58.7	13.8	27.6	43.7 ± 1.8	38.8 ± 2.8	23–53
	28–32	43		f 21[1])	9	5	9	42.9	23.8	42.9	41.8 ± 1.6	33.3 ± 2.8	18–54
	28–32	43–44	10% butter fat	m 33	3	2	28	9.1	6.1	84.8	43.9 ± 1.6	37.2 ± 2.2	23–50
	29–32	44	+1% cholesterol	f 21	0	16	5	0	76.2	23.8	41.8 ± 1.7	36.5 ± 2.6	23–46

[1]) Two of the females in this group had both cholesterol stones and amorphous pigmented stones. Each of these two animals was counted as one case of C and one case of A. Therefore, the sum of C, A and O is 23, whereas the actual number of animals in the group is 21.

[2]) Two figures connected with a vertical line on their right hand side are considered significantly different with a probability of 99 per cent.

[3]) sd of mean weight gain $= \sqrt{(\text{sd of mean initial weight})^2 + (\text{sd of mean final weight})^2}$

ether extract, evaporation, and drying. After dissolving the residue in chloroform, the LIEBERMANN-BURCHARD reaction was carried out on a suitably diluted aliquote part, and the absorbancy at 625 nm measured. For details, see (6). The cholesterol content was 0.283%, corresponding to 0,0283% of the unsupplemented butterfat diet. This amount is insignificant in comparison with the amount of cholesterol in the supplemented diet.

The content of phytosterols in the margarine fat was not determined.

Results

The results are shown in table 3.

In the experiments with margarine fat diets (exp. series 129), it is seen that addition of one per cent cholesterol to the diet increased the percentage of females having amorphous pigmented gallstones, and decreased the percentage of females having no gallstones. These changes are significant with a probability of 99 per cent[1]). The percentage of animals having amorphous pigmented gallstones was lower among the males than among the females, and addition of cholesterol did not increase the percentage of the males having this type of gallstones. An apparent decrease of the percentage of males with amorphous pigmented gallstones is of low significance.

In the experiments with butterfat diets (exp. series 130), it is seen that addition of one per cent cholesterol to the diet has reduced the frequency of cases with cholesterol gallstones among both sexes, increased the frequency of cases with amorphous pigmented gallstones among the females, and increased the frequency of cases without gallstones among the males. These changes are significant with a probability of 99 per cent.

As in the experiments with margarine fat, the percentage of animals having amorphous pigmented gallstones was lower among the males than among the females, and an apparent decrease of the percentage of males with this type of gallstones as a consequence of addition of cholesterol to the diet was of low significance.

Discussion

The finding that dietary cholesterol reduced the incidence of cholesterol gallstones among the animals on the butterfat diet might appear surprising. Obviously, the explanation of this phenomenon will require further experimentation.

In view of the fact that other workers [e. g., TOMKINS et al. (8)] have found that dietary cholesterol reduces the synthesis of cholesterol in the liver of rats, it is tempting to conjecture that the observed reduction of the incidence of cholesterol gallstones in hamsters might be causally related to reduction of hepatic cholesterol synthesis. In a study (9) in which the synthesis of cholesterol from labeled acetate in the body of young hamsters was examined, it was found that incorporation of acetate into cholesterol was greater in hamsters receiving a diet with glucose as carbohydrate (a diet greatly favouring production of cholesterol gallstones) than in hamsters receiving a diet with rice starch as carbohydrate (a diet with which the tendency to formation of gallstones is

[1]) Evaluation of the significance of observed differences in frequency is based on the method described by KOLLER (7).

low). Further, in studies (1, 10, 11) in which we analyzed the bile of young hamsters reared on diets with different influence on gallstone formation in this species, lower tendency to production of cholesterol gallstones was generally found to be associated with lower concentrations of cholesterol in the bladder bile. Future studies must clarify the question whether the concentration of cholesterol in the bile is determined by hepatic synthesis of cholesterol rather than by the concentration of cholesterol in the plasma and the amount of cholesterol deposited in the liver.

A further possibility to be considered is that dietary cholesterol might increase the production of bile acids and thereby also increase the flow of bile.

The incidence of amorphous pigmented gallstones among the animals on diets not containing added cholesterol was higher than in our previous experiments (1) with diets containing butterfat and margarine fat. The reason for this is not clear. Further, the amorphous pigmented gallstones were somewhat softer and less colored than the corresponding type of stones produced with a diet containing sucrose and lard (e. g., 12). Their amount was not sufficient for analysis. It is, therefore, not known whether the main constituents were the same as those of the previously analyzed amorphous pigmented gallstones from hamsters. In the latter case the main constituents were calcium, phosphate and glycine conjugated bile acids (13).

The fact that the amorphous pigmented gallstones observed in the present experiments were more frequent among the females than among the male hamsters agrees with our previous observations regarding this type of gallstones (10, 12, 14, and unpublished data). The mechanism by which dietary cholesterol has influenced this type of gallstones remains to be elucidated.

Summary

Groups of young hamsters (27–33 males, 21–38 females in each group) were reared for approximately 6 weeks on diets consisting of casein, 20%; glucose, 52.3%; rice starch, 12%; fat, 10%; plus salts, choline chloride and vitamins.

When the fat was *butterfat*, the incidence of cholesterol gallstones was rather high (59% among the males, 43% among the females), whereas the incidence of amorphous pigmented gallstones was low or moderate (14% among the males, 24% among the females). *Addition of 1% cholesterol* to the diet with 10% butterfat *lowered the incidence of cholesterol gallstones significantly among both sexes* (to 9% among the males and to 0% among the females), and *increased the incidence of amorphous pigmented gallstones among the females* (to 76%), whereas the incidence of amorphous pigmented gallstones among the males was not significantly changed (decreased to 6%).

When the fat was that of a *dietary margarine with a high content of linoleic acid*, the incidence of cholesterol gallstones was zero among both sexes, whereas the incidence of amorphous pigmented gallstones was moderate (22% among the males, 32% among the females). *Addition of 1% cholesterol* to the diet with 10% margarine fat did not cause any significant change of the incidence of cholesterol gallstones (increase to 4% among the males, no change among the females), but *increased the incidence of amorphous pigmented gallstones among the females* (to 85%). The incidence of amorphous pigmented gallstones among the males was not significantly changed by addition of cholesterol (decreased to 10%).

Zusammenfassung

Gruppen von jungen Hamstern (27–33 Männchen, 21–38 Weibchen in jeder Gruppe) erhielten während 42–46 Tagen eine Nahrung von Casein, 20%; Glucose, 52,3%; Reisstärke, 12%; Fett, 10%; plus Salze, Cholinchlorid und Vitamine.

Wenn das Fett aus *Butterfett* bestand, war die Inzidenz von Cholesterin-Gallensteinen recht hoch (59% unter den Männchen, 43% unter den Weibchen), während die Inzidenz von amorphen, pigmentierten Gallensteinen niedrig oder moderat war (14% unter den Männchen, 24% unter den Weibchen). *Zulage von 1% Cholesterin* zu der Butterfett-Nahrung *erniedrigte die Inzidenz von Cholesterin-Gallensteinen signifikant* (zu 9% unter den Männchen und zu 0% unter den Weibchen) und *erhöhte die Inzidenz von amorphen pigmentierten Gallensteinen signifikant unter den Weibchen* (zu 76%), während die Inzidenz von amorphen pigmentierten Gallensteinen unter den Männchen durch die Cholesterin-Zulage nicht signifikant geändert wurde (Erniedrigung zu 6%).

Wenn das Fett aus der Fettkomponente einer *linolsäurereichen Margarine* bestand, war die Inzidenz von Cholesterin-Gallensteinen gleich 0 bei den beiden Geschlechtern, während die Inzidenz von amorphen pigmentierten Gallensteinen moderat war (22% unter den Männchen, 32% unter den Weibchen). *Zulage* von *1% Cholesterin* zu der Margarinefett-Nahrung brachte keine signifikante Änderung der Inzidenz von Cholesterin-Gallensteinen hervor (Erhöhung zu 4% unter den Männchen, keine Änderung unter den Weibchen), wogegen *die Inzidenz von amorphen pigmentierten Gallensteinen unter den Weibchen signifikant* (zu 85%) *erhöht wurde.* Die Inzidenz von amorphen pigmentierten Gallensteinen unter den Männchen wurde durch die Cholesterin-Zulage nicht signifikant beeinflußt (Abnahme zu 10%).

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