From the Department of Biochemistry and Nutrition
Polytechnic Institute, Copenhagen (Denmark)

Alimentary Production of Gallstones in Hamsters

13. Influence of highly unsaturated fats and certain minerals on gallstone production*)**)

By F. CHRISTENSEN, I. PRANGE, and H. DAM

With 1 figure and 3 tables

(Received June 22, 1963)

In an earlier study (1) we have compared the influence of 3 different fats, viz. lard, soybean oil and cod liver oil, on gallstone formation in young hamsters, when these fats were incorporated at the level of 2% (at the expense of glucose) in a fat-free basal diet containing 20% casein, 62.3% glucose, and 12% rice starch as protein and carbohydrate components. The fat-free basal diet produced a very high incidence of cholesterol gallstones. The incidence of cholesterol gallstones was somewhat reduced by lard, much reduced by soybean oil, and apparently eliminated by cod liver oil. The fats, especially soybean oil and cod liver oil, were found to favor formation of amorphous pigmented gallstones.

The present communication deals with the influence of 1. cod liver oil, 2. cod liver oil from which vitamin A and a large part of other unsaponifiable constituents have been removed by adsorption on filtrol, 3. distilled ethyl esters of fatty acids from menhaden body oil, 4. soybean oil, and 5. soybean oil with added cholesterol. All the fats being incorporated at the 3% level at the expense of glucose into the same basal diet with 20% casein, 62.3% glucose and 12% rice starch.

Besides the necessary control groups receiving the fat-free basal diet, a further group on fat-free diet was introduced in which the salt mixture was supplemented with copper, manganese and iodide.

Experimental

As usual, the animals were young hamsters from our own stock colony. The experimental conditions were as described in our previous communications (e.g., 1, 2).

The fatty acid composition of the fats was analyzed by gas liquid chromatography. The fat-containing diets were made up fresh every day.

Table 1 shows the composition of the diets, Table 2 the fatty acid composition of the oils.

The filtrol-treatment of the cod liver oil was carried out by passing a 10% solution of the oil in petroleum ether through a column of filtrol and subsequent evaporation of the solvent. This treatment removed vitamin A completely as shown by the CARR-PRICE reaction, and, in the three different portions prepared, it brought the content of cholesterol down from the original level of 0.450% to 0.095%, 0.111%, and 0.093%, resp. The diet containing filtrol-treated cod liver oil was tested for oxidative destruction of the oil by

*) This work was supported by a grant from National Institutes of Health, United States Public Health Service (Grant No. A-4964-Met).

**) Herrn Prof. Dr. Dr. K. LANG zum 65. Geburtstag gewidmet.

determination of peroxide value and fatty acid composition (gas liquid chromatography of the methyl esters) immediately after incorporation of the oil into the diet and after the diet had been left in food cups in the animal room for 5.5 and 24.5 hours, resp. Very little change took place (compare Table 2).

Table 1. Composition of diets

	Basal, fat-free	3% "C.L.O."	3% "Filtrol-treated C.L.O."	3% "M.O. ethyl esters"	3% Soybean oil	3% Soybean oil + 0.1% cholesterol	"fat-free + Cu, Mn and I"
Casein, crude[1])	20	20	20	20	20	20	20
Glucose	62.3	59.3	59.3	59.3	59.3	59.2	62.3
Rice starch	12	12	12	12	12	12	12
Salt mix[2])	5	5	5	5	5	5	0
Salt mix with Cu, Mn, I[3])	0	0	0	0	0	0	5
Vitamin mix[4])	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Cod liver oil, Ph. Dan., 1948, untreated[5])	0	3	0	0	0	0	0
Cod liver oil[5]), Ph. Dan., 1948, filtrol-treated	0	0	3	0	0	0	0
Distilled ethyl esters of fatty acids from Menhaden body oil[6])	0	0	0	3	0	0	0
Soybean oil[7])	0	0	0	0	3	3	0
Cholesterol	0	0	0	0	0	0.1	0

[1]) "Dairinex", from Dansk Mejeri Industri & Export Kompagni, Stege, Denmark.

[2]) McCollum, E. V. and N. Simmonds, No. 185; J. Biol. Chem. **33**, 55 (1918) (nearly identical with salt mix no. 2, U.S.P. XIII).

[3]) Same as above plus 6.5 mg $CuSO_4$, 5 H_2O, 19.7 mg $MnSO_4$, H_2O, and 0.65 mg KI per 5 g salt mixture.

[4]) Biotin, 0.050 g; folic acid, 0.050 g; ascorbic acid, 5 g; thiamine hydrochloride, 5 g; riboflavin, 5 g; pyridoxine hydrochloride, 5 g; calcium panthotenate, 5 g; nicotinic acid, 8 g; inositol, 15 g; p-aminobenzoic acid, 35 g; vitamin K ("Synkavit", Roche), 1 g; dl-α-tocopheryl acetate ("Ephynal", Roche), 5 g; and sugar up to 500 g.

Vitamins A and D_3 were given as an aqueous solution, containing 2000 I.U. vitamin A and 200 I.U. vitamin D_3 per ml. 0.1 ml of this solution was given twice a week to each animal, corresponding to 57 I.U. vitamin A and 5.7 I.U. vitamin D_3 per animal per day.

[5]) From Northern Drug and Chemical Company Ltd., Copenhagen, Denmark.

[6]) Gift from U.S. Fish and Wildlife Service, Bureau of Commercial Fisheries, Seattle, Washington, U.S.A.

[7]) From Dansk Sojakagefabrik A/S, Copenhagen, Denmark.

Table 3 shows a summary of the experiment: number of animals at the beginning of the feeding period, maximal lenght of the feeding period, number of survivors in each group after 42 days of experimental feeding, average initial weight and weight gain during 42 days, and the number of the survivors which on autopsy were found to have the various types of gallstones recorded. The incidences in per cent of the various types of gallstones among the survivors are also represented graphically in Fig. 1. Mentioning of the gallstones in animals which died or were killed before 42 days of feeding (usually in connection with diarrhea) is made in the text in cases where it is of special importance.

The animals belonging to the same experimental series (same G no.) were fed simultaneously.

Table 2. Fatty acid composition of the dietary fats. (Gas liquid chromatography on Silicone grease and Reoplex)*)

a. As percent of methyl esters in total methyl esters. b. As percent of ethyl esters in total ethyl esters.

	C.L.O. a	Filtrol-treated C.L.O. a	Filtrol-treated C.L.O. extracted from diet 1. a	2. a	3. a	"M.O. ethyl esters" b	Soybean oil a
C_{12}^{0}	trace	trace	trace	trace	trace	trace	0
C_{14}^{0}	5.08	5.62	4.70	4.64	4.02	8.58	0
C_{15}^{0} or branched	0.73	0.68	0.59	0.63	0.47	0.66	0
C_{15}^{1} or branched		0.35	0.34	0.36	0.27		0
C_{16}^{0}	14.35	14.94	16.55	14.06	14.90	21.33	12.53
C_{16}^{1}	11.00	11.39	9.91	10.92	9.51	14.69	0
C_{16}^{2}	0	0	0	0	0	1.14	0
C_{17}^{0} or branched	1.86	1.51	0.26	0.37	0.40	1.87	0
C_{17}^{1} or branched			0.74	0.80	0.68	1.39	0
C_{18}^{0}	4.31	4.29	3.74	3.54	4.55	3.76	1.47
C_{18}^{1}	26.72	25.27	29.95	29.38	30.26	15.88	27.79
C_{18}^{2}	4.28	4.74	3.44	2.83	2.48	1.67	51.07
C_{18}^{3}	0	0	0	0	0	1.46	7.13
C_{18}^{4}	1.36	1.64	1.62	2.37	1.81		0
C_{20}^{1}	12.26	12.60	11.25	12.16	12.44	4.43	0
C_{20}^{2}?						0.46	0
C_{20}^{4}						1.07	0
C_{20}^{5}	7.47	6.89	6.48	7.24	7.07	12.85	0
C_{22}^{0} or C_{22}^{1}	4.25	3.65	5.13	4.97	5.18	0	0
C_{22}^{4}						1.48	
C_{22}^{5}						1.92	
C_{22}^{6}	6.34	6.45	5.30	5.74	5.75	5.38	0

1. Immediately after incorporation into the diet, peroxide value 7.4.
2. 5.5 hours after incorporation into the diet, peroxide value 7.1.
3. 24.5 hours after incorporation into the diet, peroxide value 9.8.

*) These determinations were carried out by Mrs. G. HØLMER, Danish Fat Research Institute.

Footnotes for Table 3:

*) f = females, m = males.

**) c = cholesterol gallstones only.
c, m = cholesterol gallstones + mixed gallstones occurring together.
c, a = cholesterol gallstones + amorphous pigmented gallstones occurring together.
c, m, a = cholesterol gallstones + mixed gallstones + amorphous pigmented gallstones occurring together.
m = mixed gallstones only.
a = amorphous pigmented gallstones only.
o = no gallstones.

Table 3. Summary of the experiment

Exp. series	Diet characteristics	Sex*) and no. of animals	Age of animals at start of feeding (days)	Maximal feeding period (days)	Initial weight (g)	Weight gain during 42 days (g)	Survivors after 42 days		Incidence of gallstones among survivors**)											
									c		c, m		c, a		c, m, a		m		a		o	
							no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
G 92	Basal, fat-free	f 16	30–35	60	33.5	13.5	15	93.8	12	80.0	2	13.3	0		0		1	6.7	0		0	
		m 15			42.3	12.7	12	80.0	9	75.0	0		0		0		1	8.3	0		2	16.7
G 92	3% C.L.O.	f 15	30–35	60	33.6	24.0	9	60.0	0		0		0		0		0		6	66.7	3	33.3
		m 16			39.4	26.9	14	87.5	0		0		0		0		0		8	57.1	6	42.9
G 92	3% filtrol-treated C.L.O.	f 16	30–35	60	33.8	30.5	13	81.3	0		0		0		0		0		3	23.1	10	76.9
		m 15			39.7	22.5	14	93.3	0		0		0		0		0		5	35.7	9	64.3
G 92	3% M.O. ethyl esters	f 15	30–35	60	34.1	24.9	15	100	0		0		0		0		0		3	20.0	12	80.0
		m 16			40.7	23.3	14	87.5	0		0		0		0		0		1	7.1	13	92.9
G 95	Basal, fat-free	f 15	30–35	48	42.9	2.2	12	80.0	11	91.7	0		0		0		0		0		1	8.3
		m 19			42.8	10.2	6	31.6	6	100.0	0		0		0		0		0		0	
G 95	3% soybean oil	f 16	30–35	49	44.4	26.6	13	81.3	0		0		0		0		0		8	61.5	5	38.5
		m 14			48.4	16.0	9	64.3	0		0		0		0		0		1	11.1	8	88.9
G 95	3% soybean oil + 0.1% cholesterol	f 14	30–35	49	42.1	21.8	9	64.3	0		0		0		0		0		5	55.6	4	44.4
		m 16			44.8	24.3	12	75.0	0		0		0		0		0		2	16.7	10	83.3
G 96	Basal, fat-free	f 31	33–39	45	52.8	8.3	20	64.5	10	50.0	2	10.0	2	10.0	0		2	10.0	1	5.0	3	15.0
		m 39			53.8	15.0	29	74.4	21	72.4	0		1	3.4	2	6.9	0		0		5	17.2
G 96	3% filtrol-treated C.L.O.	f 30	33–39	45	51.9	23.0	21	70.0	0		0		0		0		0		10	47.6	11	52.4
		m 41			55.7	19.5	25	61.0	0		0		0		0		0		3	12.0	22	88.0
G 94	Basal, fat-free	f 24	39–44	45	46.7	15.9	21	87.5	17	81.0	1	4.8	0		1	4.8	0		1	4.8	1	4.8
		m 21			53.0	15.7	20	95.2	19	95.0	0		0		1	5.0	0		0		0	
G 94	Basal, fat-free + Cu, Mn and I	f 22	39–44	45	46.0	24.5	21	95.5	14	66.7	1	4.8	2	9.5	0		1	4.8	1	4.8	2	9.5
		m 23			57.5	13.7	6	26.1	5	83.3	0		0		0		0		0		1	16.7

Results and Discussion

Fig. 1 clearly demonstrates the high incidence of cholesterol gallstones in all the groups receiving the basal diets without added fat. A low or moderate incidence of amorphous stones and "mixed stones", i.e., stones containing cholesterol and amorphous material, is also found in some of the groups fed the fat-free diet, especially among the females. Equally clear is the absence of cholesterol stones and the occurrence of amorphous stones in the survivors in all the groups receiving the unsaturated fats at the 3% level. With one, apparently accidental, exception (the group receiving filtrol-treated cod liver oil in

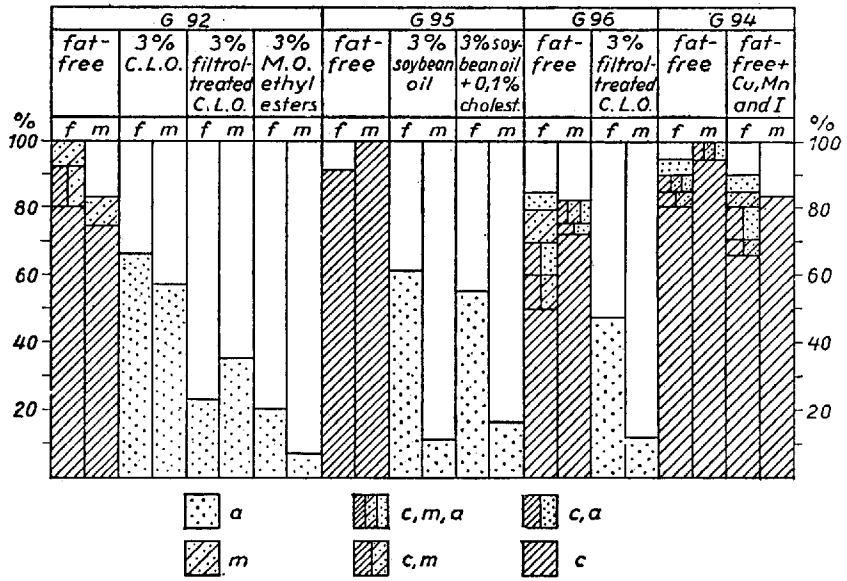

Fig. 1. Graphical representation of the incidence of gallstones among the survivors in the various groups, corresponding to Table 3.
C.L.O. = cod liver oil. M. O. ethyl esters = distilled ethyl esters of fatty acids from Menhaden body oil.

series G 92), the incidence of amorphous stones in these groups is higher among the females than among the males. This agrees with earlier observations regarding the frequency of occurrence of amorphous stones in the two sexes of hamsters (3).

Among the animals not surviving the first 42 days of the feeding period the following observation was of interest:

In the group receiving soybean oil (series G 95), one animal autopsied after only 7 days had cholesterol stones, and in one of the groups receiving filtrol-treated cod liver oil (G 96), two animals autopsied after 9 days and after 24 days had cholesterol stones. As previously observed (4), cholesterol stones can be formed very early in the feeding period. Apparently, it takes some time before the protective effect of small amounts of unsaturated fats becomes fully developed.

The occurrence of amorphous pigmented gallstones was more frequent in the groups receiving untreated cod liver oil, than in the groups receiving filtrol-treated cod liver oil or distilled ethyl esters of fatty acids from menhaden oil. This might suggest that factors in the non-saponifiable fraction increase the tendency to development of amorphous gallstones (as shown in Table 2, the fatty acid composition was essentially the same before and after the filtrol treatment). In an earlier (unpublished) experiment, ingestion of 1% cholesterol did not cause formation of cholesterol stones but increased the incidence of amorphous stones in animals fed a starch diet with 15% of a fat of low unsaturation. But the addition of 0.1% cholesterol to the soybean oil diet (series G 95) did not seem to influence the incidence of amorphous stones. Elucidation of the role of dietary non-saponifiable matter, therefore, requires further experimentation.

The animals in series G 92, G 95 and G 96 received trace elements only through impurities in the food ingredients, and, especially as far as zinc is concerned, by gnawing the zinc coated metal wires of the cages. Therefore, a group of animals receiving addition of copper, manganese and iodide was included in the present experiment (series G 94). The quantities of copper, manganese and iodide added were the same as those used in some of our earlier experiments with chicks. The amounts of copper (1.66 mg per 100 g) and, especially, that of manganese (6.4 mg per 100 g) are relatively large, that of iodide (0.5 mg per 100 g) moderate.

From Fig. 1 it is apparent that the addition of copper, manganese and iodide has not influenced the development of gallstones. As far as copper is concerned, this result is in agreement with previous experiments (5, 6) in which the effect of copper was tested with a fat-free basal diet containing sucrose as carbohydrate component. When the effect of copper was tested with a similar basal diet containing lard, some protective action against gallstone formation was observed.

Analyses of the bladder bile of animals from some of the groups of the present experiment (experimental series G 96 and G 94) will be presented in a separate communication.

Summary

Five groups of young hamsters were fed a "fat-free" basal diet containing 20% casein, 62.3% glucose and 12% rice starch as protein and carbohydrate components. Six other groups received the same basal diet in which 3% glucose was replaced by cod liver oil, filtrol-treated cod liver oil, distilled ethyl esters of the fatty acids from menhaden body oil, soybean oil, or soybean oil and 0.1% cholesterol, respectively. One of the groups fed the fat-free basal diet received an addition of 6.5 mg $CuSO_4$, 5 H_2O, 19.7 mg $MnSO_4$, H_2O, and 0.65 mg KI per 100 g diet.

Among the animals surviving the first 42 days of the feeding period, those receiving the fat-free basal diet had a very high incidence of cholesterol gallstones and a very low or moderate incidence of amorphous pigmented gallstones or "mixed stones" (i.e. stones containing both cholesterol and amorphous material in considerable amounts). The surviving animals in the groups receiving the unsaturated fats had no cholesterol gallstones but a much more pronounced tendency to formation of amorphous pigmented gallstones than the survivors in the corresponding groups fed the fat-free diet. The incidence of amorphous pigmented gallstones was higher in the group receiving untreated cod liver oil than in the groups receiving filtrol-treated cod liver oil or distilled ethyl esters of fatty

acids from menhaden oil. The addition of 0.1% cholesterol to the soybean oil diet did not alter the incidence of gallstones. In the groups fed the fat-containing diets, the incidence of amorphous pigmented gallstones was (with one exception) higher among the females than among the males.

Supplementation of the fat-free diet with copper, manganese and iodide did not alter the incidence of gallstones significally.

Zusammenfassung

Fünf Gruppen von jungen Hamstern erhielten eine fett-freie Basal-Nahrung, deren Protein- und Kohlenhydratenkomponenten aus 20% Casein, 62,3% Glucose und 12% Reisstärke bestanden. Sechs andere Gruppen erhielten dieselbe Basal-Nahrung, in welcher 3% Glucose durch Dorschlebertran, bzw. Filtrol-behandelten Dorschlebertran, distillierte Methylester der Fettsäuren von Menhaden-Körperöl, Sojabohnenöl, und Sojabohnenöl plus 0,1% Cholesterol ersetzt worden waren. In einer der fettfrei ernährten Gruppen wurde die Nahrung mit 6,5 mg $CuSO_4$, 5 H_2O, 19,7 mg $MnSO_4$, H_2O und 0,65 mg KI supplementiert.

Unter den Tieren, welche die ersten 42 Tage der Fütterungsperiode überlebten, war das Vorkommen von Gallensteinen wie unten angegeben:

In den mit der fettfreien Basal-Nahrung gefütterten Gruppen war die Incidenz von Cholesterin-Gallensteinen sehr hoch, während die Incidenz von amorphen pigmentierten oder gemischten Gallensteinen (d. h. Gallensteinen, die aus Cholesterin und amorpher Substanz in reichlicher Menge bestanden) sehr niedrig oder nur moderat war.

Die Tiere, welche die Zulage von 3% der ungesättigten Öle erhielten, hatten keine Cholesterin-Gallensteine, dagegen war die Neigung zur Bildung von amorphen pigmentierten Gallensteinen viel größer als in den entsprechenden fettfrei ernährten Gruppen. Die Incidenz von amorphen pigmentierten Gallensteinen war höher in den Gruppen, welche mit dem unbehandelten Dorschlebertran gefüttert wurden, als in denjenigen, welche Filtrol-behandelten Dorschlebertran oder Äthylester von Fettsäuren aus Menhaden-Öl erhielten. Zulage von 0,1% Cholesterin zu der Sojabohnenöl-Nahrung hatte keinen Einfluß auf die Entwicklung von Gallensteinen. Das Vorkommen von amorphen pigmentierten Gallensteinen in den mit fetthaltiger Nahrung gefütterten Gruppen war (mit einer Ausnahme) häufiger bei den Weibchen als bei den Männchen.

Zulage von Kupfer, Mangan und Iod zur fettfreien Basal-Nahrung hatte keinen sicheren Einfluß auf das Vorkommen von Gallensteinen.

References

1. Dam, H. and F. Christensen, Z. Ernährungswiss. **2**, 36 (1961). — 2. Prange, I., F. Christensen, and H. Dam, Z. Ernährungswiss. **3**, 59 (1962). — 3. Dam, H. and F. Christensen, Z. Ernährungswiss. **2**, 91 (1961). — 4. Dam, H. and F. Christensen, Z. Ernährungswiss. **2**, 154 (1962). — 5. Christensen, F. and H. Dam, Acta physiol. Scand. **31**, 75 (1953). — 6. Christensen, F. and H. Dam, IV. Internat. Congress Biochemistry, Abstracts of Communications Section 8, 73 (Vienna 1958).

Author's address:

Professor, Dr. Henrik Dam, Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen (Denmark)